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Engineering production of a novel diterpene synthase precursor in *Nicotiana benthamiana*.

Edith Forestier¹, Geoffrey D. Brown², David Harvey¹, Tony Larson¹, Ian A. Graham^{1*}

¹University of York, United Kingdom, ²University of Reading, United Kingdom

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Author contribution statement

EF designed experiments, performed experiments and analyzed data. GB, DH and TL performed experiments, analyzed data and contributed to the writing of the manuscript. EF and IG wrote the manuscript. IG contributed to the design and analysis of the study. All authors read and approved the manuscript.

Keywords

Plant diterpenes, *Nicotiana benthamiana*, GGPP, hydroxy-GGPP, MEP pathway, Metabolic Engineering

Abstract

Word count: 198

Diterpene biosynthesis commonly originates with the methylerythritol phosphate (MEP) pathway in chloroplasts, leading to the C20 substrate, geranylgeranyl pyrophosphate (GGPP).

Previous work demonstrated that over-expression of genes responsible for the first and last steps in the MEP pathway in combination with GERANYLGERANYL PYROPHOSPHATE SYNTHASE (GGPPS) and CASBENE SYNTHASE (CAS) are optimal for increasing flux through to casbene in *Nicotiana benthamiana*. When the gene responsible for the last step in the MEP pathway, 4-HYDROXY-3-METHYLBUT-2-ENYL DIPHOSPHATE REDUCTASE (HDR), is removed from this combination, casbene is still produced but at lower amounts. Here we report the unexpected finding that this reduced gene combination also results in the production of 16-hydroxy-casbene (16-OH-casbene), consistent with the presence of 16-hydroxy-geranylgeraniol phosphate (16-OH-GGPP) in the same material. Indirect evidence suggests the latter is formed as a result of elevated levels of HMBPP caused by a bottleneck at the HDR step responsible for conversion of HMBPP to DMAPP. Overexpression of geranylgeranyl synthase from *Nicotiana attenuata* produces 16-hydroxy-geranylgeranyl (16-OH-geranylgeranyl) when transiently expressed with the same reduced combination of MEP pathway genes in *N. benthamiana*.

This work highlights the importance of pathway flux control in metabolic pathway engineering and the possibility of increasing terpene diversity through synthetic biology.

Contribution to the field

Dear Editor, Many diterpenoids from plants have been shown to be bioactive with a number having pharmacological activities and hence there is much interesting in engineering their production in new host platforms. GGPP is recognised as the universal substrate for diterpenoid biosynthesis in plants. In the current manuscript we report that modifying flux through the MEP pathway can result in the production of a completely novel substrate, 16-OH-GGPP, for diterpenoid production. We present evidence that 16-OH-GGPP is formed due to a bottleneck in the MEP pathway resulting in accumulation of HMBPP and it is this pathway intermediate that results in OH-GGPP. We also show that OH-GGPP is converted by two different diterpene synthases to form novel hydroxylated diterpenes. To our knowledge, this is the first time that such a substrate for diterpene synthesis has been described and the report highlights the potential to introduce novel diversity into whole classes of diterpenoids by capitalising on the substrate promiscuity of the synthase enzymes. We expect this paper will be of broad general interest to readers of *Frontiers in Plant Science* and of particular interest to those working on engineering biology. It combines metabolic engineering and rigorous biochemical analysis and highlights the potential to increase structural diversity across different classes of diterpenoids by providing altered substrate for the first committed synthase step. We trust you will find our work suitable for publication in *Frontiers in Plant Science* and we look forward to the peer review process. Yours sincerely Ian Graham

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Studies involving human subjects

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Inclusion of identifiable human data

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In review

Data availability statement

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In review

Engineering production of a novel diterpene synthase precursor in *Nicotiana benthamiana*.

Authors: Edith C. F. Forestier¹, Geoffrey D. Brown², David Harvey¹, Tony Larson¹ and Ian A. Graham^{1*}.

1 Centre for Novel Agricultural Products, Department of Biology, University of York, Heslington, York, YO10 5DD (UK).

2 Department of Chemistry, University of Reading, Whiteknights, Reading, RG6 6AD (UK)

*Corresponding author (ian.graham@york.ac.uk)

Keywords: plant diterpenes, *Nicotiana benthamiana*, GGPP, hydroxy-GGPP, metabolic engineering.

Abstract

Diterpene biosynthesis commonly originates with the methylerythritol phosphate (MEP) pathway in chloroplasts, leading to the C₂₀ substrate, geranylgeranyl pyrophosphate (GGPP). Previous work demonstrated that over-expression of genes responsible for the first and last steps in the MEP pathway in combination with *GERANYLGERANYL PYROPHOSPHATE SYNTHASE* (*GGPPS*) and *CASBENE SYNTHASE* (*CAS*) are optimal for increasing flux through to casbene in *Nicotiana benthamiana*. When the gene responsible for the last step in the MEP pathway, *4-HYDROXY-3-METHYLBUT-2-ENYL DIPHOSPHATE REDUCTASE* (*HDR*), is removed from this combination, casbene is still produced but at lower amounts. Here we report the unexpected finding that this reduced gene combination also results in the production of 16-hydroxy-casbene (16-OH-casbene), consistent with the presence of 16-hydroxy-geranylgeranyl phosphate (16-OH-GGPP) in the same material. Indirect evidence

suggests the latter is formed as a result of elevated levels of 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) caused by a bottleneck at the HDR step responsible for conversion of HMBPP to dimethylallyl pyrophosphate (DMAPP). Overexpression of a *GERANYLLINALOOL SYNTHASE* from *Nicotiana attenuata* (*NaGLS*) produces 16-hydroxy-geranyllinalool (16-OH-geranyllinalool) when transiently expressed with the same reduced combination of MEP pathway genes in *N. benthamiana*.

This work highlights the importance of pathway flux control in metabolic pathway engineering and the possibility of increasing terpene diversity through synthetic biology.

Introduction

Many of the gem-dimethylcyclopropyl class of bioactive casbene-derived diterpenoids from plants have been shown to have pharmacological activities. Low abundance in their natural host (Hohmann et al., 2000; Johnson et al., 2008) along with their structural complexity (Jorgensen et al., 2013; Kawamura et al., 2016; Hashimoto et al., 2017) has led to efforts to engineer alternative microbial (Hill et al., 1996; Wong et al., 2018; Callari et al., 2018), algal (Mehrshahi *et al.*, 2020) or plant-based production platforms (Forestier et al., 2021). The majority of diterpenes in plants are biosynthesized in chloroplasts (Rohmer et al., 1993; McGarvey and Croteau, 1995; Lichtenthaler et al., 1997) from the five-carbon building blocks, isopentyl pyrophosphate (IPP) and dimethylallyl phosphate (DMAPP), provided by the MEP pathway and combined together in a three-to-one ratio to form the C₂₀ precursor, GGPP (Gershenzon and Croteau, 1993).

We recently developed a *Nicotiana benthamiana* platform optimised for production of casbene and derivatives by engineering flux through the MEP pathway (Forestier et al., 2021). We demonstrated how this platform could be used for production of the lathyrane jolkinol C by introduction of functionally characterised P450 oxidases (Forestier et al., 2021). The

elucidation of the biosynthetic steps from casbene to the tigliane (Kulkosky et al., 2001; Kissin and Szallasi, 2011; De Ridder et al., 2021), ingenane (Siller et al., 2010) and jatrophane (Corea et al., 2009; Hadi et al., 2013) classes of diterpenoids have also been investigated but remain to be resolved.

In our work on *N. benthamiana* to optimise flux through the MEP pathway to GGPP – the substrate for casbene production – we unexpectedly detected the novel compound, 16-hydroxy-casbene. The design of our experiments suggested that this metabolite did not arise from a hydroxylation downstream of GGPP and we therefore hypothesized that 16-hydroxy-casbene could derive from an alternative substrate. Herein we present results leading us to conclude that 16-hydroxy-GGPP can act as a novel precursor for diterpene biosynthesis.

Materials and methods

Transient expression of genes in *Nicotiana benthamiana*.

Arabidopsis thaliana cDNAs of *1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE (DXS, AT4G15560.1)*, *4-HYDROXY-3-METHYLBUT-2-ENYL DIPHOSPHATE REDUCTASE (HDR, AT4G34350.1, Phillips et al., 2008)* and *GERANYLGERANYL PYROPHOSPHATE SYNTHASE 11 (GGPPS11, AT4G36810.1, Beck et al., 2013)* coding for plastidial enzymes referenced in TAIR (The Arabidopsis Information Resource) and cDNA of *CASBENE SYNTHASE (CAS)* from *Jatropha curcas* (King et al., 2014) were cloned into pEAQ-HT (Sainsbury et al., 2009) vector as described in Forestier *et al.*, 2021. A codon optimized cDNA of *NaGLS* based on the sequence accession number XM_019410085 was synthesized by gBlock IDT with extensions allowing to clone it directly with In-Fusion® in AgeI/StuI linearized pEAQ-HT vector. Transient expression in wild-type *Nicotiana benthamiana* was performed as described in Forestier *et al.*, 2021.

Isolation and quantification of diterpenoids, GGPP and OH-GGPP

We detected both casbene and 16-OH-casbene in transiently expressed plants by extracting around 200 mg of dry material with 5 mL of hexane containing 100 µg/mL of β-caryophyllene, then sonicating for 15 min. We quantified the compounds by GC-MS as detailed in Forestier *et al.* For geranylinalool and its derivatives, we extracted around 150 mg of dry weight (DW) of infiltrated tobacco with 1 mL of ethyl acetate containing 100 mg/L of β-caryophyllene. The samples were shaken overnight at 2000 rpm on a IKA Vibrax VXR basic shaker, then centrifuged and 100 µL of the supernatant was used directly for GC-MS.

We isolated GGPP and 16-OH-GGPP by adapting the protocol described by Nagel *et al.* (Nagel *et al.*, 2014). Approximately 750 mg of ground dry material was extracted with 15 mL of methanol/H₂O (7:3, v/v) and sonicated for 30 minutes. We then added 5 mL of water to the mixture, centrifuged for 3 min at 2000 g and filtered through Whatman filter paper grade 1 and cotton. The cleared extracts were passed through Chromabond HX RA columns, pre-conditioned with 5 mL of methanol and 5 mL of water, and compounds were eluted with 3 mL of ammonium formate 1M in methanol. Each eluate was dried under a stream of nitrogen and re-dissolved in 250 µL of water/methanol (1:1). We transferred 100 µL into glass HPLC vials and 2 µL aliquots were analysed by LC-MS as described by Catania *et al.* (Catania *et al.*, 2018). Additional high-resolution mass spectral data were obtained on a parallel LC interfaced to a Thermo Orbitrap Fusion mass spectrometer, operating in –ESI mode at 500000 (FWHM) resolution for MS1 data, with MS2 data collected at 120000 resolution using stepped collision energies between 20-60 units in both HCD and CID modes.

Accumulation and purification of compounds for NMR spectroscopy.

To identify 16-OH-casbene, we ground 4.9 g of dry material obtained from 10 full-grown plants infiltrated with *DXS*, *GGPPS* and *CAS*, and extracted with 100 mL of hexane. After 1h

of sonication and 2 days shaking, the extract was centrifuged for 3 min at 2000 g, filtered through Whatman paper grade 1 and cotton, and evaporated to obtain 350 mg of oily residue. The residue was resuspended in 10 mL of hexane/ethyl acetate (70:30, v/v) and purified through a 40 g Bucchi silica column on a PuriFlash® 4250 system (Interchim). We used the same method of flash chromatography as described in King *et al.* 2014 (King et al., 2014) to fractionate the extract into 80 samples. GC-MS was used to identify the fraction containing our compound of interest, and 2.6 mg of this was obtained after evaporation, at sufficient purity for direct ¹H NMR analysis on a Bruker AVIII 700 MHz instrument equipped with a cryoprobe. For 16-OH-geranylinalool, we infiltrated 40 young plants with *DXS*, *GGPPS* and *NaGLS*, which provided 10 g of dry material after freeze-drying and grinding. We extracted this with 150 mL of ethyl acetate and left to shake for 5 days on a rotary shaker. After centrifugation and filtration as detailed above, we reduced the volume down to 1 mL before re-suspending in 9 mL of hexane/ethyl acetate and purifying with the same column and method as described above. The fractions of interest were combined and dried to obtain 16.4 mg of extract that was further purified with a reverse phase column (C18-HQ 5 µm 250x10 mm (Interchim)) to remove the pigment content. The reverse phase column was first equilibrated with solvent A – mix of water/acetonitrile (95:5, v/v) – for 8 column volumes (CV), before injecting the extract, diluted in 2.5 mL of the same solvent, into a 5 mL injection loop. The separation method consisted of 1 CV of solvent A, followed by a gradient of 9 CV, to reach 100% acetonitrile (solvent B). This solvent was maintained for a further 10 CV, and the entire run was carried out at a flow rate of 3 mL/min. We used an in-line connected Advion Expression compact mass spectrometer (CMS), which enabled product isolation guided by mass spectra. For the samples used in Figures 4 and S3, we additionally used UPLC-MS (**Figure S6**) to evaluate compounds ions. Fragmentation of 16-OH-geranylinalool (Compound 2 in Figure S6) showed two main ions at m/z 271 and m/z 289 (**Figure S6H**), which we used to select our compound of interest

on the Puriflash-CMS. We collected one fraction, which after evaporation contained 4.6 mg of sufficiently pure metabolite for NMR identification.

Results

Transient expression of *1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE (DXS)* with *GERANYLGERANYL PYROPHOSPHATE SYNTHASE (GGPPS)* and *CASBENE SYNTHASE (CAS)* in *Nicotiana benthamiana* can produce metabolites in addition to casbene.

In previous work, we tested the transient co-expression of different MEP pathway genes and *GGPPS* from *Arabidopsis thaliana* with *CAS* from *Jatropha curcas* to evaluate the best combination for the highest production of casbene. We determined that the combination of *DXS* (catalysing the first step in the MEP pathway), *HDR* (4-hydroxy-3-methylbut-2-enyl diphosphate reductase, catalysing the last step), *GGPPS* and *CAS* resulted in an up to 5-fold increase in casbene production, compared to *CAS* expression alone (Forestier et al., 2021). Omitting *HDR* from this combination resulted in lower production of casbene (Forestier et al., 2021). Further inspection of the total ion chromatograms of plant extracts from this reduced gene combination identified three additional peaks (**Figure 1A**), compared to co-expression of *DXS*, *HDR*, *GGPPS* and *CAS* which only produced casbene (**Figure 1B**).

The largest of the three additional peaks was present in sufficient amount to allow its identification as 16-hydroxy-casbene (16-OH-casbene) by NMR spectroscopy (**Figure S1**). 16-OH-casbene was present at approximately 30% of casbene levels when *HDR* was absent from the gene combination (**Figure S2**).

The fact that 16-OH-casbene is only produced when *HDR* is omitted from the gene combination used to increase flux into the C₂₀ GGPP precursor for casbene production, led us

to investigate the possibility that the hydroxyl group at the 16-position of the precursor is also hydroxylated. GGPP is formed by head-to-tail condensations between one molecule of DMAPP and three molecules of IPP (Ogura and Koyama, 1998), with the chain-starter DMAPP ending up distal to the pyrophosphate group (**Figure 2A**). In the MEP pathway, the immediate precursor to DMAPP is (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) (**Figure 1C**), which has the same structure as DMAPP, but with a hydroxyl functionality at position 4 (**Figures 2A and 2B**). This hydroxyl group would appear at the 16-position of GGPP after chain-extension by IPP if HMBPP is accepted by GGPPS as an alternative chain-starter to DMAPP (**Figure 2B**). We considered therefore that 16-OH-GGPP could be formed if there were an excess of HMBPP, caused by the over-expression of *DXS* and *GGPPS* with insufficient conversion to DMAPP and/or IPP due to this being dependent on an endogenous *HDR*. 16-OH-GGPP could then be further incorporated into the casbene backbone assuming that *CAS* accepts 16-OH-GGPP as substrate for production of 16-OH-casbene (**Figure 2B**).

Detection of GGPP and putative 16-OH-GGPP *in planta* by transient over-expression of *DXS* + *GGPPS* and *DXS* + *HDR* + *GGPPS*

To establish if 16-OH-GGPP accumulates depending on the gene combination, either *DXS* + *GGPPS* or *DXS* + *HDR* + *GGPPS* were transiently expressed in *N. benthamiana* and C₂₀ prenyl diphosphate intermediates were extracted as described by Nagel et al., 2014. UPLC-MS/MS negative mode analysis of methanolic extracts was used to detect GGPP by selecting the m/z range 449-450 and by comparison with an authentic GGPP standard (**Figure 3A-C**). In the absence of a 16-OH-GGPP standard, we predicted that since m/z 449.2 represents the [M-H]⁻ ion for GGPP, hydroxylated forms should be detectable at an added mass of 16; *i.e* at 465.2. Hydroxylated forms of prenyl diphosphates would be more hydrophilic and therefore elute at

an earlier retention time compared to GGPP in reverse phase chromatographic separation. A clear peak at m/z 465.2, was detected at the earlier retention time of 2.5 min, in the extract of material over-expressing *DXS + GGPPS* (**Figure 3D**), consistent with 16-OH-GGPP. High-resolution mass spectrometry analysis revealed a m/z of 449.1869 for GGPP ($[M-H]^-$ theoretical 449.1864; error 1.11 ppm) and m/z of 465.1819 for 16-OH-GGPP ($[M-H]^-$ theoretical 465.1813; error 1.29 ppm). Both peaks generated a common 158.9252 m/z MS₂ fragment, identified as the diagnostic pyrophosphate group ion, $[P_2O_6H]^-$). We were unable to detect the m/z 465.2 peak in the gene combination of *DXS + HDR + GGPPS* (**Figure 3E**), consistent with the hypothesis that OH-GGPP is produced when HMBPP reduction is limiting due to lack of HDR activity.

Transient expression in *N. benthamiana* of a *GERANYLLINALOOL SYNTHASE* from *Nicotiana attenuata* (*NaGLS*) results in production of both geranylinalool and 16-hydroxy-geranylinalool when co-expressed with *DXS + GGPPS* but only geranylinalool when *HDR* is included in the gene combination.

To further explore if 16-OH-GGPP could be used by other diterpene synthases we transiently expressed the *Nicotiana attenuata* geranylinalool synthase (*NaGLS*) in *N. benthamiana*, alone or in combination with *DXS + HDR + GGPPS* (**Figure 4 and S3**). This resulted in accumulation of geranylinalool in both cases. Co-expression of *NaGLS + DXS + GGPPS* produced geranylinalool but also two additional peaks with retention times (R_t) of 30.0 min and 31.6 min (**Figure 4A and S3E**). We used flash chromatography to purify the compound giving rise to the larger peak at R_t 30.0 min in sufficient quantities to permit its identification by NMR spectroscopy as 16-OH-geranylinalool (**Figure S4**). This novel natural product represented up

to 25% of the geranylinalool peak (**Figure 4C**). This is comparable amount of 16-OH-casbene to casbene (30%) with the same reduced gene combination (**Figure S2**).

Despite obvious parallels with the regio-isomeric 17-OH-geranylinalool (**Figure S5**), the precursor of the insecticidal diterpene glycosides in many *Nicotiana* species (Snook et al., 1997; Jassbi et al., 2010; Falara et al., 2014), there is no indication that 16-OH-geranylinalool could be involved in the biosynthetic pathway to 17-OH-diterpenes. A recent work actually demonstrated that two cytochrome P450s from *N. attenuata* are responsible for the 17-hydroxylation of geranylinalool (Li et al., 2021).

We did not detect any other hydroxy geranylinalool compounds apart from 16-OH-geranylinalool with the *DXS* + *GGPPS* + *NaGLS* gene combination, providing further evidence that 16-OH-geranylinalool is derived from a direct conversion of 16-OH-GGPP.

Discussion

This work provides evidence for the formation of 16-OH-GGPP when the flux through the MEP pathway in *N. benthamiana* is altered. Both casbene synthase from *Jatropha curcas* and geranylinalool synthase from *Nicotiana attenuata* result in production of 16-hydroxylated versions of their usual diterpene products when transiently expressed in *N. benthamiana* producing 16-OH-GGPP. The detection of additional minor compounds from both enzymes suggests that additional products may also arise when 16-OH-GGPP is used as substrate. We hypothesize that 16-OH-GGPP is formed through the action of *A. thaliana* GGPP synthase when HMBPP levels are elevated due to increased flux through the MEP pathway and a bottleneck exists at the HDR step. When the HDR enzyme, which reduces the hydroxy group in HMBPP to make DMAPP, is co-expressed with *DXS* and *GGPPS*, neither 16-OH-GGPP nor 16-OH-diterpenes are detected. Taken together the evidence presented supports formation of 16-OH-casbene or 16-OH-geranylinalool by promiscuous diterpene synthases acting on 16-

OH-GGPP rather than P450 based hydroxylation of casbene or geranylinalool. The fact that formation of these 16-hydroxylated compounds is exclusively associated with overexpression of the first step in the MEP pathway combined with omission of the last step points to the bottleneck at the HDR step giving rise to 16-OH-GGPP via a plausible route. Interestingly, in *E. coli*, overproduction of HMBPP is cytotoxic and removal of this effect is achieved by activation of *IspG*, the gene encoding the *HDR* equivalent in plants (Li et al., 2017). The transient expression approach we use *in-planta* may have by-passed such regulation if indeed it is important in *N. benthamiana*.

There are precedents from the terpenoid literature for the formation of more highly oxidized precursors, which are then accepted as alternatives to the normal substrate in a known biosynthetic pathway. Thus, 2,3-oxidosqualene, the usual precursor of triterpenes and sterols, can undergo a second oxidization by the endogenous squalene epoxidase to form dioxidosqualene, when it accumulates in yeast (Salmon et al., 2016). Research has shown that a mutated triterpene synthase actually prefers this double-oxygenated substrate to the normal 2,3-oxidosqualene, leading to the production of unusual triterpenes that incorporate an additional oxygen atom in the fifth ring (Salmon et al., 2016). There are also examples of synthetic chemistry work focusing on obtaining analogues of the sesquiterpene precursor farnesyl pyrophosphate (Dolence and Dale Poulter, 1996; Placzek and Gibbs, 2011) or even (Z,E,E)-geranylgeranyl pyrophosphate (Minutolo et al., 2006), demonstrating the interest of alternative substrates for terpenoid production.

It is perhaps unlikely that 16-OH-GGPP is a significant substrate in nature when the MEP and diterpene biosynthetic pathways are subject to their normal mechanisms of regulation. However, the substantial level of 16-hydroxylated diterpenes with native diterpenes synthases in transient expression systems might suggest that 16-OH-diterpenes can become more biologically relevant under abnormal circumstances, when such regulation is compromised.

In terms of engineering biology this work demonstrates the importance of regulating flux through biosynthetic pathways to ensure intermediates do not accumulate as the promiscuity of substrate specificity can result in the production of unexpected end products. On the other hand, this example shows that the generation of a novel GGPP substrate can open the possibility of entirely new diterpenes that could be further modified and evaluated in terms of their bioactivity.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contribution

EF designed experiments, performed experiments and analyzed data. GB, DH and TL performed experiments, analyzed data and contributed to the writing of the manuscript. EF and IG wrote the manuscript. IG contributed to the design and analysis of the study. All authors read and approved the manuscript.

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Figures legends

Figure 1 GC-MS total ion chromatograms (TIC) showing the diterpene profile of *N. benthamiana* infiltrated with casbene synthase (CAS) and different precursor genes. (A) Co-expression of *DXS* + *GGPPS* + *CAS*. (B) Co-expression of *DXS* + *HDR* + *GGPPS* + *CAS*. (C) Simplified diagram of the MEP pathway plus geranylgeranyl pyrophosphate synthase (*GGPPS*). MEP pathway enzymes are *DXS* (1-deoxy-D-xylulose 5-phosphate synthase), *DXR* (1-deoxy-D-xylulose 5-phosphate reductoisomerase), *MCT* (2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase), *CMK* (4-(cytidine 5'diphospho)-2-C-methyl-D-erythritol kinase), *MDS* (2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase), *HDS* (4-hydroxy-3-methylbut-2-enyl-diphosphate synthase), *HDR* (4-hydroxy-3-methylbut-2-enyl diphosphate reductase) and *IPPI* (isopentenyl diphosphate Δ -isomerase). 1, casbene; 2, 16-hydroxy-casbene; 3 and 4, undetermined compounds.

Figure 2 Theoretical formation of GGPP/casbene and OH-GGPP/16-OH-casbene. (A) Condensation of prenyl diphosphates DMAPP and IPP to form GGPP and cyclisation by casbene synthase to form casbene. (B) Condensation of HMBPP and IPP to form OH-GGPP and cyclisation by casbene synthase to form 16-OH-casbene.

Figure 3 UPLC-MS chromatograms showing the prenyl diphosphate profile of *N. benthamiana* infiltrated with different diterpene precursor genes. (A) Authentic standard of GGPP, m/z 449-450. (B) Co-expression of *DXS* + *GGPPS*, m/z 449-450. (C) Co-expression of *DXS* + *HDR* + *GGPPS*, m/z 449-450. (D) Co-expression of *DXS* + *GGPPS*, m/z 465-466. (E) Co-expression of *DXS* + *HDR* + *GGPPS*, m/z 465-466. (F) Mass spectrum of GGPP (1) and putative OH-GGPP (2).

Figure 4 *NaGLS* produces both geranylinalool and 16-hydroxy-geranylinalool in *N. benthamiana* (A) GC-MS TIC of extract from *N. benthamiana* infiltrated with *DXS* + *GGPPS* + *NaGLS*. (B) Extract from material infiltrated with *DXS* + *HDR* + *GGPPS* + *NaGLS*. (C) Quantities of compounds 1, 2 and 3 in extracts from the different gene combinations (ng/mg DW \pm standard deviation, n = 5). Symbols show significant differences between treatment means ($P < 0.05$, F-test and T-test). 1, geranylinalool; 2, 16-hydroxy-geranylinalool; 3, undetermined.

Figure 1.TIFF

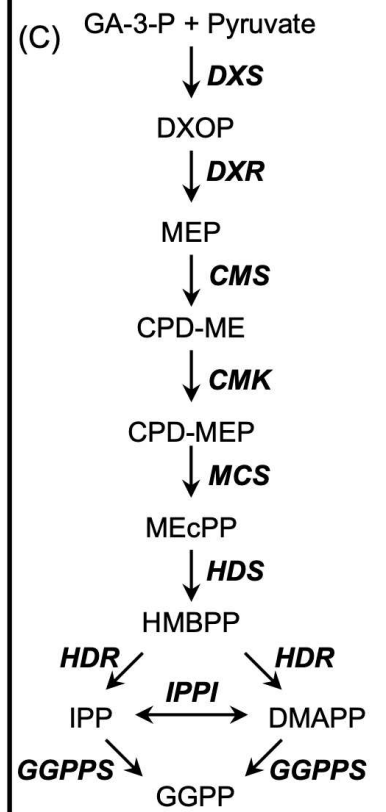
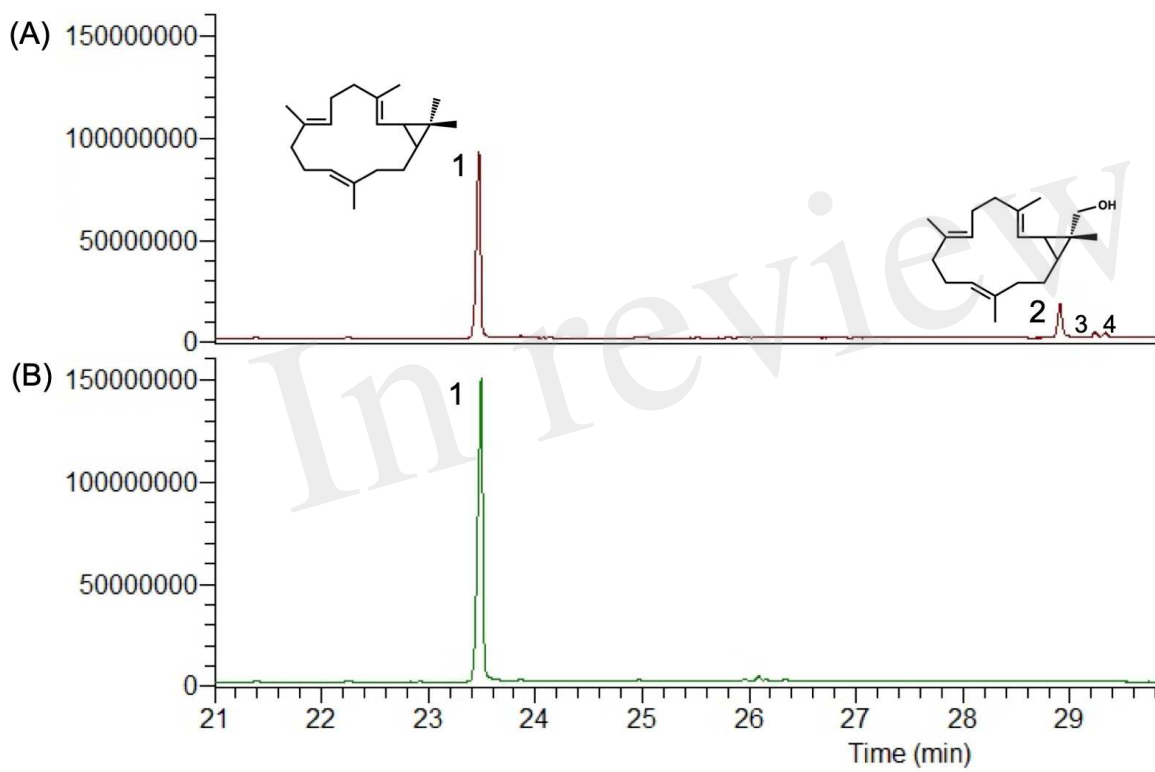


Figure 2.TIFF

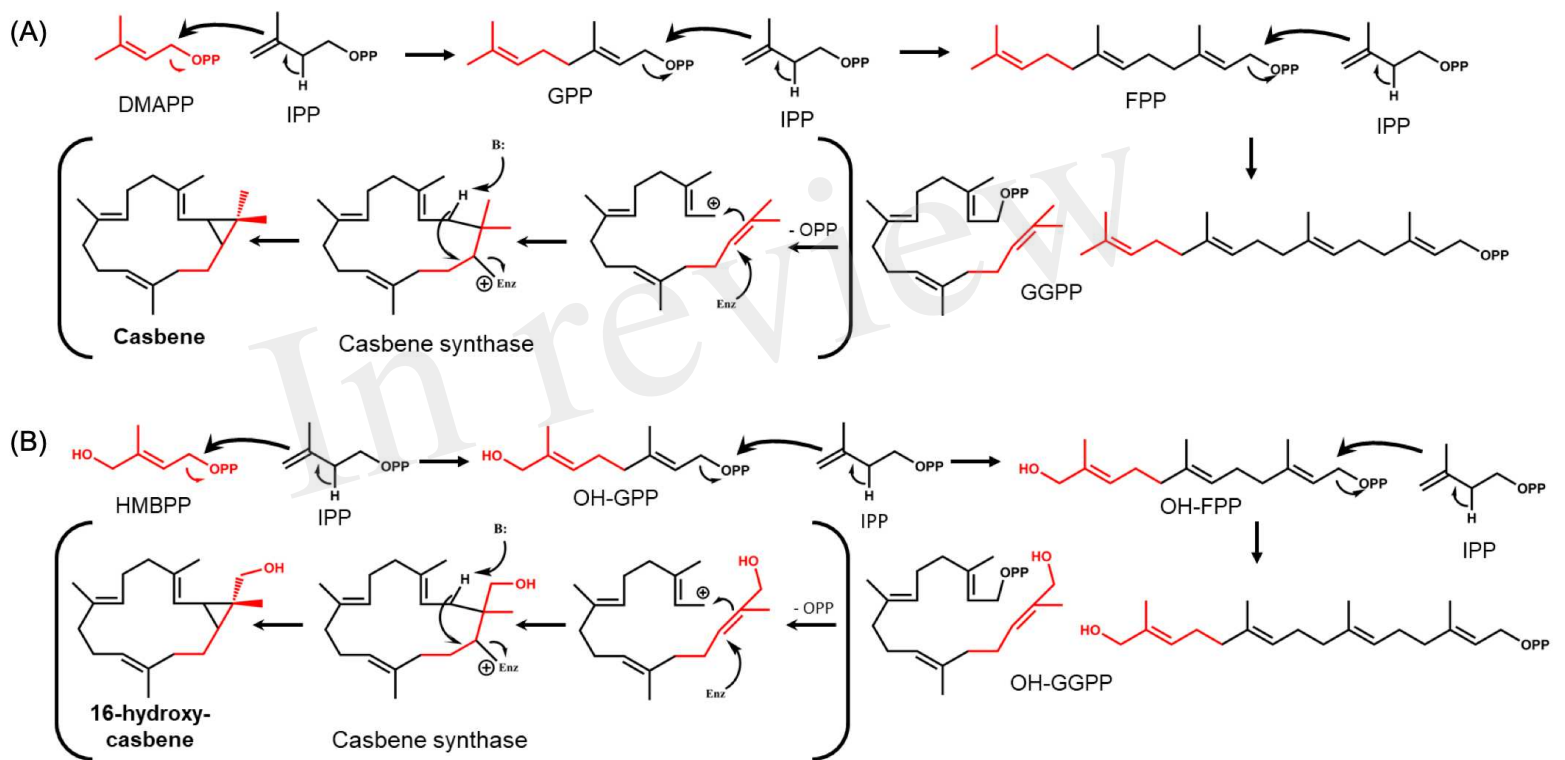


Figure 3.TIFF

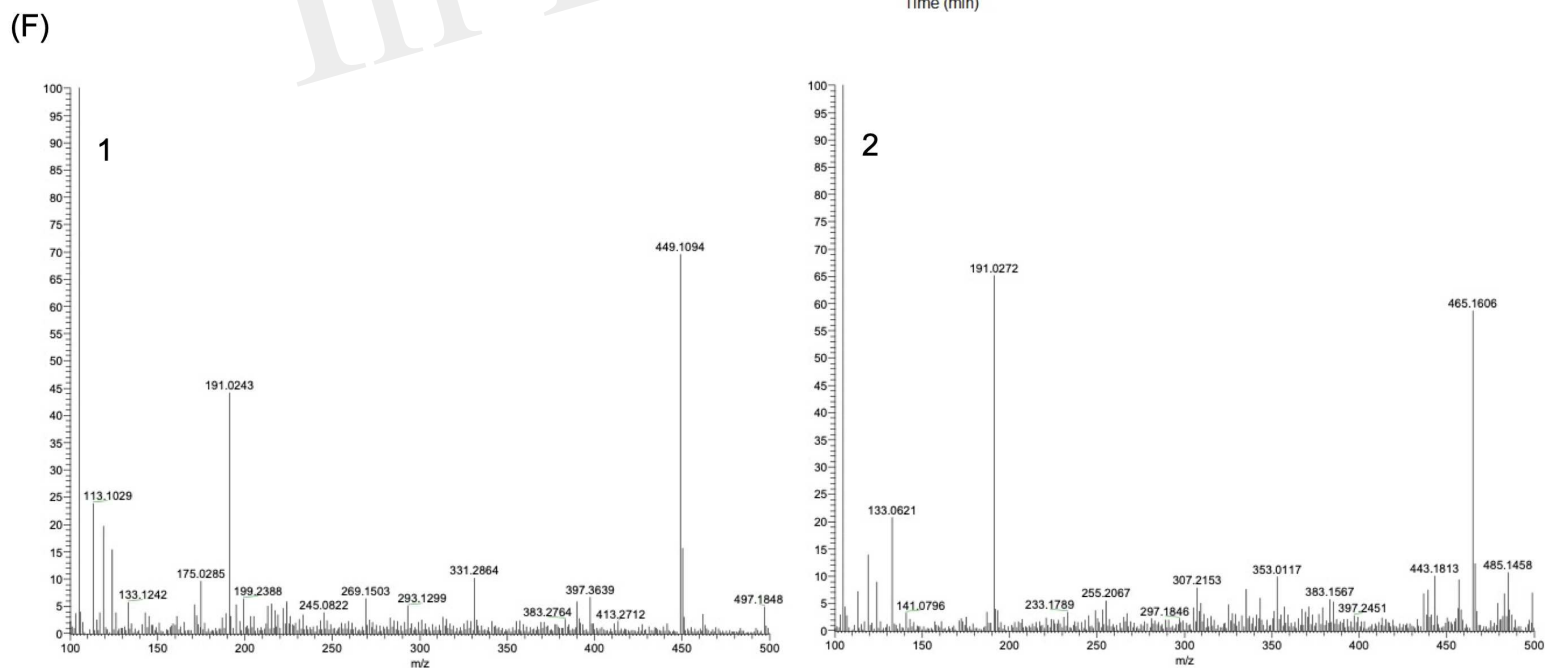
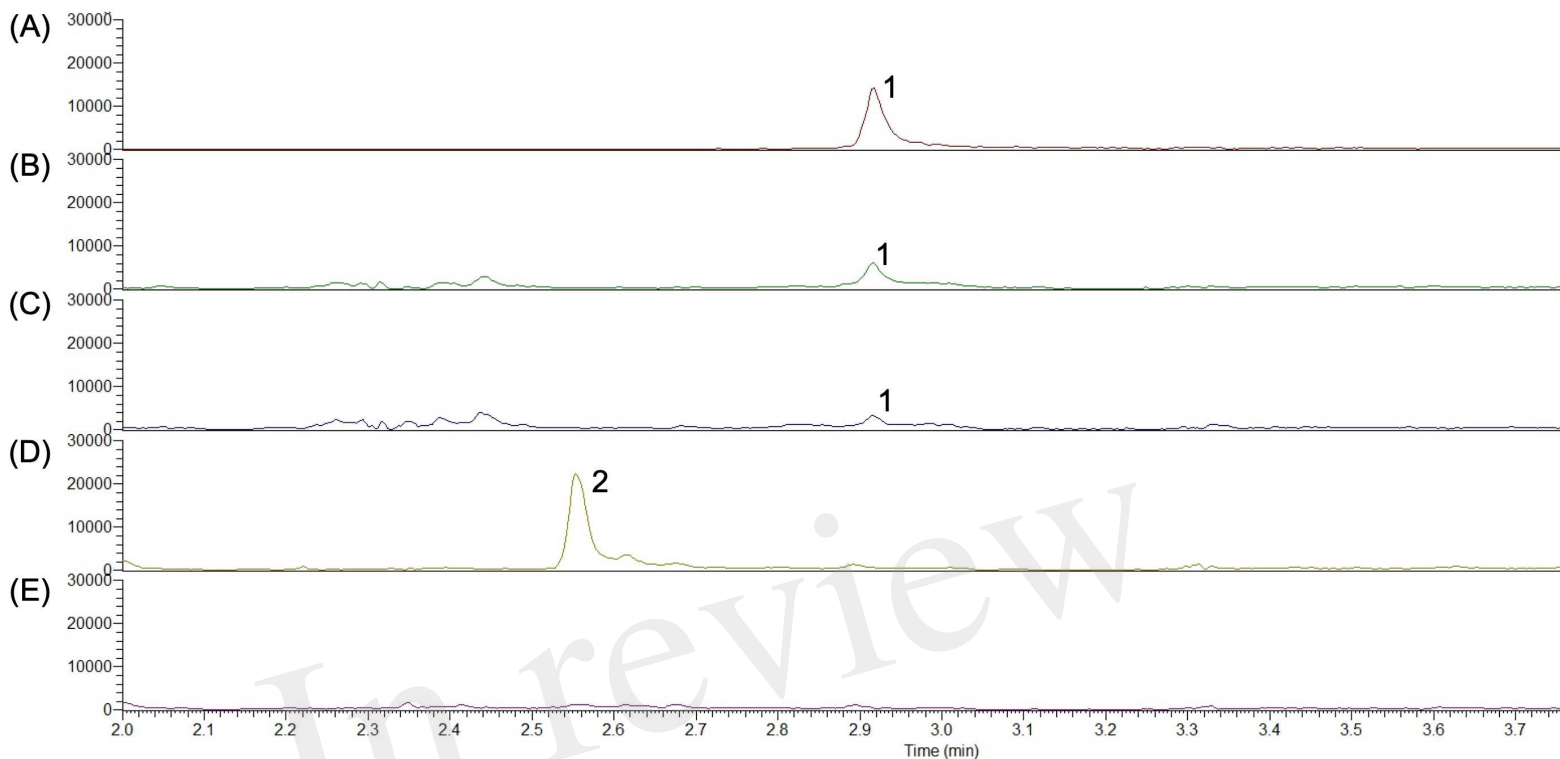


Figure 4.TIFF

