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Developing a *Nicotiana benthamiana* transgenic platform for high-value diterpene production and candidate gene evaluation

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Introduction

Plant diterpenes containing a gem-dimethylcyclopropane subunit, mostly found in species of the *Euphorbiaceae*, are of much interest across various industrial sectors including pharmaceuticals due to their bioactivity and structural complexity (Durán-Peña et al., 2014). However, the low abundance in the natural host and difficulties in chemical synthesis owing to high structural complexity often limit the development of industrial applications for these compounds (Andersen-Ranberg et al., 2016). Ingénol mebutate from *Euphorbia peplus* and tigilanol tiglate from *Euphorbia peplus* are examples of casbene-derived gem-dimethylcyclopropane diterpenes that exemplify the fact that even when these compounds are developed as products, the supply chain remains challenging for industry. Ingénol mebutate is a licensed treatment for actinic keratosis (Picato | 44(0)1904328505; fax: +44(0)1904328505; email ian.graham@york.ac.uk) due to its bioactivity and structural complexity (Durán-Peña et al., 2014). However, the low abundance in the natural host and difficulties in chemical synthesis owing to high structural complexity often limit the development of industrial applications for these compounds (Andersen-Ranberg et al., 2016). Developing new sustainable production platforms for high value diterpenoids would improve the supply chains of existing diterpene-derived drugs and provide the confidence needed to exploit the huge potential that this class of compounds has to offer.

*Nicotiana benthamiana* represents a well-established heterologous expression system to address this issue. Transient foreign gene expression mediated by *Agrobacterium tumefaciens* infiltration using either syringe or vacuum infiltration (Kapila et al., 1997; Reed et al., 2017) results in production of recombinant proteins or metabolites (Hasan et al., 2014; McCormick et al., 1999; Whaley et al., 2011). Companies such as Leaf Expression System (Norwich, UK) or Kentucky BioProcessing Inc (Owensboro, KY, USA) have scaled-up this *N. benthamiana* platform for production of antibodies, antigens and enzymes ([https://kentuckybioprocessing.com](https://kentuckybioprocessing.com), [https://www.leafexpressionsystems.com](https://www.leafexpressionsystems.com)). Transient gene expression in *N. benthamiana* is a routine and valuable tool for functional characterization of genes involved in plant metabolism including diterpenoids (Andersen-Ranberg et al., 2010) but obtained solely from the extraction from seeds of *Fontainea picrosperma*, a sub-canopy tree from a restricted area of Queensland rainforest (Lamont et al., 2016). Developing new sustainable production platforms for high value diterpenoids would improve the supply chains of existing diterpene-derived drugs and provide the confidence needed to exploit the huge potential that this class of compounds has to offer.

Summary

To engineer *Nicotiana benthamiana* to produce novel diterpenoids, we first aimed to increase production of the diterpenoid precursor geranylgeranyl pyrophosphate (GGPP) by up-regulation of key genes of the non-mevalonate (MEP) pathway sourced from *Arabidopsis thaliana*. We used transient expression to evaluate combinations of the eight MEP pathway genes plus GGPP synthase and a *Jatropha curcas casbene synthase* (*JcCAS*) to identify an optimal combination for production of casbene from GGPP. *AtDXS* and *AtHDR* together with *AtGGPPS* and *JcCAS* gave a 410% increase in casbene production compared to transient expression of *JcCAS* alone. This combination was cloned into a single construct using the MoClo toolkit, and stably integrated into the *N. benthamiana* genome. We also created multi-gene constructs for stable transformation of two *J. curcas cytochrome* *P450 genes*, *JcCYP726A20* and *JcCYP71D495* that produce the more complex diterpenoid jolkinol C from casbene when expressed transiently with *JcCAS* in *N. benthamiana*. Stable transformation of *JcCYP726A20*, *JcCYP71D495* and *JcCAS* did not produce any detectable jolkinol C until these genes were co-transformed with the optimal set of precursor-pathway genes. One such stable homozygous line was used to evaluate by transient expression the involvement of an ‘alkenal reductase’-like family of four genes in the further conversion of jolkinol C, leading to the demonstration that one of these performs reduction of the 12,13-double bond in jolkinol C. This work highlights the need to optimize precursor supply for production of complex diterpenoids in stable transformants and the value of such lines for novel gene discovery.
et al., 2016; King et al., 2014, 2016; Reed and Osbourne, 2018), but scaling up the approach to produce significant amounts of end product is not routine, with one exception being the production of the triterpene β-amyrin at mg/g leaf DW amounts (Reed et al., 2017; Stephenson et al., 2018). Vacuum infiltration requires substantial upstream work such as growing large volumes of A. tumefaciens and this can become more of a limiting factor when multiple genes need to be expressed as is the case for production of end products of complex metabolic pathways.

We reasoned that stable transformation of N. benthamiana to produce either a valuable end product or a key intermediate would simplify the production process as once stable lines are obtained they could be maintained as seeds and grown at scale. We targeted production of jokolin C, a member of the lathyrane class of casbene-derived diterpenes and a presumed intermediate of both ingenol mebutate and tigilanol tiglate described above (King et al., 2014, 2016; Luo et al., 2016). The production of such stable transformants producing intermediates in complex biochemical pathways could also possibly serve as a valuable tool for functional characterization by transient expression of candidate genes associated with the latter stages of such pathways.

Our engineering approach aimed to (i) optimize the flux of carbon from pyruvate and glyceraldehyde 3-P of primary metabolism through the MEP pathway to the diterpene precursor geranylgeranyl pyrophosphate (GGPP) (Gershenzon and Croteau, 2018) and (ii) combine this with addition of a casbene synthase (CAS) which cyclizes GGPP into casbene (Dueber et al., 1978) and two cytochrome P450s that oxidize casbene to produce an intermediate that undergoes non-enzymatic ring closure to produce jokolin C (King et al., 2016; Figure 1). We initially used transient expression to identify enzymatic steps of the MEP pathway that would increase GGPP as determined by production of casbene yield, then combined this novel combination of genes with the casbene oxidizing enzymes. This work reports on the successful production of jokolin C in stable homozygous transformants of N. benthamiana and how we then exploited these to determine the function of a novel jokolin C modifying enzyme.

Results and discussion

Transient expression in Nicotiana benthamiana to determine the optimal combination of MEP pathway genes for production of casbene

Previous reports have shown that DXS, the first committed enzyme of the MEP pathway, is critical in the synthesis of IPP and DMAPP in many plants (Estévez et al., 2001; Gong et al., 2006; Lois et al., 2000; Morris, 2006). For example, when the diterpene synthase CEMB RATRIEN-OL SYNTHASE was expressed in combination with DXS and GGPPS, there was a significant increase in all combinatorial tests, we included a Jatropha curcas CASBENE SYNTHASE (JcCAS) gene also under control of the CAMV35SS promoter in pEAG-HT and monitored casbene levels as an indirect measure of GGPP production.

We transiently infiltrated A. tumefaciens cultures carrying distinct plasmid constructs to test individual genes and various gene combinations by transient expression. This revealed that of the individual genes, only DXS resulted in an increase in casbene and the optimal combination of MEP pathway genes was AToDXS and AThHDR together with AtGGPPS and JcCAS (Figure 2). This combination of four genes gave a 410% increase in casbene production compared to transient expression of JcCAS alone (Figure 2), with AThHDR contributing 140% of this increase. To the best of our knowledge, this is the first demonstration of this association of these three MEP pathway genes can greatly increase the quantity of GGPP precursor.

Remarkably, addition of either AThHDR or AThMD5 to the AtDXS, AtGGPPS and JcCAS combination resulted in a decrease rather than an increase in casbene production, with levels falling back to those found for expression of the JcCAS gene alone after the addition of AThDXS (Figure 2). This dominant negative effect of AThDS on casbene production is found in various gene combinations including with the JcCAS alone (Figure S1). In E. coli, overexpression of ispG—encoding the native HDS enzyme—resulted in overproduction of HMBPP, which could cause cytotoxicity by interfering with the synthesis of nucleotides and proteins (Li et al., 2017). Activation of the ispH gene encoding the E. coli HDR enzyme was then able to eliminate the cytotoxic effect of ispG. A similar phenomenon may be occurring when AThDXS is overexpressed in the N. benthamiana transient expression system, as supported by the observation that the dominant negative effect of AThHS on casbene production is removed when the gene is co-expressed with AThHDR, which removes the toxic intermediate and increases the flux towards GGPP production. However, addition of HDS to the DXS + HDR + GGPPS combination does not significantly increase the amount of casbene produced and thus we did not include HDS in the optimal combination of MEP pathway genes.

Development of a single vector multi-gene system for transient up-regulation of casbene precursors

Having established the optimal combination of MEP pathway genes for casbene production using separate vectors, we next wanted to express these in a single vector with promoters of moderate strength. The aim was to use these multigene vectors subsequently for stableexpression, choosing promoters other than CaMV35S, so as to avoid triggering gene silencing in future stable transgenic lines (Elmayan and Vaucheret, 1996; Mishiba et al., 2005). We used the MoCLO modular cloning system (Engler et al., 2014; Weber et al., 2011) as it offers multiple options of expression cassette with different terminator regions and different promoters. We classified the promoters into two groups, A and B, established by Engler and co-workers on the basis of GFP expression (Table S2). The strength of the promoters was measured, and this method has been proved to be a quantitative reporter of gene expression (Sobolewski et al., 2005). Using this criterion, we estimated that promoters of group A were able to
provide between 5 and 15% of relative fluorescence compared to the reference construct 35S:GFP. Promoters of group B produced GFP fluorescence of between 25% and 45% as compared to the same reference. Level 2 (L2) MoClo vectors were assembled with distinct promoter and terminator sequences in different gene constructs to avoid homology-dependent gene silencing when integrated in the genome (Park et al., 1996). We produced four L2 constructs: two with AtDXS and AtGGPPS under the control of group A or B promoters (referred to as A-2 and B-2) and two with AtDXS, AtGGPPS and AtHDR driven by the same promoter groups (referred to as A-3 and B-3; Figure 3a). The Bar gene conferring Basta (phosphinothricin) resistance was included in all constructs as they were designed for both transient and stable transformation. Each L2 construct was tested by transient expression in N. benthamiana by co-infiltration with a separate pEAQ-HT vector containing 35S-JcCAS in order to evaluate casbene production when compared with the infiltration of 35S-JcCAS alone (Figure 3b). All L2 constructs combined to 35S-JcCAS produced significantly more casbene than single infiltration of the latter. Maximum production of casbene of 3.9 \( \mu \text{g/mg dry weight} \) was achieved by infiltration of the three gene L2 construct with the group A promoter plus 35S-

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**Figure 2** Casbene content in *N. benthamiana* co-expressing CAS and individual MEP pathway genes plus GGPPS. Casbene content (µg/mg DW ± standard deviation, n = 3). Letters show similarity or significant differences between treatment means (P < 0.05, F-test and T-test).

**JcCAS.** This represents a 485% increase in casbene compared to that produced upon infiltration of the 35S-JcCAS. We consistently found that both the two gene and three gene L2 constructs under the control of group B promoters gave lower levels of casbene production compared to the group A promoters, suggesting that higher expression is not always better in this transient expression system.

**Engineering stable production of casbene-derived diterpenoids in *N. benthamiana***

Having demonstrated that we could produce elevated levels of casbene in the transient expression platform, we decided to establish whether a similar result could be achieved by stable transformation of *N. benthamiana* and, if possible, to use this same system for production of casbene-derived diterpenoids, such as jolkinol C. Previous work in our laboratory had shown that transient expression of *N. benthamiana* with pEAQ-HT vectors containing the individual *J. curcas* genes, CYP726A20 and CYP71D495 that encode cytochrome P450 oxidase enzymes, together with pEAQ-HT::JcCAS, enabled the production of jolkinol C and *epi*-jolkinol C, which are proposed intermediates in the biosynthetic pathways to various bioactive casbene-derived diterpenoids (Figure 1; King et al., 2014, 2016). We used the MoClo modular cloning system to generate gene constructs with group A and B promoter variants of JcCYP726A20, JcCYP71D495 and JcCAS plus the *NptII* cassette which confers kanamycin resistance in plants. We named these gene constructs A-CP and B-CP (“CP” for CAS-P450s; Figure S2a). Prior to stable transformation, we evaluated these two constructs by transient expression in *N. benthamiana* and found that they both enabled production of jolkinol C and *epi*-jolkinol C (Figure S2b). The gene constructs driven by group A promoters produced significantly more jolkinol C than those driven by group B promoters, and more than the combination of genes expressed on separate pEAQ-HT vectors, which is consistent with the transient expression of the various MEP pathway genes constructs and casbene production.

Since both A-CP and B-CP constructs functioned in transient expression, they were used for stable transformation of *N. benthamiana* using the *A. tumefaciens* leaf disc transformation method (Horsch et al., 1989). Single transformants carrying either A-CP or B-CP constructs and co-transformants carrying either A-CP or B-CP and one of A-2, A-3, B-2 or B-3 were produced. To avoid the risk of gene silencing due to identical transgene components, co-transformations were conducted between A-2 or A-3 with B-CP and B-2 or B-3 with A-CP (Figure S3). Neither jolkinol C nor *epi*-jolkinol C were detectable in any of the 18 single transformants expressing the JcCYP726A20, JcCYP71D495 and JcCAS, which contrasts with the results of transient expression. It is noteworthy, however, that an intermediate identified by NMR as 6,9-dihydroxy-5-ketocasbene was detected in the A-CP single transformants (Figures 4b and Figure S4). This compound differs from the putative direct precursor of jolkinol C, 6-hydroxy-5,9-diketocasbene (King et al., 2016), in terms of the extent of oxidation at the C-9 position. We propose that 6,9-dihydroxy-5-ketocasbene is the product of incomplete oxidation by JcCYP71D495, resulting in a hydroxyl rather than a keto-group at C-9. 6,9-Dihydroxy-5-ketocasbene is unable to participate in the same spontaneous aldol reaction forming jolkinol C as 6-hydroxy-5,9-diketocasbene and we therefore propose that this causes it to accumulate in the A-CP transformants. It is not immediately obvious why JcCYP71D495 should only be catalysing partial oxidation at the C-9 position, but accumulation of this compound has previously been observed for the same set of genes, when expressed in *S. cerevisiae* (Wong et al., 2018). No pathway intermediates or end-products were detectable in the B-CP transformants.

Co-transformation with dual selection on kanamycin and Basta was four times less efficient than single transformation but still resulted in 27 T0 lines, distributed unevenly across the 4 gene vector combinations (Figure S3). Qualitative analyses of jolkinol C and *epi*-jolkinol C together with their pathway intermediates revealed that 15 of the 27 T0 co-transformants had detectable amounts of jolkinols (Figure 4c and Table 1). The most consistent combination for jolkinol and *epi*-jolkinol production was from the B-2/A-CP co-transformant class for which 15 out of 15 primary transformants contained jolkinols. The addition of HDR did not result in more jolkinols in the stable lines, but larger numbers of independent transformants would need to be evaluated before concluding that this step is not limiting in provision of the casbene precursor.

Among the B-2/A-CP co-transformants, the 5 lines showing a relative high content of jolkinol C (symbol +++ in Table 1) displayed morphological abnormalities including narrowed flower corolla and smaller seed pods (Figure S5a); nevertheless, the majority of the transgenic lines produced sufficient quantities of viable seeds to perform segregation analyses.

**Development of a diterpenoid transgenic platform for gene candidate evaluation**

We used segregation ratios for the basta and kanamycin selectable marker genes in T1 and T2 progeny of independent transformants to determine copy number and zygosity. This identified three independent transformants (number 1, 3 and 4) that carry a single copy of the B-2 and A-CP cassettes (Table 1). We identified the homozygous lines by segregating the T2 generation and analysed the casbene derivatives content (Figure S6). We selected the n4 B-2/A-CP line for its higher content of jolkinol C in the T2, hereinafter referred to as NbJolk-C, for further analysis and advanced this through to the T3 and T4 generations, at which stage it presented a distinct growth phenotype compared to WT (Figure S5b). Despite slower germination and growth, plus a more upright appearance in the first weeks of development, NbJolk-C produced leaves, flowers and viable seeds.

Next we tested whether the T3 and T4 NbJolk-C material could be used as a transient expression platform to investigate the function of other genes from the *Jatropha curcas* diterpenoid
biosynthesis gene cluster that contains \( JcCYP726A20 \), \( JcCYP71D495 \) essential for production of jolkinol-C (Figure S7; King et al., 2016). We transiently expressed four 'alkenal reductase'-like genes from this cluster in combination and individually in \( NbJolK-C \) and discovered that the alkenal reductase 3-like gene results in production of two new compounds (Figure 5). These were identified by NMR spectroscopy as 12,13-dihydro-jolkinol C and 12,13-dihydro-epi-jolkinol C (Figure S8), leading us to conclude that the \( J. \) curcas alkenal reductase 3-like gene encodes a double bond reductase enzyme that can reduce the C12-C13 double bond present in jolkinol C and epi-jolkinol C.

Biosynthesis of ingenanes, tiglanes and jatrophanes has been proposed in the literature to involve a lathyrane intermediate (Evans and Taylor, 1983; Schmidt, 1987). It is noteworthy that while lathyranes such as jolkinol C contain a C12-C13 double bond, this position is reduced in these other classes (Durán-Peña et al., 2014; Evans and Taylor, 1983). We can therefore speculate that the activity we report herein for the \( J. \) curcas alkenal reductase 3-like gene represents a crucial step in the biosynthesis of these more complex classes of diterpenoids derived from a lathyrane backbone.

**Conclusion**

Transient expression, in various combinations, of the entire suite of MEP pathway genes from \( A. \) thaliana allowed us to define an optimal combination of three genes, \( DXS \), \( HDR \) and \( GGPPS \), for production of casbene in \( N. \) benthamiana. Our finding that overexpression of \( HDR \) rescues the dominant negative effect on casbene production of the preceding enzyme in the MEP pathway, HDS, mirrors what was found in \( E. \) coli where it is understood that the HDR enzyme equivalent removes the cytotoxic intermediate and product of HDS, HMBPP (Li et al., 2017). The very positive effect of overexpression of \( AtHDR \) on casbene production could therefore be due to reduction in HMBPP levels instead of or in addition to the HDR step being rate-limiting. Transient expression also allowed us to select an optimal set of gene promoters for casbene production and, interestingly, we found that promoters that drive expression at intermediate rather than high levels (as determined by GFP fluorescence) were most effective at increasing flux to casbene through the MEP pathway. Placing the chosen MEP pathway gene constructs in a single vector also proved to increase casbene production compared to transient expression of casbene synthase alone. The resulting vector, carrying the optimal set of MEP pathway genes, was then used for stable transformation of \( N. \) benthamiana. The ability to up-regulate the MEP pathway and direct flux to casbene was demonstrated to be essential when it comes to engineering production of casbene-derived diterpenoids such as jolkinol C and epi-jolkinol C in stable transgenic lines of \( N. \) benthamiana, highlighting the importance of optimizing substrate supply in metabolic engineering of complex diterpenoids.
diterpenoids in stable production platforms. Such platforms may prove valuable for production of bioactive diterpenoids but the pathways for production of many of these remain to be fully elucidated and will involve a process of step-by-step gene discovery. We demonstrate that an *N. benthamiana* line engineered to produce jolkinol C and epijolkinol C can be a valuable tool when used in combination with transient expression for candidate gene function determination. This approach was used to reveal the function of the enzyme responsible for the double bond reduction at the C12-C13 position on jolkinol C and epijolkinol C, which could be an important step in the biosynthesis of more complex diterpenes including ingenol mebutate and tigilanol tiglate. Our discovery could therefore contribute to engineering the production of medicinal compounds in heterologous systems.

**Material and methods**

**Transient expression of genes in *Nicotiana benthamiana***

cDNAs from *Arabidopsis thaliana* plastidial MEP genes and plastidial GGPPS11 (Beck et al., 2013) were prepared from total RNA samples using Superscript III reverse transcriptase (Invitrogen) and random hexamer primers. Accession numbers and references for these genes can be found in Table S1 (Phillips et al., 2008a,b; Ruiz-Sola et al., 2016). The open reading frames (ORFs) of these genes were subsequently amplified with Phusion PFu polymerase (New England Biolabs) using primers designed on NEBuilder for Gibson assembly and detailed in Table S1. Casbene synthase, cytochrome P450 genes CYP726A20 and CYP71D495 and the four alkenal reductases from *Jatropha curcas* have been amplified in previous work (King et al., 2014) and were already available in pEAQ-HT vectors (Sainsbury et al., 2009). ORFs from *Arabidopsis* genes were cloned with NEB Gibson Assembly Mastermix according to the manufacturer’s protocol in pEAQ-HT vector, allowing each gene to be positioned under the control of an improved cauliflower mosaic virus (CAMV) 35S promoter (Sainsbury and Lomonossoff, 2008).

For assembly with Modular Cloning (MoClo), ORFs were first domesticated, that is removal of the restriction sites BsaI and BpiI when necessary. The domesticated coding sequences (CDS) were cloned in level 1/C0 and level 0 vectors using MoClo Tool Kit (Addgene) (Weber et al., 2011; Werner et al., 2012), following the long protocol described by the manufacturer. CDS were then combined to different promoters and terminators provided by the MoClo Plant Parts kit (Addgene) (Engler et al., 2014) in the level 1 vector. Genes coding for *NptII* (kanamycin resistance cassette) and *Bar* (bialaphos/glufosinate/Basta resistance cassette) were also available in the Plant Parts kit. The genes obtained were finally assembled in the level 2 vector intended for transient and stable expression (see Table S3 for details on the transcriptional units).

The expression vectors were then transformed into *Agrobacterium tumefaciens* LBA4404 using the freeze thaw method (Höfgen and Willmitzer, 1986). A pEAQ-HT vector with eGFP was also created in previous work (King et al., 2016) to visualize and delimitate the infiltrated areas of the leaves when co-transformed with candidate genes. *A. tumefaciens* cultures were initially grown to an OD between 2 and 3 in YEB media (5 g/L beef extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose and 0.5 g/L MgCl₂), pelleted at 4000 g for 15 min and resuspended in pre-infiltration media (10 mM MgCl₂, 200 μM acetosyringone and 0.015%) at an OD of 5 to be left for 1–2 h. For mixed
infiltrations, the same amount of cells were added such that each strain was present at the same density of 0.2 OD in the final infiltration volume. Vacuum infiltration of *N. benthamiana* plants was performed by dipping plants into the infiltration media (10 mM MgCl₂, 200 µM acetylsyringone, 0.015% Silwet L-77 plus *A. tumefaciens*) in a degassing chamber at 50 mBar for 60 s. Five days after *Agrobacterium* infiltration, leaves showing GFP signal under UV were harvested, freeze-dried and ground for 30 s with a steel bead at 30 Hz in a Retsch homogenizer in order to perform metabolomic extraction.

**Isolation and quantification of diterpenoids**

To detect and quantify the production of casbene in transiently expressed plants, around 200 mg of dry material was extracted with 5 mL of hexane containing 100 µg/mL of β-caryophyllene then sonicated for 15 min. 100 µL of the extracts were used for GC-MS analysis and 2 µL were injected in a Leco Pegasus IV GC-TOF instrument. The GC oven was fitted with a Restek RTX-5SIL MS capillary column (30m, 0.25-mmID, 0.25 mm df). The oven temperature was set at 100°C for 2 min and then increased to 300°C at a rate of 5°C min⁻¹. Mass spectral data were acquired over the m/z range of 50 to 450 in positive electron ionization mode at −70 eV.

For the quantification of casbene and jolkinols in stable and transiently transformed plants, ca. 250 mg of dry material was extracted with 1 mL of ethyl acetate containing 10 µg/mL of β-caryophyllene and 20 µg/mL of phorbol myristate acetate (PMA). After an overnight shaking at 2200 rpm on an IKA Vibrax VXR basic shaker, the samples were centrifuged and 100 µL of the supernatant was used directly for GC-MS, while the rest was

### Table 1 Qualitative detection of oxidized casbene derivatives in the 2G T0 co-transformant lines and estimation of the copy number of each gene cassette

| Condition | T0 line | 6HSK | 6,9dHSK | Epi-jol | Jolkinol C | Basta | Kan |
|-----------|---------|------|---------|--------|------------|-------|-----|
| A-2/B-CP  | n°1     | –    | –       | –      | –          | <1    | 1   |
|           | n°2     | –    | –       | –      | –          | 2     | >3  |
| B-2/A-CP  | n°1     | +++  | ++      | +      | +          | 1     | 1   |
|           | n°2     | ++   | +++     | +      | +          | 1     | 3   |
|           | n°3     | ++   | +++     | +      | +          | 1     | 1   |
|           | n°4     | +++  | +++     | +      | +          | 1     | 1   |
|           | n°5     | +++  | +++     | +      | +          | 1     | 2   |
|           | n°6     | ++   | +++     | +      | +          | Sterile |     |
|           | n°7     | +    | +       | –      | –          | Sterile |     |
|           | n°8     | +    | +       | +      | +          | > 3   | 1   |
|           | n°9     | +    | +       | –      | –          | 1     | 1   |
|           | n°10    | +    | +       | ++     | +++        | < 1   | 3   |
|           | n°11    | +    | +++     | +      | +++        | < 1   | > 3 |
|           | n°12    | +    | +++     | +      | +++        | < 1   | 1-2 |
|           | n°13    | +    | +++     | +      | +++        | < 1   | 2   |
|           | n°14    | ++   | ++      | +      | +          | < 1   | 3   |
|           | n°15    | +    | ++      | +++    | +          | < 1   | 2   |
| A-3/B-CP  | n°1     | –    | –       | –      | –          | 2     | 1-2 |
|           | n°2     | –    | –       | –      | –          | 1     | 1   |
|           | n°3     | –    | –       | –      | –          | 2     | 2   |
|           | n°4     | –    | –       | –      | –          | 1     | 1   |
| B-3/A-CP  | n°1     | –    | –       | –      | –          | 1     | 1   |
|           | n°2     | –    | –       | –      | –          | 1     | 1   |
|           | n°3     | ++   | +       | –      | –          | < 1   | 1   |
|           | n°4     | –    | –       | –      | –          | Sterile |     |
|           | n°5     | +    | +       | +      | +          | 1-2   | <1  |
|           | n°6     | +++  | +++     | +      | +          | Sterile |     |

6HSK, 6-hydroxy-S-ketocasbene; 6,9dHSK, 6,9-dihydroxy-S-ketocasbene; epi-jol, epi-jolkinol C.

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**Figure 5** Use of jolkinol C producing platform to evaluate candidate gene function. A stable *N. benthamiana* jolkinol C producing transgenic line was used to assay the function by transient expression of four candidate alkenal reductase genes identified on a *J. curcas* genes cluster (King et al., 2016). In all cases, mass spectrometry (UPLC-MS) is shown at base peak m/z 301. (a) Empty pEAQ-HT vector control, (b) co-expression of all four alkenal reductase genes in separate pEAQ-HT vectors, (c) pEAQ-HT::alkenal reductase 1, (d) pEAQ-HT::alkenal reductase 2, (e) pEAQ-HT::alkenal reductase 3, (f) pEAQ-HT::alkenal reductase 4 and (g) schematic presentation of the conversion of jolkinol C and epi-jolkinol C to 12,13-dihydro-jolkinol C and 12,13-dihydro-epi-jolkinol C, respectively. 1. 12,13-dihydro-jolkinol C; 2. 12,13-dihydro-epi-jolkinol C; 3. 6,9-dihydroxy-S-ketocasbene.
evaporated in a GeneVac EZ-2 plus and resuspended in 250 µL of methanol for UPLC-MS analysis. A 2 µL aliquot was analysed in the Waters Acquity™ UPLC using an Acquity UPLC® BEH C18 column (Waters, 1.7 µm, 2.1 x 100 mm) kept at 60°C. Mobile phases A and B were water with 5% methanol + 0.1% formic acid and methanol + 0.1% formic acid, respectively. A flow rate of 0.5 mL/min was used. The gradient profile was as follows: 30 s at 40% B; a linear gradient lasting 25 min from 40% B to 100% B, then held for 5 min; and a final step of 40% B maintained for 2 min. Mass spectral data were acquired over the m/z range of 100–1000 in positive polarity mode using an APCl source.

Cassbene was quantified by determination of the total ion chromatogram (TIC) peak area and comparison to the peak area of the internal standard, β-caryophyllene. Jolkinol C, epi-jolkinol C, 6-hydroxy-5-ketocasbene, 6,9-dihydroxy-5-ketocasbene, 12,13-dihydro-jolkinol C and 12,13-dihydro-epi-jolkinol C were quantified and/or detected by determination of their main ion peak area (m/z 299 for jolkinol C and epi-jolkinol C; m/z 285 or 303 for 6-hydroxy-5-ketocasbene; m/z 301 or 319 for 6,9-dihydroxy-5-ketocasbene, 12,13-dihydro-jolkinol C and 12,13-epi-dihydro-jolkinol C) and comparison with the main ion base peak area of the internal standard PMA (m/z 389).

Accumulation and purification of compounds for NMR spectroscopy

For 6,9-dihydroxy-5-ketocasbene, we vacuum infiltrated 48 N. benthamiana WT plants with Agrobacterium strains containing plasmids for overexpression of precursor genes, JcCas, JcCYP726A20 and JcCYP71D49S to obtain 6.1 g of freeze-dried material. This material was extracted with 15 volumes of ethyl acetate during 5 days on a gentle rotary shaker. The extract was dried on a rotary evaporator to yield 480 mg of green oily residue. This material was extracted with 15 volumes of ethyl acetate during 5 days on a gentle rotary shaker. The extract was dried on a rotary evaporator to yield 480 mg of green oily residue, resuspended in 10 mL of hexane/ethyl acetate (70:30, v/v) and subjected to one round of flash chromatography on a PuriFlash® 4250 system (Interchim). We used a 40 g Bucli silica column and a hexane/ethyl acetate gradient as described in the King et al., 2014 to fractionate the extract into 80 samples. Fractions were analysed by UPLC-MS and those containing 6,9-dihydroxy-5-ketocasbene were combined and dried to yield 0.23 mg of compound. This was sufficiently pure to allow an 1H NMR analysis to be recorded in CDC13 with a Bruker AVIII 700 MHz spectrometer instrument.

For 12,13-dihydro-jolkinol C and 12,13-dihydro-epi-jolkinol C, we applied the same procedure described above. We used WT tobacco to over-express the same genes and the alkenal reductase 3 or double-bond reductase DBR. We infiltrated 96 plants to obtain 21 g of freeze-dried material leading to 1.15 g of green oily residue. The fractions obtained from the flash chromatography allowed accumulation of 0.23 mg and 0.14 mg of the metabolites later identified as 12,13-dihydro-jolkinol C and 12,13-dihydro-epi-jolkinol C, respectively.

Stable transformation of Nicotiana benthamiana

Nicotiana benthamiana stable transformation was performed following the leaf discs method (Horsch et al., 1989). Leaves from 6-week-old N. benthamiana were first sterilized in 10% bleach for 10 min and then rinsed 4–5 times in sterile distilled water (Clemente, 2006). Discs were cut with a sterile cork borer of 1 cm diameter and soaked in an Agrobacterium co-cultivation solution consisting of 4.3 g/L of MS medium M0221 (Duchefa), 30 g/L of anhydroglucose, 100 mg/L of myo-inositol, 0.5 mg/L of the vitamins nicotinic acid, thiamine-HCl and pyridoxine, 2 mg/L of glycine and few drops of KOH 1N to adjust the pH to 5.7–5.8. Agrobacterium LBA4404 (Hoekema et al., 1983) containing vectors of interest were grown in preliminary culture for 2 days in YEB media then centrifuged and washed with 1 mL of 10mM MgSO4 before being resuspended in 1 mL of the co-cultivation solution described above. Leaf discs infected with Agrobacterium were then dried on sterile blotting paper and incubated for 3–4 days onto solid co-cultivation medium containing 0.1 mg/L of 1-Naphthaleneacetic acid (NAA) and 1 mg/L of 6-Benzylaminopurine (BAP). After this incubation period, discs were transferred on the same medium supplemented with the plant selection agent (100 mg/L for kanamycin and 5 mg/L for glufosinate) and 500 mg/L of cefotaxime to eliminate the bacteria. Discs were transferred every 2–3 weeks onto fresh co-cultivation plates to promote and improve appearance of buds and calli. Shoots started to appear 30–40 days after transformation and were transplanted into sterile pots containing rooting medium (2.65 g/L of modified MS n°4 M0238 from Duchefa, 825 mg/L of NH4NO3 30 g/L of sucrose, 100 mg/L of myo-inositol, 0.5 mg/L of the same 3 vitamins as the co-cultivation medium, a few drops of KOH to adjust the pH at 5.7–5.8 and 6 g/L of agar). The doses of kanamycin and glufosinate were doubled in this medium to eliminate the non-transformed explants. Roots started developing 15–30 days after transfer, allowing the transformed seedlings to be put into soil. Primary transformants were tested by PCR and on their metabolic content to confirm the success of the transformation.

Seeds of the subsequent generations were sown on a germination medium (4.4 g/L MS medium M0221 from Duchefa, 10 g/L sucrose, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid and pyridoxine, 1 mg/L thiamine, few drops of KOH to adjust at pH 5.7–5.8 and 6 g/L agar) containing 500 mg/L kanamycin and 10 mg/L glufosinate to perform segregation tests and estimate the copy number of each transgene.

Following germination and appropriate selection, WT and transgenic seedlings were transferred to F2 + 5 seed and modular compost (Levingston Advance) and cultivated in a growth chamber under white fluorescent lamps set at 22°C during the day (16 h) and 20°C during the night (8 h).

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

EF designed experiments, performed experiments and analysed data. TC, AD, AC, AK and GD performed experiments and analysed data. EF and IG wrote the manuscript. IG contributed to the conception, design and analysis of the study. All authors read and approved the manuscript.
References

Andersen-Ranberg, J., Kongstad, K.T., Nielsen, M.T., Jensen, N.B., Pateraki, I., Bach, S.S., Hamberger, B. et al. (2016) Expanding the landscape of diterpene structural diversity through stereochemically controlled combinatorial biosynthesis. Angew. Chem. Int. Ed 55, 2142–2146.

Beck, G., Coman, D., Herren, E., Ruiz-Sola, M.A., Rodríguez-Concepción, M., Gruss, W. and Vranová, E (2013) Characterization of the GGPP synthase gene family in Arabidopsis thaliana. Plant Mol. Biol. 82, 393–416.

Botella-Pavia, P., Besumbe, Ö., Phillips, M.A., Carretero-Paulet, L., Boronat, A. and Rodríguez-Concepción, M. (2004) Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors: Role of HDR for carotenoid biosynthesis. Plant J. 40, 188–199.

Brückner, K. and Tissera, A. (2013) High-level diterpene production by transient expression in Nicotiana benthamiana. Plant Methods 9, 46.

Clemente, T. (2006) Nicotiana (Nicotiana tabacum, Nicotiana benthamiana). AgroBact. Protocol. 143–154.

Dueber, M.T., Adof, W. and West, C.A. (1978) Biosynthesis of the diterpene phytoalexin casbene: partial purification and characterization of casbene synthetase from ricinus communis. Plant Physiol. 62, 598–603.

Durán-Peña, M.J. Botobul Ares, J.M., Collado, I.G. and Hernández-Galán, R. (2014) Biologically active diterpenes containing a gem-dimethylcyclopropane subunit: an intriguing source of PKC modulators. Nat. Prod. Rep. 31, 940–952.

Elmayan, T. and Vaucheret, H. (1996) Expression of single copies of a strongly expressed 35Stransgene can be silenced post-transcriptionally. Plant J. 7, 787–797.

Engler, C., Youles, M., Gruetznner, R., Ehret, T.-M., Werner, S., Jones, J.D.G. et al. (2014) A golden gate modular cloning toolbox for plants. ACS Synth. Biol. 3, 839–843.

Estévez, J.M., Cantero, A., Reindl, A., Reichler, S. and Willmitzer, L. (1988) Storage of competent cells for Agrobacterium tumefaciens binary plant vector strategy based on separation of vir- and T-region of the Ti-plasmid. Planta Med. 56, 179–180.

Evans, F.J., Taylor, S.E. et al. (1983) Pro-inflammatory, tumour-promoting and anti-tumour diterpenes of the plant families Euphorbiaceae and Thymelaeaceae. In Fortschritte der Chemie organischer Naturstoffe Progress in the Chemistry of Organic Natural Products Fortschritte der Chemie organischer Naturstoffe/Progress in the Chemistry of Organic Natural Products (Buchanan, J.G., Crews, P., Epe, B., Evans, F.J., Hanke, F.J. and Manes, L.V., eds), pp. 1–99. Vienna: Springer.

Gershenzon, J. and Croteau, R. (2018) Terpenoid biosynthesis: the basic pathway and formation of monoterpenes, sesquiterpenes, and diterpenes. In Moore, T. S. (Ed.), Lipid Metabolism in Plants. Chapter 11. Boca Raton, Florida CRC Press, pp. 339–388. First edition in 1993.

Gong, Y., Liao, Z., Guo, B., Sun, X. and Tang, K. (2006) Molecular cloning and expression profile analysis of Ginkgo biloba DKS gene encoding 1-Deoxy-d-xylulose-5-phosphate synthase, the first committed enzyme of the MEP metabolic network: implications for the control of the tuber life cycle. J. Exp. Bot. 57, 3007–3018.

Park, Y.-D., Papp, I., Moscone, E.A., Iglesias, V.A., Vaucheret, H., Matzke, A.M. and Matzke, M.A. (1996) Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity. Plant J. 9, 183–194.

Puyet, H. and Lomonossoff, G.P. (2013) The phiAQ vector series: the easy and quick way to produce recombinant proteins in plants. Plant Mol. Biol. 83, 51–58.

Phillips, M.A., D’Auria, J.C., Gershenzon, J. and Pichersky, E. (2008) The Arabidopsis thaliana Type I isopentenyl diphosphate isomerase targets to multiple subcellular compartments and have overlapping functions in isoprenoid biosynthesis. Plant Cell Online, 20, 677–696.

Phillips, M., Leon, P., Boronat, A. and Rodríguez-Concepcion, M. (2008) The plastidal MEP pathway: unified nomenclature and resources. Trends Plant Sci. 13, 619–623.

PicatoEuropean Medicines Agency. https://www.ema.europa.eu/en/medicines/human/referrals/picato

Reed, J. and Osbourn, A. (2018) Engineering terpenoid production through transient expression in Nicotiana benthamiana. Plant Cell Rep. 37, 1431–1441.

Reed, J., Stephenson, M.J., Miettinen, K., Brouwer, B., Leveau, A., Brett, P., Goss, R.J.M. et al. (2017) A translational synthetic biology platform for rapid access to gram-scale quantities of novel drug-like molecules. Metab. Eng. 42, 185–193.

Ridder, T.R.D., Campbell, J.E., Burke-Schwarz, C., Clegg, D., Elliott, E.L., Geller, S. et al. (2020) Randomized controlled clinical study evaluating the efficacy and safety of intratumoral treatment of canine mast cell tumors with tigilanol tiglate (EBC-46). J. Vet. Intern. Med. 35(1), 415–429.

Ruiz-Sola, M.A., Coman, D., Beck, G., Barja, M.V., Colinas, M., Graf, A. et al. (2016) Arabidopsis GERANYLGERANYL DIPHOSPHATE SYNTHASE 11 is a hub isoform required for the production of most photosynthesis-related isoprenoids. New Phytol. 209, 252–264.
Sainsbury, F. and Lomonossoff, G.P. (2008) Extremely high-level and rapid transient protein production in plants without the use of viral replication. *Plant Physiol.* 148, 1212–1218.

Sainsbury, F., Thuenemann, E.C. and Lomonossoff, G.P. (2009) pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. *Plant Biotechnol. J.* 7, 682–693.

Schmidt, R.J. (1987) The biosynthesis of tigliane and related diterpenoids: an intriguing problem. *Bot. J. Linn. Soc.* 94, 221–230.

Sobolev, M.R., Oaks, J. and Halford, W.P. (2005) Green fluorescent protein is a quantitative reporter of gene expression in individual eukaryotic cells. *FASEB J.* 19, 440–442.

Stephenson, M.J., Reed, J., Brouwer, B. and Osbourn, A. (2018) Transient expression in *nicotiana benthamiana* leaves for triterpene production at a preparative scale. *J. Vis. Exp.* 138 (58169).

Weber, E., Engler, C., Gruetzner, R., Werner, S. and Marillonnet, S. (2011) A modular cloning system for standardized assembly of multigene constructs. *PLoS One* 6, e16765.

Werner, S., Engler, C., Weber, E., Gruetzner, R. and Marillonnet, S. (2012) Fast track assembly of multigene constructs using Golden Gate cloning and the MoClo system. *Bioengineered* 3, 38–43.

Whaley, K.J., Hiatt, A. and Zeill, L. (2011) Emerging antibody products and *Nicotiana* manufacturing. *Human Vaccines* 7, 349–356.

Wong, J., de Rond, T., d’Espaux, L., van der Horst, C., Dev, I., Rios-Solis, L., Kirby, J. et al. (2018) High-titer production of lathyrane diterpenoids from sugar by engineered *Saccharomyces cerevisiae*. *Metab. Eng.* 45, 142–148.

**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.