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Research Article

(+)-Valencene production in *Nicotiana benthamiana* is increased by down-regulation of competing pathways

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Plant sesquiterpenes, such as (+)-valencene, artemisinin, and farnesene are valuable chemicals for use as aromatics, pharmaceuticals, and biofuels. Plant-based production systems for terpenoids critically depend on the availability of farnesyl diphosphate (FPP). Currently, these systems show insufficient yields, due to the competition for FPP of newly introduced pathways with endogenous ones. In this study, for the first time an RNAi strategy aiming at silencing of endogenous pathways for increased (+)-valencene production was employed. Firstly, a transient production system for (+)-valencene in *Nicotiana benthamiana* was set up using agroinfiltration. Secondly, silencing of the endogenous 5-*epi*-aristolochene synthase (*EAS*) and squalene synthase (*SQS*) that compete for the FPP pool was deployed. This resulted in a *N. benthamiana* plant that produces (+)-valencene as a prevalent volatile with a 2.8-fold increased yield. Finally, the size of the FPP pool was increased by overexpression of enzymes that are rate-limiting in FPP biosynthesis. Combined with silencing of *EAS* and *SQS*, no further increase of (+)-valencene production was observed, but emission of farnesol. Formation of farnesol, which is a breakdown product of FPP, indicates that overproducing sesquiterpenes is no longer limited by FPP availability in the cytosol. This study shows that metabolic engineering of plants can effectively be used for increased production of desired products in plants.

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1 Introduction

Terpenoids form a large family of compounds with a wide variety of bioactivities. Plants make many terpenes as secondary metabolites, which serve plants to operate ecological interactions with, for example insects [1, 2]. Metabolic engineering of terpenes in plants has been initiated from the late 1990s. Ectopic expression of terpene biosynthetic pathways in plants has been explored for several reasons. In line with the ecological roles of terpenes, attraction of carnivorous arthropods that protect the plant from arthropod infestation has been achieved in *Arabidopsis* and maize by engineering terpenoid biosynthesis [3–5]. More relevant for industry, many terpenes have applications as a flavor, fragrance, or as a pharmaceutical ingredient. However, plant resources for commercially interesting terpenes do not always match the market demand, for which reason there is a drive to find alternative plant production platforms. Hence, fast growing plants like *Nicotiana* species have been deployed to produce (precursors of) pharmaceutically active terpenes such as artemisinin [6–9], triterpenes [10], or fragrance molecules such as patchoulol [9] and limonene [11].

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Abbreviations: CnVS, *Callitropsis nootkatensis* (+)-valencene synthase; **EAS,** 5-*epi*-aristolochene synthase; **FPP,** farnesyl diphosphate; **FPS,** farnesyl diphosphate synthase; **SQS,** squalene synthase; **tHMGR,** truncated 3-hydroxy-3-methylglutaryl-CoA reductase

Ectopic expression of pathways in plants can be achieved by stable transformation of the plant. Plant transformation requires the regeneration of single cells that contain the ectopic DNA into full plants. Such transgenic plants can be directly used for production applications, but the investment in time needed for generation of such stable transformants often prohibits combinatorial biosynthesis approaches, where multiple combinations of genes may need to be tested in parallel. A transient expression technology by agroinfiltration has been developed for the model plant *Nicotiana benthamiana*. While originally developed for transient expression of heterologous protein such as antibodies [12], this system has also been successfully applied to metabolite production, for example for discovery of genes involved in glucosinolate biosynthesis [13], for production of poly-unsaturated fatty acids [14] and to unravel genes involved in sesquiterpene lactone biosynthesis [15].

In the cytosol, sesquiterpenes are synthesized from isoprenoid precursors produced by the mevalonate pathway [16]. The mevalonate pathway starts from acetyl-CoA and leads to formation of isopentenyl diphosphate (IPP) and dimethyl allyl diphosphate (DMAPP). Farnesyl diphosphate synthase (FPS) then condenses DMAPP and IPP into farnesyl diphosphate (FPP). FPP is used by terpene synthases to form terpene hydrocarbons, which can be further modified by oxidation into a wide range of terpenoids. The cytosolic FPP pool is the common precursor for sesquiterpenes, but also for triterpenoids and sterols [16].

Straightforward ectopic expression of sesquiterpene synthases in plant cells often leads to disappointingly low yields, which do not exceed the levels of endogenously present sesquiterpenes [17]. This has inspired a number of strategies to achieve higher sesquiterpene levels. By redirecting sesquiterpene synthases to other subcellular compartments such as mitochondria [3], that have an endogenous FPP pool, or by localizing both the terpene synthase and an FPP synthase to the plastids [9], levels of ectopically produced sesquiterpene have been strongly increased. Alternatively, the key regulatory enzyme of the mevalonate pathway, the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), has been overexpressed together with the sesquiterpene synthase, which resulted in a strong increase in sesquiterpene production [4, 6].

A strategy that has been proven very successful with regard to metabolic engineering of terpene production in micro-organisms like yeast, is to downregulate competing pathways. In yeast, downregulation of the steroid biosynthetic pathway by tight control of the expression of squalene synthase (SQS) ERG9 has led to significantly increased production of sesquiterpenes [18, 19]. With the availability of genome sequences for *N. benthamiana* [14, 20], it has become possible to find targets for suppression of endogenous genes in this model plant. Suppression can be transiently achieved by agroinfiltration of constructs coding for RNAi, as demonstrated for production of fatty acids [14].

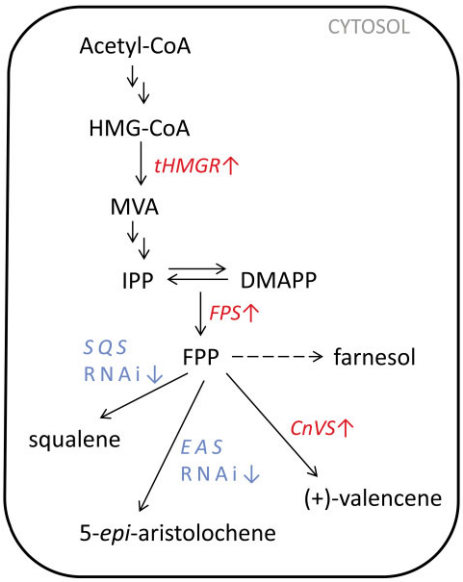

Figure 1. Scheme of the metabolic engineering steps for (+)-valencene production in *N. benthamiana*. Overexpression and silencing constructs used in the study are schematically presented. The truncated *A. thaliana* 3-hydroxy-3-methylglutaryl-CoA reductase (*tHMGR*), *A. thaliana* farnesyl diphosphate synthase (*FPS*), and *C. nootkatensis* (+)-valencene synthase (*CnVS*) were overexpressed. The endogenous squalene synthase (*SQS*) and the 5-*epi*-aristolochene synthase (*EAS*) were silenced using RNAi hairpin constructs (MVA, mevalonic acid; IPP, isopentenyl diphosphate; DMAPP, dimethyl allyl diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA).

In this work we describe the application of combined gene silencing and overexpression to enhance production of sesquiterpenes in *N. benthamiana* (Fig. 1). We enhance FPP availability in the cytosol of *N. benthamiana* by overexpression of truncated 3-hydroxy-3-methylglutaryl-CoA reductase (tHMGR) and FPS. The second strategy we employ is silencing of endogenous genes of *N. benthamiana* that use FPP as substrate, SQS and 5-*epi*-aristolochene synthase (EAS). We apply these strategies for production of the citrus aromatic sesquiterpene (+)-valencene. Recently, we described a new valencene synthase from Nootka cypress (*Callitropsis nootkatensis*) for biotechnological production of (+)-valencene in *Rhodobacter sphaeroides* [21]. In this study, we aimed to optimize (+)-valencene production using the same gene in a plant platform.

2 Materials and methods

2.1 Plasmid constructs for silencing of endogenous squalene synthase and 5-*epi*-aristolochene synthase

N. benthamiana EAS gene (SGN-U509263) and *SQS* gene (SGN-U507215) were identified in the *N. benthamiana*

Unigenes database (Sol Genomics Network) by homology search using the *Nicotiana tabacum* EAS (GenBank L04680.1) [22] and SQS (GenBank U60057.1) [23] as query. Two primer pairs were designed for each gene to amplify a region between 200 and 300 bp using a proofreading Phusion High-fidelity DNA polymerase (Finnzymes). PCR primers used for amplification are listed in Supporting information, Table S1. PCR products were cloned into the pCR®8/GW/TOPO vector (Invitrogen), transformed into OneShot TOP10 *Escherichia coli* competent cells (Invitrogen) and selected on LB plates supplemented with spectinomycin (100 μg/mL). The inserts were then subcloned into the pK7GWIWG2(II) vector [24] by gateway LR reaction (Gateway LR clonase enzyme mix; Invitrogen) and colonies were selected on LB medium supplemented with chloramphenicol (50 μg/mL). The four plasmids obtained by this procedure were named EasRNAi1, EasRNAi2, SqsRNAi1, SqsRNAi2 (Supporting information, Table S2). Correct insertion was confirmed with DETT sequencing (GE Healthcare). A control silencing construct was prepared in the same manner containing a fragment of the firefly luciferase gene (primers listed in Supporting information, Table S1) to yield a vector named LucRNAi.

2.2 Cloning of valencene synthase, truncated 3-hydroxy-3-methylglutaryl-CoA reductase, and farnesyl diphosphate synthase

C. nootkatensis (+)-valencene synthase (*CnVS*, GenBank JX040471) [21] was cloned into the *Bam*HI and *Not*I restriction sites of ImpactVector pIV1A-1.1 (www.impactvector.com) with cytoplasmic targeting. By this procedure *CnVS* was positioned between the Rubisco small subunit RbcS1 promotor and RbcS1 terminator from *Chrysanthemum morifolium* [25]. Correct insertion of genes into Impact vectors was confirmed by restriction analysis and DETT sequencing (GE Healthcare). The full expression cassette was then transferred to the pBIN + Dest [26] vector by gateway LR reaction (Gateway LR clonase enzyme mix; Invitrogen). *tHMGR* from *Arabidopsis thaliana* (GenBank J04537) lacking the N-terminal membrane binding domain involved in the post-transcriptional regulation and *FPS2* from *A. thaliana* (GenBank NM_117823) were also cloned into the pImpactVector pIV1A-1.1 using *Bam*HI and *Not*I restriction sites and subcloned into the pBIN + Dest vector, as described above. The full list of constructs used in this study is given in Supporting information, Table S2.

2.3 Transient gene expression in *N. benthamiana*

Silencing and overexpression constructs for agroinfiltration were transferred to *Agrobacterium tumefaciens* strain Agl-1 by electroporation. Transformed Agl-1 strains were grown at 28°C at 250 rpm for 24 h in LB medium supplemented with 50 μg/mL kanamycin and 40 μg/mL rifampicin for pBIN + Dest-based constructs and 100 μg/mL spectinomycin and 40 μg/mL rifampicin for pK7GWIWG2(II)-based plasmids. The cultures were centrifuged at 4000*g* for 20 min at 20°C and the cells were resuspended in 10 mL infiltration medium (10 mM MES, pH 5.6, 10 mM $MgCl_2$, 100 μM acetosyringone) by rolling on a roller bank for 3 h at room temperature and consequently diluted to a OD_{600} of 0.5. For co-infiltration, the Agrobacterium cultures were mixed so that the gene load within one experiment remains constant.

N. benthamiana plants for transient transformation were grown in the greenhouse in soil with a minimum of 16 h of light per day (28°C in the day and 25°C at night). Infiltration was performed by slowly injecting the bacterial suspension into 5-wk-old *N. benthamiana* plants by pressing a 5 mL syringe without the metal needle against the abaxial side of the leaf. Three individual plants were injected for each construct combination. The plants were grown for another 5 days in the greenhouse and then the leaves were collected for analysis. Negative infiltrations using Agrobacterium cultures containing empty vectors were used in every series of agroinfiltration experiments. Overall volatile emission can vary among experiments, therefore statistical comparison was always made with control plants that were infiltrated and grown at the same time as treated plants.

2.4 Headspace volatile trapping

For each treatment, three freshly cut *N. benthamiana* leaves from different plants were weighed, and immediately placed in a 10-mL glass beaker filled with water. Each leaf was placed in a separate 0.5-L sealed glass container jar. Volatiles emitted by the leaves were collected on stainless steel cartridges (Markes, Llantrisant, UK) filled with 200 mg Tenax TA (20/35 mesh; Grace-Alltech) using a headspace system similar as described by Snoeren et al. [27]. Volatiles were trapped by sucking air out of the jar at a rate of 100 mL/min for 24 h at 21°C in a light regime of 16 h of light and 8 h of darkness. The amount of (+)-valencene and 5-*epi*-aristolochene emitted was quantified for each treatment by averaging the peak area of these volatiles in three biological replicates per g leaf per 24 h.

2.5 Thermo-desorption GC–MS

Headspace samples were analyzed on a thermodesorption GC–MS system similar as described by Snoeren et al. [27]. Before thermodesorption, cartridges were flushed with helium at 50 mL/min for 20 min to remove moisture and oxygen. After flushing, collected volatiles were desorbed from the cartridges at 220°C (Ultra; Markes) for 5 min with a helium flow of 30 mL/min with a split ratio of 1:1. The released compounds were focused on an electrically cooled sorbent trap (Unity; Markes) at a temperature

of 10°C. Volatiles were then injected on the analytical column (ZB-5MSi, 30 m × 0.25 mm ID, 1.0 μm – film thickness, Zebron; Phenomenex) by ballistically heating the cold trap to 250°C for 3 min with using a split flow of 40 mL/min. The GC temperature program started at 60°C (3 min hold) and then linearly rose with 10°C/min to 280°C (3 min hold) with a constant helium flow of 1 mL/min. The column effluent was ionized by electron impact at 70 eV. Mass scanning was done from 33 to 280 *m/z* with a scan time of 5 scans/s. Ion source and transfer line temperature were set to 250 and 275°C, respectively. Results were analyzed using Xcalibur Software (Thermo, Waltham). The identity of (+)-valencene was confirmed by comparing the mass spectra and retention time with those of the authentic (+)-valencene standard (Fluka).

2.6 Gene expression analysis of *EAS* and *SQS* upon silencing

RNA was isolated from *N. benthamiana* leaves by the RNeasy plant mini kit (Qiagen). For each treatment three plants were sampled. One microgram of total RNA was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad, Veenendaal, The Netherlands) after DNAseI treatment (Invitrogen). Primers designed for real-time PCR analysis of EAS and SQS gene expression are given in Supporting information, Table S1. The transcript level of actin was used as endogenous reference gene, using previously described primers nbact-8 and nbact-226 [28]. Real-time PCRs were carried out in a total volume of 20 μL containing 10 μL of iQ SYBR Green Supermix (Bio-Rad), 0.3 μM of forward and reverse primer and cDNA corresponding to 20 ng RNA in a MyiQ real-time PCR instrument (Bio-Rad). The following PCR program was used: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The PCR efficiency was checked for the real-time assays for EAS, SQS, and actin detection using a calibration dilution curve and slope calculation and was shown to be 95, 94, and 92%, respectively. No unspecific amplification was observed by the melting curve analysis. Relative gene expression was calculated as: $2^{-\Delta C_t}$, where $\Delta C_t = C_t$ (EAS/SQS) – C_t actin.

3 Results

3.1 Ectopic production of (+)-valencene in *N. benthamiana*

The native open reading frame of valencene synthase (*CnVS*) from Nootka cypress [21] was expressed under control of the Rubisco small subunit gene promoter in a

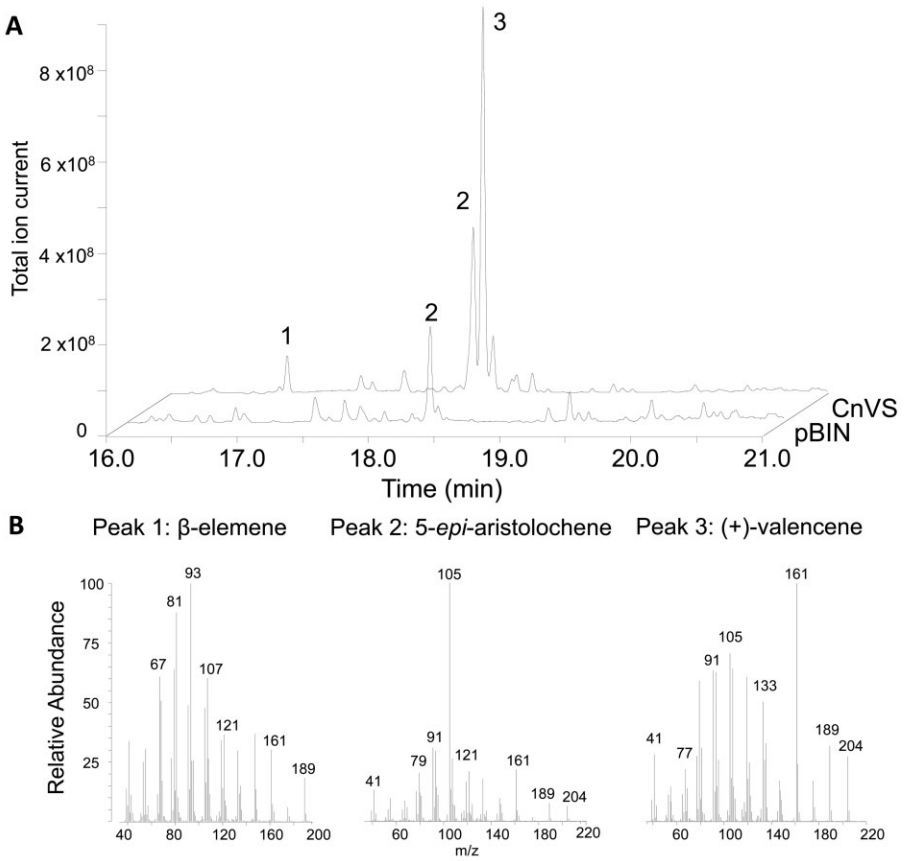

Figure 2. Production of (+)-valencene by agroinfiltration in *N. benthamiana*. The volatiles produced by *N. benthamiana* leaves infiltrated with *CnVS* and empty pBIN vector were collected on tenax-filled steel cartridges and analyzed by thermo-desorption GC–MS. (**A**) GC–MS chromatograms of *CnVS* infiltrated plants and plants infiltrated with the empty pBIN vector. (**B**) Mass spectra of β-elemene (peak 1), 5-*epi*-aristolochene (peak 2) and (+)-valencene (peak 3) in *CnVS* infiltrated plants are shown.

transient assay using agroinfiltration in *N. benthamiana*. After 5 days, the headspace of the leaves was sampled. The predominant sesquiterpene found in the plants infiltrated with empty pBIN vector was found to be 5-*epi*-aristolochene (retention time 18.30 min). Two new peaks were detected in the headspace of *N. benthamiana* expressing *CnVS* (Fig. 2). The peak at the retention time of 18.38 min was identified as (+)-valencene by comparison of the retention time and the mass spectrum to the authentic standard. The second peak at the retention time of 16.89 min was identified to be β-elemene, a Cope rearrangement product of germacrene A, which is a known side product of CnVS [21]. The amount of produced valencene was 0.70 ± 0.13 μg (+)-valencene per g leaf per 24 h.

3.2 Overexpression of tHMGR and FPS leads to higher production of (+)-valencene but also of the endogenous sesquiterpene 5-*epi*-aristolochene

To further increase (+)-valencene production in *N. benthamiana tHMGR* and *FPS* from *A. thaliana* were co-expressed with *CnVS* in *N. benthamiana* (Fig. 3A). (+)-Valencene production was increased 2.9-fold ($p<0.01$) by this approach while the amount of endogenous 5-*epi*-aristolochene in the headspace increased 37-fold ($p<0.0001$), compared with plants infiltrated with *CnVS* alone. Thus, overexpression of genes involved in FPP biosynthesis strongly enhanced sesquiterpene biosynthesis, but only a fraction of the FPP was channeled into production of additional (+)-valencene.

3.3 Silencing of endogenous competing pathways in *N. benthamiana*

The FPP pool in the cytosol is consumed by SQS, which is the first dedicated step in the biosynthesis of sterols and triterpenes in plants [16]. FPP is also consumed for the synthesis of sesquiterpenes of which 5-*epi*-aristolochene produced by the EAS is the predominant product in *N. benthamiana*. Therefore, a silencing strategy was devised based on the silencing of *SQS* and *EAS*. For *SQS* only one homolog was identified among the *N. benthamiana* unigenes (SGN-U507215) with a nucleotide identity of 97% to the *N. tabacum* SQS. For *EAS*, two *N. benthamiana* unigenes SGN-U509263 and SGN-U517646 with 94 and 92% nucleotide identity to the tobacco *EAS* gene were identified. Two silencing constructs for both *EAS* and *SQS* genes were designed. Fragments of 200–300 bp

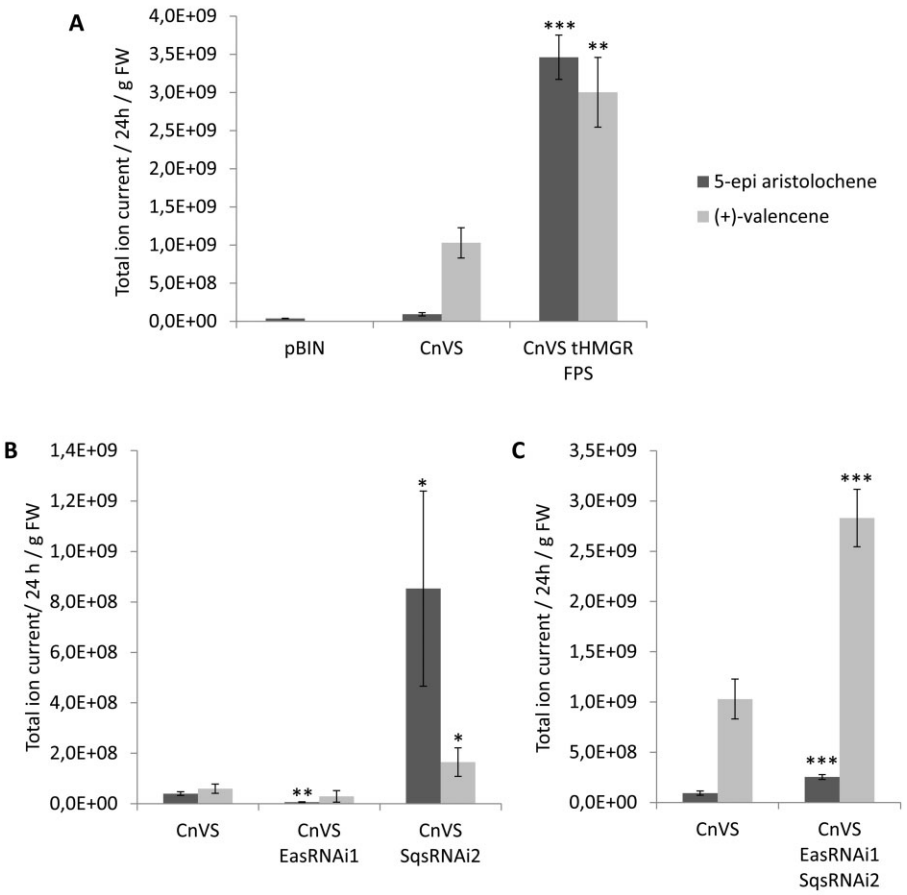

Figure 3. (+)-valencene emission in *N. benthamiana* leaves upon expression of *CnVS*. The amounts of 5-*epi*-aristolochene and (+)-valencene in the headspace of *N. benthamiana* (as quantified by the total ion current measured on GC–MS) are shown for (**A**) plants infiltrated with an empty pBIN vector, *CnVS* and plants co-infiltrated with *tHMGR*, *FPS* and *CnVS*, (**B**) plants where *CnVS* was co-infiltrated with individual silencing constructs EasRNAi1 and SqsRNAi2, and (**C**) plants where *CnVS* was co-infiltrated with both EasRNAi1 and SqsRNAi2 silencing constructs. The mean ± SD for three biological replicates are shown. The asterisks denote statistical difference between the *CnVS* infiltration and other treatments (two-tailed Student's *t*-test): *$p<0.05$; **$p<0.01$; ***$p<0.001$; $n = 3$.

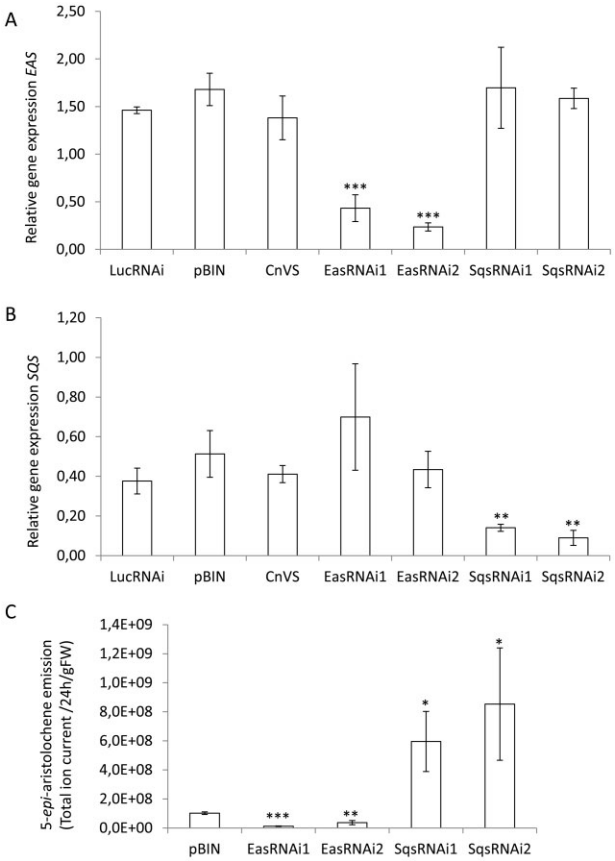

Figure 4. Gene expression of *EAS* and *SQS* and 5-*epi*-aristolochene emission in *N. benthamiana* leaves infiltrated with silencing constructs. Gene expression of (**A**) *EAS* and (**B**) *SQS* was monitored by real-time PCR. Relative gene expression compared to the actin gene is shown. The asterisks denote statistical difference between the LucRNAi infiltration and other treatments. The amount of 5-*epi*-aristolochene (as quantified by the total ion current measured on GC–MS) in the headspace of *N. benthamiana* plants infiltrated with silencing constructs EasRNAi1, EasRNAi2, SqsRNAi1, and SqsRNAi2 is shown in panel (**C**). The asterisks denote statistical difference between the empty pBIN infiltration and other treatments. For all plots, the mean ± SD for three biological replicates is shown. Two tailed Student's *t*-test was used: *$p<0.05$; **$p<0.01$; ***$p<0.001$, $n = 3$.

were cloned into the pK7GWIWG2(II) vector to create four RNAi hairpin constructs: EasRNAi1 and EasRNAi2 aimed at silencing *EAS*, and SqsRNAi1 and SqsRNAi2 aimed at silencing *SQS*. These constructs were infiltrated separately into the leaves of *N. benthamiana* and the effect on gene expression level of *SQS* and *EAS* and the 5-*epi*-aristolochene production in the headspace was studied.

The effect of silencing on the transcript level of *EAS* and *SQS* was probed by real-time PCR. Relative gene expression compared with the expression level of the actin gene is shown in Fig. 4. Upon gene silencing of *EAS* we observed 3.4-fold ($p<0.001$) and 6.2-fold ($p<0.001$) reduction in the level of *EAS* transcript for EasRNAi1 and EasRNAi2 silencing constructs compared with plants infiltrated with the LucRNAi construct, respectively (Fig. 4A). The LucRNAi silencing construct contains a fragment of the firefly luciferase gene, which should trigger the silencing mechanism without targeting an endogenous transcript. Expression of *SQS* was decreased 2.7-fold ($p<0.01$) and 4.2-fold ($p<0.01$) upon silencing with constructs SqsRNAi1 and SqsRNAi2, respectively (Fig. 4B). No significant changes in *EAS* and *SQS* gene expression were observed upon expression of *CnVS* or upon infiltration of empty pBIN vector.

The headspace of plants infiltrated with pBIN, EasRNAi1, EasRNAi2, SqsRNAi1, and SqsRNAi2 was analyzed. The effect of the infiltration of the silencing constructs on the 5-*epi*-aristolochene production is shown in Fig. 4C. Upon silencing of *EAS*, the amount of 5-*epi*-aristolochene was found to be 8.4-fold ($p<0.001$) and 2.8-fold ($p<0.01$) decreased for constructs EasRNAi1 and EasRNAi2, respectively. The extent of *EAS* silencing by the two RNAi constructs at the RNA level did therefore not correlate with their effectiveness in reducing 5-*epi*-aristolochene production. Upon expression of the hairpin constructs for the silencing of *SQS*, the 5-*epi*-aristolochene production increased by 5.8-fold ($p<0.05$) and 8.3-fold ($p<0.05$). Constructs EasRNAi1 and SqsRNAi2 were used in subsequent experiments to assess their effect on (+)-valencene production.

3.4 Simultaneous silencing of *EAS* and *SQS* increases (+)-valencene production

The EasRNAi1 and SqsRNAi2 silencing constructs were co-infiltrated in *N. benthamiana* together with *CnVS* and (+)-valencene production in the headspace was measured. Infiltration of the EasRNAi1 together with *CnVS* did not result in a significant change in (+)-valencene levels (Fig. 3B), compared with the infiltration of *CnVS* alone, although the amount of 5-*epi*-aristolochene decreased 6.4-fold ($p<0.01$). Upon co-infiltration of SqsRNAi2 and *CnVS* (+)-valencene production increased 2.8-fold ($p<0.05$), while the amount of 5-*epi*-aristolochene increased 21-fold ($p<0.05$).

Subsequently, both EasRNAi1 and SqsRNAi2 constructs were infiltrated together with *CnVS* (Fig. 3C) and (+)-valencene amounts were compared with the production of (+)-valencene upon boosting of the FPP pool via overexpression of *tHMGR* and *FPS* (Fig. 3A). The silencing approach led to a 2.7-fold increase of (+)-valencene production ($p<0.001$) and the overexpression of tHMGR and FPS resulted in a 2.9-fold increase in (+)-valencene production, therefore both approaches increased (+)-valencene production in *N. benthamiana* at comparable levels. However, expression of *CnVS*, in combination with silencing by EasRNAi1 and SqsRNAi2 results in only a 2.7-fold ($p<0.001$) increase in the amount of 5-*epi*-aristolochene in the headspace compared with infiltration of *CnVS* alone. In plants co-infiltrated with *CnVS*, *tHMGR*,

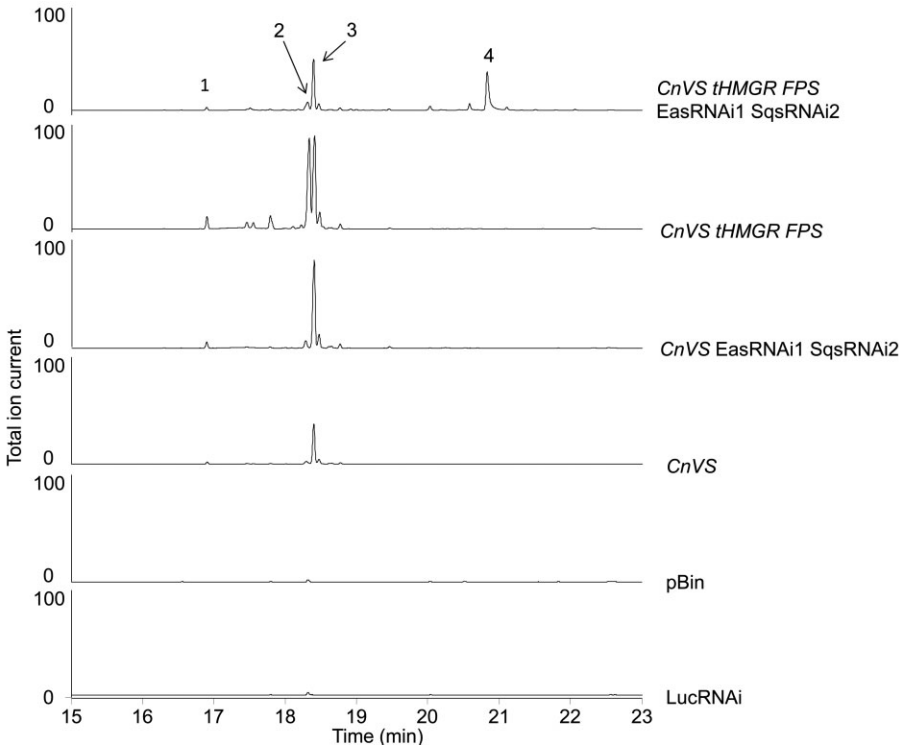

Figure 5. GC–MS chromatograms of *N. benthamiana* headspace using different engineering approaches. GC–MS total ion current chromatograms of *N. benthamiana* headspace for different construct combinations are shown. The peaks 1, 2, 3, and 4 correspond to β-elemene, 5-*epi*-aristolochene, (+)-valencene, and farnesol, respectively. All chromatograms are shown to scale (scale 100 = TIC 2.5E9).

and *FPS*, the amount of 5-*epi*-aristolochene in the headspace increased 37-fold ($p<0.0001$) compared to plants infiltrated with *CnVS* only. The preferred approach to specifically induce (+)-valencene emission, preventing induction of 5-*epi*-aristolochene, is by the silencing approach.

3.5 Combining the overexpression and silencing approaches for increased terpene production

To further increase the (+)-valencene production, both overexpression of tHMGR and FPS to increase the FPP pool and simultaneous silencing of the *SQS* and the *EAS* was used. The amount of 5-*epi*-aristolochene and (+)-valencene in these plants did not exceed the amounts obtained by the overexpression and silencing approach individually. However, a large new peak was identified at the retention time of 20.83 as farnesol (Fig. 5). No production of farnesol was observed in any of the other construct combinations.

4 Discussion

4.1 Silencing of endogenous pathways results in prevalent emission of (+)-valencene by *N. benthamiana*

In this work, the use of RNAi technology to promote sesquiterpene biosynthesis by plants is described for the first time. For producing terpenes in plants, earlier work has focused on ectopic overexpression of terpene synthases [11, 17, 29], targeting of terpene synthases to subcellular organelles where a large pool of FPP is present [3, 6, 9] and overexpression of enzymes that catalyze rate-limiting steps in the biosynthesis of FPP. In this work, we have established a *N. benthamiana* based production system for the aromatic sesquiterpene (+)-valencene. By simple ectopic expression of *CnVS* without an artificial subcellular localization signal, the amount of emitted (+)-valencene hardly exceeded that of the endogenous sesquiterpene 5-*epi*-aristolochene.

A strategy to divert carbon flux towards (+)-valencene biosynthesis was developed by repressing endogenous pathways that compete for the FPP pool present in the cytoplasm of the plant cell with the heterologously introduced enzyme. Two enzymes that tap from the pool of cytoplasmic FPP were chosen in *N. benthamiana*, one is SQS that catalyses the first dedicated step in the production of triterpenes and steroids. The other, EAS, catalyzes the formation of 5-*epi*-aristolochene, the major sesquiterpene emitted by *N. benthamiana*. Apparently, also these two endogenous enzymes are competing for the same pool of FPP, since downregulation of the *SQS* resulted in a significant increase in 5-*epi*-aristolochene emission (Fig. 4C).

The silencing strategy was deployed to enhance (+)-valencene emission. When *EAS* was silenced, together with ectopic overexpression of *CnVS*, 5-*epi*-aristolochene emission was significantly reduced, while no change in

(+)-valencene emission was observed. Apparently, FPP that becomes available by silencing of *EAS* is not at the disposal of the introduced CnVS but is likely consumed by SQS. The silencing of *SQS* resulted in an increase of (+)-valencene emission by 2.8-fold, but most FPP that became available through *SQS* silencing was used to increase 5-*epi*-aristolochene emission (Fig. 3B). Silencing of both *EAS* and *SQS* resulted eventually in a similar increase in (+)-valencene emission, but the main advantage of the silencing approach was that the emitted ratio of desired valencene to endogenous 5-*epi*-aristolochene side product was much more favorable (Fig. 3C). In plants where *CnVS* was co-infiltrated together with EasRNAi1 and SqsRNAi2 constructs, (+)-valencene was emitted as by far the most prevalent volatile. This may offer an advantage when testing the biological effect of expressing heterologous terpenes, for instance on insect repellency. For testing biological activity, it is essential that endogenous volatile components such as 5-*epi*-aristolochene are not increased as they might interfere with the interpretation of the results of the bioassays. In case of using *N. benthamiana* as a production system for terpenes biosynthesis more pure products also have the advantage of easier purification from the plants.

4.2 Farnesol as a novel metabolite in *N. benthamiana*

In follow up experiments, the gene silencing approach was combined with the overexpression of enzymes that are rate-limiting in the production of FPP. Co-infiltration of *tHMGR* and *FPS* with *CnVS* lead to an equal enhancement of (+)-valencene production compared with the gene silencing approach, while, emission of 5-*epi*-aristolochene was strongly enhanced (Fig. 5). When both approaches were combined, no further improvement in (+)-valencene production was achieved. In a yeast strain optimized for producing the sesquiterpene amorphadiene, overexpression of tHMGR improved amorphadiene production approximately fivefold and downregulation of the yeast SQS using a methionine repressible promoter led to an additional twofold increase in amorphadiene production [19]. We do not observe a comparable additive effect in plants. Surprisingly, these plants showed emission of farnesol, which can be considered another outlet for the FPP pool. Farnesol was not observed in any other of the treatments, and is not known to be a volatile emitted by *N. benthamiana*. In animal cells, farnesol could internally arise from FPP by removal of the diphosphate group through action of an FPP-specific diphosphatase [30]. In that system, the FPPase activity increased under conditions of increased metabolic flow through the isoprenoid pathway. A dose-dependent formation of farnesol was previously observed in *N. tabacum* cv bright yellow-2 cell suspensions that were treated with squalestatin-1, an inhibitor of SQS [31]. The authors propose that also in plants farnesol could be formed directly from FPP by hydrolysis catalyzed by a FPP diphosphatase, analogous to mammalian cells. Farnesol formation could be detrimental for plants, since in high concentrations it causes cell death [32]. Although the leaves of *N. benthamiana* producing farnesol did not show any necrosis, clearly emission of farnesol would not be beneficial for a production system for terpenes.

4.3 Further possibilities for improved (+)-valencene production in *N. benthamiana*

The novel formation of farnesol in the combined approach, and the failure of (+)-valencene emission in this approach to rise beyond the levels reached by either gene silencing of competing pathway genes or by de-bottlenecking FPP supply indicates that a further increase in production of (+)-valencene is no longer dependent on FPP availability. This suggests that now the activity of CnVS is limiting. Indeed, comparison of kinetic constants of CnVS to EAS from *N. tabacum* and SQS from yeast shows that CnVS is a slow enzyme. The turnover rate k_{cat} of CnVS is 0.2 min^{-1} [21], while for EAS, this value is 2.9 min^{-1} [33] and for yeast SQS is even 200 min^{-1} [34]. A possible alternative explanation could be that the protein expression level of CnVS is sub-optimal. Clearly, the formation of farnesol shows that the current strategy of overproducing sesquiterpenes is no longer limited by the FPP availability in the cytosol. Further improvements could be achieved by increasing the CnVS protein levels, or by improving its activity.

The use of viral silencing-suppressor protein p19 from the tomato bushy stunt virus is common in agroinfiltration assays since it enhances the expression of the transgenes [35]. Since p19 interferes with hairpin silencing we did not use it in our experiments. Use of V2 viral silencing-suppressor protein from *tomato yellow leaf curl virus* [36] was shown to support transgene overexpression and the same time allow hairpin RNA-based silencing of endogenes [14], and could therefore be used in the future to further improve production of (+)-valencene in *N. benthamiana*.

4.4 Outlook on plant platforms for terpene production

Transient silencing of *SQS* and *EAS* did not cause a visual phenotype in the leaves of *N. benthamiana*. To apply the same strategy to stable plant transformation, several options can be envisioned for controlled silencing of *SQS* and *EAS*, for example by using promoters that are tissue specific or activated in later stages of development. Alternatively, an inducible promoter could be used for a metabolic switch to production of heterologous terpenes. The transient assays however provide us with a rapid system of testing several gene combinations and can be used to find the most efficient gene combinations and to define metabolic bottlenecks.

The production of sequiterpenes in microbial platforms has been strongly developed in recent years [19, 37, 38]. The improvements were based on discovery and transfer of plant terpene biosynthetic pathways to microbes, and also by using extensive metabolic engineering and synthetic biology approaches to modify the microbial metabolism for optimal carbon channeling into the terpene pathways. Besides the production of plant terpenes in microbes, engineering of heterologous terpene biosynthesis in plants remains interesting. The production of heterologous terpenes can enhance plant protection mechanisms [3, 4], but plants can also be used to produce high value terpenoids, such as artemisinin [6, 8]. Plant production systems have several advantages compared with the microbial systems, such as internal membrane systems, which enable efficient expression of membrane-anchored proteins, cell compartmentalization, which is essential for the biosynthesis of complex isoprenoid-derived phytochemicals such as indol alkaloids [39] and availability of specialized storage organs for terpenoids such as lacticifers and trichomes. At the same time, expensive fermentation setups are not required. However, the availability of metabolic engineering tools is needed to promote heterologous production of terpenes in plants. With the increasing availability of plant genome sequences the endogenous metabolism of the host plant can be manipulated, which adds an additional valuable tool for metabolic engineering of plants.

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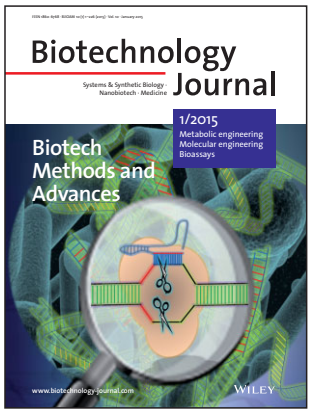

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