Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Plant Biology

Engineering *Nicotiana benthamiana* as a platform for natural product biosynthesis

D. Golubova[1,a], C. Tansley[1,2,a], H. Su[1] and N. J. Patron[1,2]

Nicotiana benthamiana is a model plant, widely used for research. The susceptibility of young plants to *Agrobacterium tumefaciens* has been utilised for transient gene expression, enabling the production of recombinant proteins at laboratory and commercial scales. More recently, this technique has been used for the rapid prototyping of synthetic genetic circuits and for the elucidation and reconstruction of metabolic pathways. In the last few years, many complex metabolic pathways have been successfully reconstructed in this species. In addition, the availability of improved genomic resources and efficient gene editing tools have enabled the application of sophisticated metabolic engineering approaches to increase the purity and yield of target compounds. In this review, we discuss recent advances in the use of *N. benthamiana* for understanding and engineering plant metabolism, as well as efforts to improve the utility of this species as a production chassis for natural products.

Addresses
[1] Earlham Institute, Norwich Research Park, Norwich, NR4 7UZ, UK
[2] Department of Plant Sciences, University of Cambridge, Cambridge, CB2 3EA, UK

Corresponding author: Patron, NJ (njp56@cam.ac.uk)
[a] These authors contributed equally.

Current Opinion in Plant Biology 2024, **81:**102611

This review comes from a themed issue on **Physiology and metabolism 2024**

Edited by **Vincent Courdavault** and **Anne Osbourn**

For a complete overview see the Issue and the Editorial

Available online xxx

https://doi.org/10.1016/j.pbi.2024.102611

1369-5266/© 2024 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

Introduction

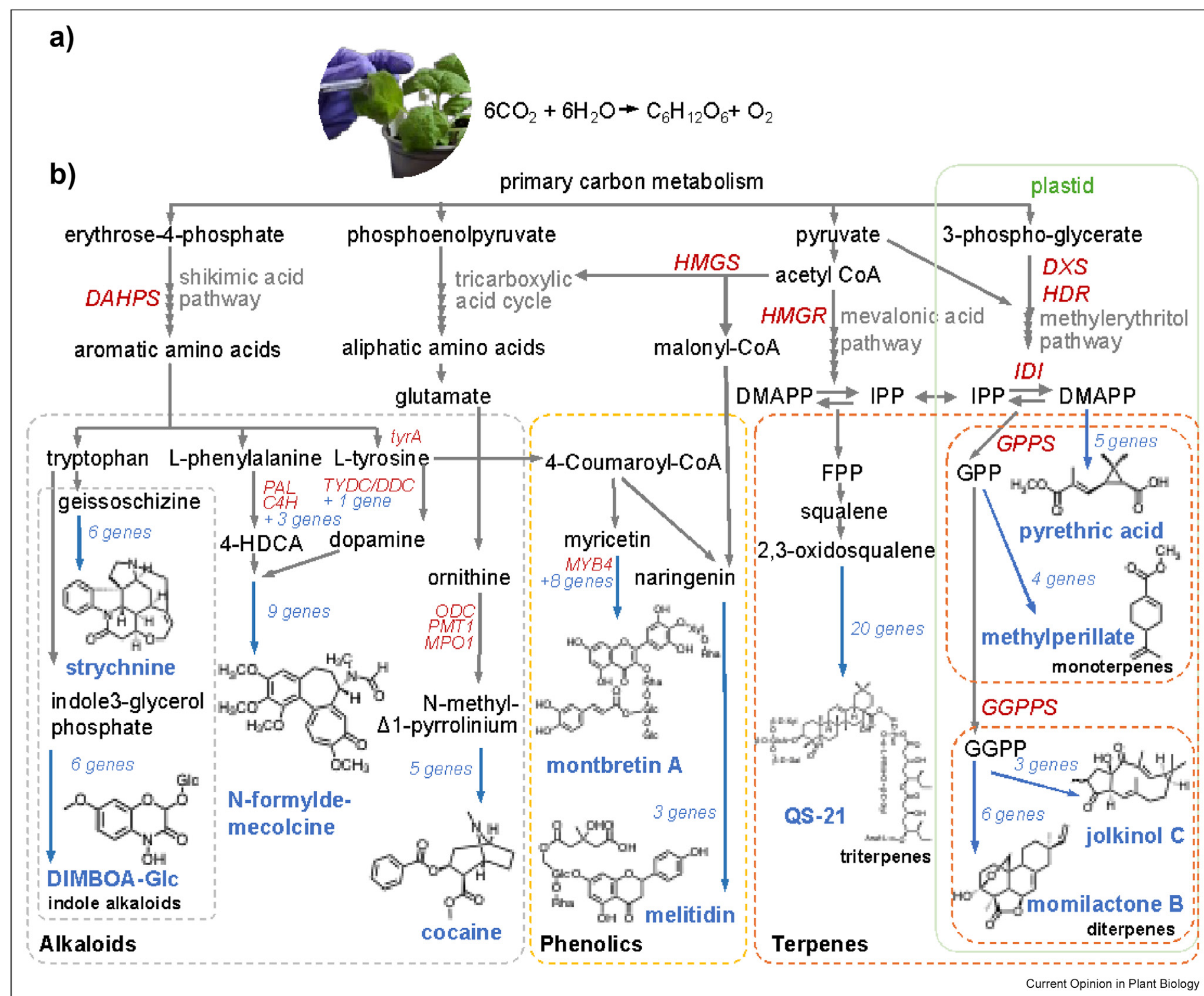
Many organisms produce metabolites with properties that are of interest to health and industry. However, these molecules sometimes occur in complex mixtures or within a limited number of cell types reducing accessibility. Furthermore, complex stereochemistry can limit the feasibility of chemical synthesis. Consequently, global demands can place unsustainable pressure on the species in which they occur. For example, the extraction of paclitaxel from the bark of the Pacific yew (*Taxus brevifolia*) led to a 30% decline in population, resulting in the species being listed as 'near threatened' [1].

Reconstruction of plant metabolic pathways in microbial hosts provides an alternative route for production. This can enable high yields and allow enzymes from different species to be combined into novel pathways to produce new-to-nature molecules. However, as an emerging industry, it remains difficult to predict commercial competitiveness. Furthermore, the sustainability of some of the feedstocks used to cultivate industrial microbes has been questioned [2]. Photosynthetic chassis, including plants, provides an attractive alternative. Advantages include accessibility to metabolic precursors derived from the products of photosynthesis, the presence of multiple subcellular compartments, and that most enzymes are functional when heterologously expressed [3]. In contrast, genetic manipulation is often more laborious. Here, we discuss the recent explosion in the use of *Nicotiana benthamiana* for the elucidation and reconstruction of metabolic pathways, with a focus on advances in metabolic engineering to improve the utility of this species as a production chassis.

Nicotiana benthamiana, a plant chassis for pathway elucidation and reconstruction

N. benthamiana is an Australian relative of field tobacco (*Nicotiana tabacum*), utilised for decades as a model species for fundamental research. The widely utilised 'LAB' strain descended from a single collection of specific ecotype that carries a disruptive insertion in its RNA-dependent RNA polymerase (Rdr1) gene, weakening its immunity [4]. A prized feature of *N. benthamiana* is its amenability to rapid, transient expression using a technique known as agroinfiltration, in which strains of *Agrobacterium tumefaciens* carrying genes or pathways of interest are injected into leaves (Figure 1a). This results in high-level expression after just a few days. Recent efforts to optimise agroinfiltration include the development of an 'E-platform' for whole-plant vacuum infiltration [5] and targeted mutagenesis of genes involved in immunity and flowering to maximise biomass [6,7]. In addition, viral vectors that self-replicate following agroinfiltration have also been used to increase yields [8,9].

Figure 1



Examples of plant biosynthetic pathways reconstructed in *Nicotiana benthamiana*. a) The infiltration of *Agrobacterium* strains carrying pathway genes into leaf tissues enables the conversion of endogenous metabolites, themselves derived from the products of photosynthesis, to target compounds. **b)** Examples of multigene heterologous pathways reconstructed in *Nicotiana benthamiana*. Black/grey text indicates endogenous metabolites/pathways; red text indicates endogenous enzymes targeted for metabolic engineering by over expression of endogenous or heterologous isoforms; blue text indicates heterologous pathways and products. C4H, cinnamate 4-hydroxylase; DAHPS, 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase; DXR, 1-deoxy-d-xylulose5-phosphate reductase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; GGPPS, geranylgeranyl pyrophosphate synthase; GPPS, geranyl pyrophosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl coenzyme (a reductase); HMGS, 3-hydroxy-3-methylglutaryl-coenzyme A synthase; IDI, isopentenyl diphosphate isomerase; MPO, N-methylputrescine oxidase; MYB4, R2R3-subfamily transcription factor; ODC, ornithine decarboxylase; PMT, putrescine N-methyltransferase; PAL, phenylalanine ammonia lyase; tyrA, prephenate dehydrogenase; TYDC/DDC, L-tyrosine/L-DOPA decarboxylase; DXS, deoxyxylulose 5-phosphate synthase.

The use of *N. benthamiana* has now expanded to the elucidation and reconstitution of increasingly complex biosynthetic pathways (Figure 1b). The ability to simultaneously co-infiltrate multiple *Agrobacterium* strains facilitates the reconstruction of such pathways, negating the requirement to assemble large DNA constructs; candidates for unknown pathway steps can be tested simply by substituting single strains [10]. To date, several classes of molecules have been successfully produced in *N. benthamiana*. Recent achievements in the production of monoterpenoids include methylperillate, valued as a functionalised substrate for the production of terephthalic acid [11], and the insecticidal pyrethric acid [12]. Examples of diterpenoid production include jolkinol C [13], the anticancer paclitaxel [14], and the allelopathic momilactone B [15]. Examples of triterpenoids

include immunostimulant triterpene glycosides [16,17], the early biosynthetic steps of limonoids and tetranortriterpenoids used as bioinsecticides [18]. Reported yields of terpenoids differ considerably: while yields of heterologously produced momilactone B were far higher (167 μg/g dry weight (DW)) than those obtained from rice husks [15], yields of QS21 were three to five times lower than those reported for *Quillaja saponaria* (soap bark tree) [17]. It is important to note, however, that transient expression takes only a few days; thus, biomass production is far greater than from slow-growing tree species.

Successfully expressed phenolic compounds include the phytotoxic sorgoleone [19], the terpenophenolic cannabigerolic acid [20], and the acylated flavonol glycosides, montbretin A [21,22] and melitidin [23]. *N. benthamiana* has also been utilised to elucidate the pathway of a large number of alkaloids including strychnine [24], the benzoxazinoid 2-(2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-d-glucopyranose [25], the colchicine precursor N-formyldemecolcine [26], cocaine [27], diosgenin [28], and piperine [29].

The power of agroinfiltration to facilitate the screening gene combinations was particularly well highlighted by the identification of auxiliary genes to alleviate metabolic bottlenecks in the production of the glucosinolate, glucoraphanin [30]. The advantages of co-infiltration have also been used to enable the production of new-to-nature compounds. Combining genes from different species enabled the production of novel ß-amyrin-based triterpenes [31], whereas co-infiltrating unnatural substrates enabled the production of novel monoterpene indole alkaloids [32].

Scaling up: transient vs. transgenic expression

Large-scale facilities for the scale-up of *N. benthamiana* agroinfiltration have been developed. However, these typically require an artificially lit and heated or cooled plant growth facility, peat-free media or substrate for plant cultivation, feedstocks for culturing *A. tumefaciens* strains, and specialised infrastructures for moving plants for infiltration [33]. It remains uncertain if such platforms will be economically viable for the production of natural products that are not of exceptionally high commercial value.

In contrast, stable transgenics take a considerable amount of time to produce and characterise, and the regulatory processes for field growth of transgenic plants remain challenging. However, field cultivation is comparatively cheap and easy to scale. Transgenic approaches often seek to integrate transgenes into the nuclear genome. To facilitate transmission to progeny generations, it is desirable to integrate transgenes as a single multigenic cassette. Furthermore, to avoid silencing, plants with single-integration loci are preferred. This means that yields of target products are considerably lower (per unit of leaf tissue) than those obtained by transient infiltration [34]. Although *N. benthamiana* is a relatively small species, closely related species such as *N. tabacum* accumulate considerable biomass. Ultimately, to compare the commercial feasibility of transient and field production, calculations of yield per unit of input cost are likley to be a more useful measure than μg/g. This is similarly true for comparisons to microbial production, for which different units (e.g. g/L) are used to report yields.

A further challenge of stable transgenics is controlling gene expression. Strong, constitutive expression of heterologous metabolic pathways can impact plant growth and development, as observed in lines producing fatty-acid-based insect sex pheromones [35]. While these impacts can be overcome by the use of inducible promoters, it is challenging to maintain high yields [34]. The same study also noted that the assembly of pathway genes onto a multigenic construct altered the relative gene expression of pathway genes, which then affected the yields of the target products [34]. Furthermore, coassembly of genes onto single cassettes requires access to a diversity of regulatory elements to avoid gene silencing; constructs with repeated elements were observed to reduce yields of monoterpene indole alkaloids [36].

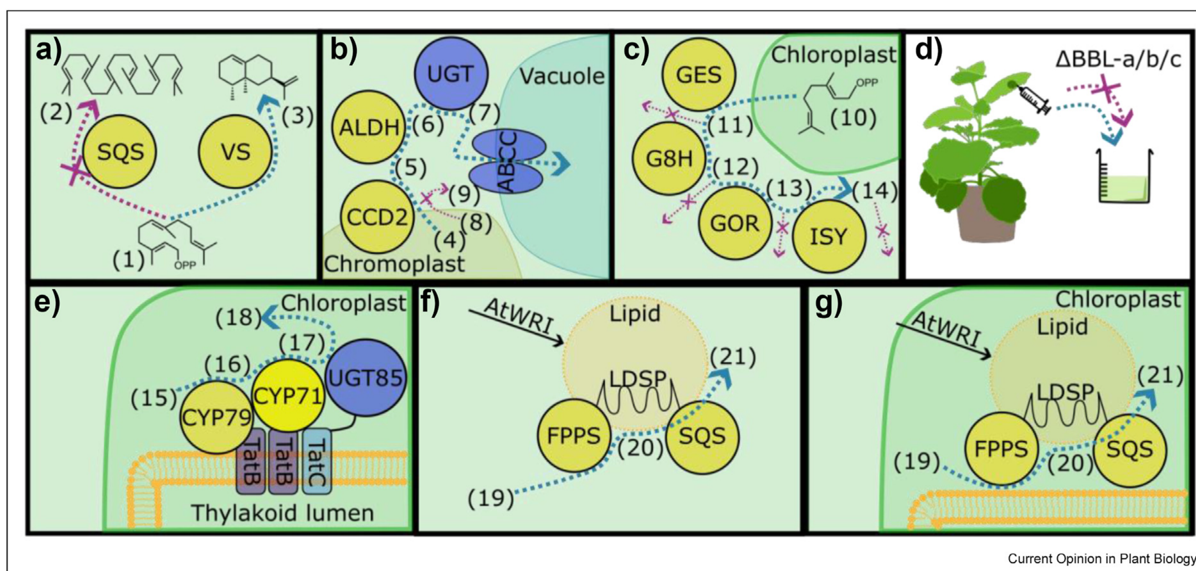
Transplastomic plants, in which transgenes are integrated into plastid genomes, exploit the high copy-number of plastid genomes. This has been utilised to achieve high yields of recombinant proteins. Importantly, Schmidt et al. demonstrated that production of bacterial cellulase from transplastomic *N. tabacum* plants reduced costs by three orders of magnitude over current cell-culture methods [37]. However, plastid production of natural products may be limited by the fact that plastids contain only a subset of plant metabolic pathway precursors. This may be overcome by the expression of additional enzymes to enable a wider range of pathways to be reconstructed. For example, the normally cytosolic artemisinin biosynthetic pathway was reconstructed in transplastomic *N. benthamiana* plants by expressing a farnesyl pyrophosphate synthase (FPPS) to enable the pathway to utilise C_5 isoprenoid products of the plastidial methylerythritol phosphate (MEP) pathway [38]. The concentration of artemisinic acid in transplastomic plants reached up to 1.2% DW, favourable to yields obtained from *Artemisia annua* (0.01−1% DW) [38]. However, transient expression experiments in which diterpene biosynthesis was rerouted from the chloroplast to the cytosol to make use of the high-flux mevalonate pathway, improved yields of momilactone B [15]. Further, redirecting cytosolic enzymes from the strictosidine biosynthesis pathway to the plastid reduced yield [39]. These data indicate that while the copy number of the plastid genome may be

advantageous compared to nuclear encoded transgenes, metabolic flux and enzyme environment are critical for maximising yields.

Metabolic engineering to increase precursor availability

Recent years have also seen advances in *N. benthamiana* metabolic engineering. This includes the application of technologies such as RNAi and gene editing to manipulate endogenous metabolism (Figure 2 a-d) and synthetic biology strategies to engineer pathway localisation (Figure 2 e-g). The conservation of plant central metabolism means engineering the production of precursors is rarely required to produce plant natural products in *N. benthamiana*. However, precursor availability can be yield-limiting. Knocking down the expression of endogenous pathways that compete for the same precursor has been shown to increase yields of target molecules [40,41] (Figure 2a). Similarly, co-expressing genes encoding rate-limiting steps in the mevalonate pathway increased production of triterpenes and sesquiterpenes by increasing the pool of C_5 precursors [31,42]. Further increases in the yields of triterpenes and sesquiterpenes have been achieved using a translational fusion of FPPS and 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGR), or by expression of a feedback-insensitive HMGR [31,43]. Similarly, overexpression of Arabidopsis deoxyxylulose 5-phosphate synthase and hydroxymethylbutenyl diphosphate reductase (HDR) increased yields of linalool, casbene, and lathyrane (jolkinol C) in *N. benthamiana* by increasing the pool of C_5 precursors from the plastidial MEP pathway [13,42].

Figure 2



Metabolic engineering strategies to increase the yield and purity of target compounds in *Nicotiana benthamiana*. Panels A–D illustrate the manipulation of endogenous metabolism using gene silencing or editing approaches. Panels E–G illustrate synthetic scaffolding of pathways. Blue arrows represent the desired reactions, and purple arrows represent unwanted reactions. **a)** Silencing the gene encoding squalene synthase (SQS), which competes for the pool of (1) farnesyl pyrophosphate (FPP) to produce (2) squalene, increased the yields of (3) (+)-valencene from heterologous expression of *Callitropsis nootkatensis* valencene synthase (VS) (Cankar et al., 2014). **b)** Crocin production was engineered by heterologous expression of *Crocus sativus* carotenoid cleavage dioxygenase 2 (CCD2) to convert (4) zeaxanthin to (5) crocetin dialdehyde, an aldehyde dehydrogenase (ALDHH3I1) to convert (5) to (6) crocetin, and a UDP-glycosyltransferase (UGT91P3) to convert (6) to (7) crocins. CCD2 can also utilise (8) lutein as an alternative substrate resulting in the accumulation of (9) 3-OH-ϵ-apo-8′-carotenal. This unwanted reaction was reduced by mutating endogenous zeaxanthin epoxidases (ZEP1 and ZEP2) to reduce the production of (8) lutein [43]. **c)** In the early iridoid pathway (10), geranyl pyrophosphate is converted to (14) *cis–trans* nepetalactol via (11) geraniol, (12) 8-hydroxygeraniol, and (13) 8-oxogeranial by the action of geraniol synthase (GES), geraniol 8-oxidase (G8H), and iridoid synthase (ISY). Reconstruction of the *Catheranthus roseus* early iridoid pathway in *N. benthamiana* resulted in the accumulation of pathway intermediates with unwanted pentose- and hexose-sugar-modifications. This derivatisation was reduced in plant lines with loss-of-function mutations in endogenous UGTs (Dudley et al., 2022). **d)** Loss-of-function mutations in endogenous berberine bridge-like enzymes (BBLs) reduced the accumulation of alkaloids including nicotine, which can complicate product purification from *N. benthamiana* [51]. **e)** In *Sorghum bicolour* (18) Dhurrin is biosynthesised from (15) tyrosine via (16) p-hydroxyphenylacetaldoxime and (17) p-hydroxymandelonitrile by two cytochrome p450s (CYP79 and CYP71) and a UDP-glucosyltransferase (UGT85). Fusing these enzymes to *Arabidopsis thaliana* twin arginine translocation proteins B and C (AtTatB and AtTatC) created a synthetic metabolon in the thylakoid membrane, increasing yields and reducing product derivatisation [42]. **f)** (21) Squalene is produced from (19) isopentyl pyrophosphate to (21) squalene via (20) farnesylpyrophosphate by farnesyl pyrophosphate synthase (FPPS) and squalene synthase (SQS). The accumulation of squalene was increased by tag-mediated scaffolding of these enzymes on lipid-droplets produced via the expression of the *Arabidopsis thaliana* WRINKLED1 (AtWRI1) transcription factor. **g)** When reconstructed in chloroplasts, these lipid droplets reduced the negative effects of squalene production on photosynthesis [53].

Coexpression of an MYB transcription factor from montbretia (*Crocosmia × crocosmiiflora*) to upregulate the phenylpropanoid pathway together with a flavonol synthase and a flavonol 3′,5′-hydroxylase enhanced production of the aglycone precursor myricetin [21]. Similarly, Selma et al. used a Cas9 activator system (dCas9EV2.1) to selectively upregulate pathways demonstrating increased production of naringenin, eriodictyol, kaempferol, and quercetin [44]. Finally, maximising precursor flux into a heterologous pathway was enabled by fusing pathway enzymes to Tat proteins, anchoring them to the thylakoid to make synthetic metabolons. This led to a five-fold increase in dhurrin yields [45] (Figure 2e).

The availability of the correct precursors is also important to avoid nonspecific reactions. For example, when reconstructing casbene biosynthesis, it was noted that removal of HDR leads to a bottleneck that results in the incorporation of 4-hydroxy-3-methyl-but-2-enyl pyrophosphate to make 16-hydroxy-geranyllinalool, reducing yield [46]. Furthermore, glycosylation of montbretin A was found to be nonspecific when reconstructed in *N. benthamiana*, but coexpression of three genes from the shikimate shunt of the general phenylpropanoid pathway enabled production of the caffeoyl-CoA group required for montbretin A activity [22]. Genome editing was used to engineer *N. benthamiana* carotenoid-biosynthesis to predominantly accumulate zeaxanthin as precursor for crocin production, evading the promiscuity of the first enzyme of the native pathway, *Crocus sativus* carotenoid cleavage dioxygenase 2, for alternative substrates [8] (Figure 2b). Similar approaches have been proposed for increasing yields of the methionine-derived glucosinolate, glucoraphanin, as *N. benthamiana* produces more leucine and isoleucine-rich glucosinolates [30].

Strategies to engineer the accumulation and purity of target compounds

An often-reported difficulty of expressing natural products in *N. benthamiana* is the derivatization of pathway intermediates and target compounds. An early report demonstrated the action of endogenous glycosyltransferases on artemisinic acid to make artemisinic acid-12-b-diglucoside [43]. Subsequently, modifications including glutathione conjugates, cysteine conjugates, hydroxylation, and glycosides have been detected when producing monoterpenes, monoterpene-indole alkaloids, diterpenes, and sesquiterpenes [39,47–50]. This may also affect a wider range of compounds: although studies of *N. benthamiana* UDP-glycosyltransferases (UGTs) are limited, UGTs with high promiscuity to a range of substrates from different chemical classes have been identified in other plant species [51]. UGTs are primarily cytosolic, and relocating the dhurrin biosynthetic pathway to the plastids reduced derivatization five-fold [45]. A reduction in the derivatization of early intermediates of monoterpene indole alkaloid biosynthesis was observed in *N. benthamiana* lines with loss-of-function mutations in endogenous UGTs [39] (Figure 2c). *N. benthamiana* also produces alkaloids including nicotine that are likely to complicate purification of target compounds. The production of the thaumatin II protein required a chromatographic step to reduce alkaloids to acceptable levels [52], and separation from natural products is likely to be even more complex. To address this, genome editing has been used to produce low-nicotine lines of *N. benthamiana* and *N. tabacum* [53,54] (Figure 2d). Multiplexed genome engineering combined with further characterisation of *N. benthamiana* enzyme families may therefore provide routes for further improvements to this production chassis.

Engineering of *N. benthamiana* has also focussed on increasing the ability to store target compounds. For example, Delatte et al. increased the production of lipid bodies by expression of a triglyceride-synthesising enzyme, diacylglycerol acyltransferase-1 (AtDGAT1), from Arabidopsis together with transcription factors (AtWRI1 and AtOLE1) to upregulate fatty-acid biosynthesis [55]. When expressed with pathway genes, this strategy enabled a two-fold increase in stored a-barbatene and (*E*)-b-caryophyllene. Bibik et al. also expressed AtWRI1 to form lipid droplets, combining this with the fusion of squalene biosynthetic enzymes (farnesyl-pyrophosphate farnesyltransferase and squalene synthase) to a lipid-droplet surface protein from the microalga *Nannochloropsis oceanica* to target them to the lipid droplets for direct storage of squalene [56] (Figure 2 f). Heterologous expression of some pathways has been shown to be detrimental to photosynthesis [56,57]. Relocating the lipid-droplet strategy to the chloroplast reduced this impact [56,57] (Figure 2g). Other groups found that the number of lipid bodies in *N. benthamiana* could be increased by expression of a WRI1 homologue from *Perilla frutescens*, which may be utilised for further yield increases [58].

Conclusions

In the last few years, *N. benthamiana* has been utilised for the elucidation of numerous complex plant biosynthetic pathways (Figure 1). In particular, agroinfiltration has facilitated the testing and comparison of candidate genes for unknown pathway steps. However, the feasibility of agroinfiltration as a scalable platform for commercial production of natural products largely remains untested. To date, only a few molecules have been produced at a scale sufficient to enable purification and technoeconomic feasibility assessments are limited. Importantly, variability in the methods and units used to quantify product yields, combined with a limited understanding of production costs make it challenging to

compare production in *N. benthamiana* to other production chassis.

Other plant species being explored as scalable bioproduction chassis include the bryophytes *Marchantia polymorpha* and *Physcomitrella patens* [59,60]. These platforms offer the advantage of rapid biomass accumulation. However, although transformation methods are established and are faster than for many vascular plants, the timelines are still lengthy, compared to agroinfiltration, which makes them less attractive for prototyping and pathway elucidation. As the number and types of pathways reconstituted in *N. benthamiana* have increased, issues such as unwanted derivatization have become more apparent. The last few years have seen a focus on metabolic engineering to increase the yield, purity, and accumulation of target products (Figure 2). The availability of multiomic resources for *N. benthamiana* is likely to lead to further rapid advances [61]. However, there is still a pressing need for better molecular toolkits that enable the precise control of gene expression and efficient gene-editing to increase the throughput of metabolic engineering cycles.

Author contributions

NJP and CTwere responsible for conceptualisation. DG and CT were responsible for graphics. All authors contributed to writing and editing. NJP and CT were responsible for supervision. NJP was responsible for fundraising.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data were used for the research described in the article.

Acknowledgements

The authors acknowledge the financial support of the Department of Plant Sciences, University of Cambridge; UK Research and Innovation Engineering Biology awards BB/W014173/1 and BB/Y007751/1; Biotechnology and Biological Sciences Research Council Earlham Institute Strategic Programme Grant DECODE (BBX011089/1) and its constituent work package BBS/E/ER/230002B; DG is supported by a John Innes Foundation PhD scholarship; HS is supported by the BBSRC Norwich Research Park Doctoral Training Partnership Scholarship (BB/T008717/1 Project No. 2578291).

References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Howes M-JR, Quave CL, Collemare J, Tatsis EC, Twilley D, Lulekal E, Farlow A, Li L, Cazar M-E, Leaman DJ, *et al.*: **Molecules from nature: reconciling biodiversity conservation and global healthcare imperatives for sustainable use of medicinal plants and fungi**. *Plants People Planet* 2020, **2**:463–481.

2. Wendisch VF, Brito LF, Gil Lopez M, Hennig G, Pfeifenschneider J, Sgobba E, Veldmann KH: **The flexible feedstock concept in industrial biotechnology: metabolic engineering of *Escherichia coli*, *Corynebacterium glutamicum*, *Pseudomonas*, *Bacillus* and yeast strains for access to alternative carbon sources**. *J Biotechnol* 2016, **234**: 139–157.

3. Stephenson MJ, Reed J, Patron NJ, Lomonossoff GP, Osbourn A: **Engineering tobacco for plant natural product production**. In *Comprehensive natural products III*. Edited by Liu H-W (ben), Begley TP, Elsevier; 2020:244–262.

4. Bally J, Nakasugi K, Jia F, Jung H, Ho SYW, Wong M, Paul CM, Naim F, Wood CC, Crowhurst RN, *et al.*: **The extremophile Nicotiana benthamiana has traded viral defence for early vigour**. *Nat Plants* 2015, **1**, 15165.

5. Yao X, Wuzhang K, Peng B, Chen T, Zhang Y, Liu H, Li L, Fu X, Tang K: **Engineering the expression of plant secondary metabolites-genistein and scutellarin through an efficient transient production platform in *Nicotiana benthamiana* L.** *Front Plant Sci* 2022, **13**, 994792.

6. ** Dodds I, Chen C, Buscaill P, van der Hoorn RAL: **Depletion of the NbCORE receptor drastically improves agroinfiltration productivity in older *Nicotiana benthamiana* plants**. *Plant Biotechnol J* 2023, **21**:1103–1105.
This study describes the production of *Nicotiana benthamiana* lines with a mutation in the gene responsible for perception of the cold shock protein (CSP) of *Agrobacterium tumefaciens*. This mutation abolished age-related immunity and led to a drastic increase in transient expression, enabling the infiltration of older plants with more biomass.

7. De Paola C, Garcia-Carpintero V, Vazquez-Vilar M, Kaminski K, Fernandez-Del-Carmen A, Sierro N, Ivanov NV, Giuliano G, Waterhouse P, Orzaez D: **Comparative analysis of the squamosa promoter binding-like (SPL) gene family in *Nicotiana benthamiana* and *Nicotiana tabacum***. *Plant Sci* 2023, **335**, 111797.

8. * Demurtas OC, Sulli M, Ferrante P, Mini P, Martí M, Aragonés V, Daròs J-A, Giuliano G: **Production of saffron apocarotenoids in *Nicotiana benthamiana* plants genome-edited to accumulate zeaxanthin precursor**. *Metabolites* 2023, **13**.
Metabolic engineering of carotenoid biosynthesis to produce *Nicotiana benthamiana* lines with higher zeaxanthin content, used to increase heterologous biosynthesis of crocins.

9. Martí M, Diretto G, Aragonés V, Frusciante S, Ahrazem O, Gómez-Gómez L, Daròs J-A: **Efficient production of saffron crocins and picrocrocin in *Nicotiana benthamiana* using a virus-driven system**. *Metab Eng* 2020, **61**:238–250.

10. * Carlson ED, Rajniak J, Sattely ES: **Multiplicity of the *Agrobacterium* infection of *Nicotiana benthamiana* for transient DNA delivery**. *ACS Synth Biol* 2023, **12**:2329–2338.
This manuscript analysed the Poisson probability distribution of T-DNA transfer from *Agrobacterium tumefaciens* to leaf pavement cells during agroinfiltration. This enabled control of transfection and the identification of experimental regimes where the probability of near-simultaneous delivery of >20 unique T-DNAs remains high throughout the leaf.

11. Jongedijk E, Müller S, van Dijk ADJ, Schijlen E, Champagne A, Boutry M, Levisson M, van der Krol S, Bouwmeester H, Beekwilder J: **Novel routes towards bioplastics from plants: elucidation of the methylperillate biosynthesis pathway from *Salvia dorisiana* trichomes**. *J Exp Bot* 2020, **71**:3052–3065.

12. Xu H, Li W, Schilmiller AL, van Eekelen H, de Vos RCH, Jongsma MA, Pichersky E: **Pyrethric acid of natural pyrethrin insecticide: complete pathway elucidation and reconstitution in *Nicotiana benthamiana***. *New Phytol* 2019, **223**:751–765.

13. * Forestier ECF, Czechowski T, Cording AC, Gilday AD, King AJ, Brown GD, Graham IA: **Developing a *Nicotiana benthamiana* transgenic platform for high-value diterpene production and candidate gene evaluation**. *Plant Biotechnol J* 2021, **19**: 1614–1623.
Combinatorial metabolic engineering of the nonmevalonate pathway to identify optimal combinations for optimising precursor supply for the production of complex diterpenoids in *Nicotiana benthamiana*.

14. Zhang Y, Wiese L, Fang H, Alseekh S, Perez de Souza L, Scossa F, Molloy J, Christmann M, Fernie AR: **Synthetic biology identifies the minimal gene set required for paclitaxel biosynthesis in a plant chassis**. *Mol Plant* 2023, **16**:1951–1961.

15. ** De La Peña R, Sattely ES: **Rerouting plant terpene biosynthesis enables momilactone pathway elucidation**. *Nat Chem Biol* 2021, **17**:205–212.
This work describes the rerouting of diterpene biosynthesis to utilise the high-flux, cytosolic mevalonate pathway. This was used to reconstitute the pathway for momilactone B biosynthesis, which was then purified and used to validate its allelopathic activity. It was also used to improve yields of heterologously produced forskolin and taxadiene.

16. Reed J, Orme A, El-Demerdash A, Owen C, Martin LBB, Misra RC, Kikuchi S, Rejzek M, Martin AC, Harkess A, *et al.*: **Elucidation of the pathway for biosynthesis of saponin adjuvants from the soapbark tree**. *Science* 2023, **379**:1252–1264.

17. * Martin LBB, Kikuchi S, Rejzek M, Owen C, Reed J, Orme A, Misra RC, El-Demerdash A, Hill L, Hodgson H, *et al.*: **Complete biosynthesis of the potent vaccine adjuvant QS-21**. *Nat Chem Biol* 2024, https://doi.org/10.1038/s41589-023-01538-5.
The reconstitution of a 20-step pathway for the production of the vaccine adjuvant, QS-21, in *Nicotiana benthamiana*

18. Hodgson H, De La Peña R, Stephenson MJ, Thimmappa R, Vincent JL, Sattely ES, Osbourn A: **Identification of key enzymes responsible for protolimonoid biosynthesis in plants: opening the door to azadirachtin production**. *Proc Natl Acad Sci U S A* 2019, **116**:17096–17104.

19. Pan Z, Bajsa-Hirschel J, Vaughn JN, Rimando AM, Baerson SR, Duke SO: **In vivo assembly of the sorgoleone biosynthetic pathway and its impact on agroinfiltrated leaves of *Nicotiana benthamiana***. *New Phytol* 2021, **230**:683–697.

20. Berman P, de Haro LA, Jozwiak A, Panda S, Pinkas Z, Dong Y, Cveticanin J, Barbole R, Livne R, Scherf T, *et al.*: **Parallel evolution of cannabinoid biosynthesis**. *Nat Plants* 2023, **9**:817–831.

21. Irmisch S, Jancsik S, Man Saint Yuen M, Madilao LL, Bohlmann J: **Complete Biosynthesis of the anti-diabetic plant metabolite Montbretin A**. *Plant Physiol* 2020, **184**:97–109.

22. Kruse LH, Sunstrum FG, Garcia D, López Pérez G, Jancsik S, Bohlmann J, Irmisch S: **Improved production of the antidiabetic metabolite montbretin A in *Nicotiana benthamiana*: discovery, characterization, and use of *Crocosmia shikimate* shunt genes**. *Plant J* 2024, **117**:766–785.

23. Shen S, Wang S, Yang C, Wang C, Zhou Q, Zhou S, Zhang R, Li Y, Wang Z, Dai L, *et al.*: **Elucidation of the melitidin biosynthesis pathway in pummelo**. *J Integr Plant Biol* 2023, **65**: 2505–2518.

24. * Hong B, Grzech D, Caputi L, Sonawane P, López CER, Kamileen MO, Hernández Lozada NJ, Grabe V, O'Connor SE: **Biosynthesis of strychnine**. *Nature* 2022, **607**:617–622.
The use of *Nicotiana benthamiana* as a platform for the elucidation and reconstitution of the biosynthesis pathway for the neurotoxin, strychnine.

25. Florean M, Luck K, Hong B, Nakamura Y, O'Connor SE, Köllner TG: **Reinventing metabolic pathways: independent evolution of benzoxazinoids in flowering plants**. *Proc Natl Acad Sci U S A* 2023, **120**, e2307981120.

26. * Nett RS, Lau W, Sattely ES: **Discovery and engineering of colchicine alkaloid biosynthesis**. *Nature* 2020, **584**:148–153.
The elucidation and reconstruction of a 16-gene biosynthetic pathway for N-formyldemecolcine from the amino acids phenylalanine and tyrosine.

27. * Wang Y-J, Huang J-P, Tian T, Yan Y, Chen Y, Yang J, Chen J, Gu Y-C, Huang S-X: **Discovery and engineering of the cocaine biosynthetic pathway**. *J Am Chem Soc* 2022, **144**: 22000–22007.
Reconstruction of cocaine biosynthesis in *Nicotiana benthamiana*, including the characterisation of two previously unidentified genes necessary for the biosynthesis of the tropane skeleton.

28. Yin X, Liu J, Kou C, Lu J, Zhang H, Song W, Li Y, Xue Z, Hua X: **Deciphering the network of cholesterol biosynthesis in *Paris polyphylla* laid a base for efficient diosgenin production in plant chassis**. *Metab Eng* 2023, **76**:232–246.

29. Lv Y, Zhu J, Huang S, Xing X, Zhou S, Yao H, Yang Z, Liu L, Huang S, Miao Y, *et al.*: **Metabolome profiling and transcriptome analysis filling the early crucial missing steps of piperine biosynthesis in *Piper nigrum* L.** *Plant J* 2024, **117**: 107–120.

30. Barnum CR, Endelman BJ, Ornelas IJ, Pignolet RM, Shih PM: **Optimization of heterologous glucoraphanin production *in planta***. *ACS Synth Biol* 2022, **11**:1865–1873.

31. Reed J, Stephenson MJ, Miettinen K, Brouwer B, Leveau A, Brett P, Goss RJM, Goossens A, O'Connell MA, Osbourn A: **A translational synthetic biology platform for rapid access to gram-scale quantities of novel drug-like molecules**. *Metab Eng* 2017, **42**:185–193.

32. Boccia M, Grzech D, Lopes AA, O'Connor SE, Caputi L: **Directed biosynthesis of New to nature alkaloids in a heterologous *Nicotiana benthamiana* expression host**. *Front Plant Sci* 2022, **13**, 919443.

33. Chen Q, Lai H, Hurtado J, Stahnke J, Leuzinger K, Dent M: **Agroinfiltration as an effective and scalable strategy of gene delivery for production of pharmaceutical proteins**. *Adv Tech Biol Med* 2013, **1**:103.

34. * Kallam K, Moreno-Giménez E, Mateos-Fernández R, Tansley C, Gianoglio S, Orzaez D, Patron N: **Tunable control of insect pheromone biosynthesis in *Nicotiana benthamiana***. *Plant Biotechnol J* 2023, **21**:1440–1453.
The use of synthetic genetic elements and construct topology to tune the expression of pathway genes, optimising the yield of heterologous expressed insect sex pheromones. This paper also includes a comparison of yields obtained using transient and transgenic approaches.

35. Mateos-Fernández R, Moreno-Giménez E, Gianoglio S, Quijano-Rubio A, Gavaldá-García J, Estellés L, Rubert A, Rambla JL, Vazquez-Vilar M, Huet E, *et al.*: **Production of volatile moth sex pheromones in transgenic *Nicotiana benthamiana* Plants**. *Biodes Res* 2021, **2021**, 9891082.

36. Grzech D, Hong B, Caputi L, Sonawane PD, O'Connor SE: **Engineering the biosynthesis of late-Stage vinblastine precursors precondylocarpine acetate, catharanthine, tabersonine in *Nicotiana benthamiana***. *ACS Synth Biol* 2023, **12**:27–34.

37. Schmidt JA, McGrath JM, Hanson MR, Long SP, Ahner BA: **Field-grown tobacco plants maintain robust growth while accumulating large quantities of a bacterial cellulase in chloroplasts**. *Nat Plants* 2019, **5**:715–721.

38. Fuentes P, Zhou F, Erban A, Karcher D, Kopka J, Bock R: **A new synthetic biology approach allows transfer of an entire metabolic pathway from a medicinal plant to a biomass crop**. *Elife* 2016, **5**.

39. * Dudley QM, Jo S, Guerrero DAS, Chhetry M, Smedley MA, Harwood WA, Sherden NH, O'Connor SE, Caputi L, Patron NJ: **Reconstitution of monoterpene indole alkaloid biosynthesis in genome engineered *Nicotiana benthamiana***. *Commun Biol* 2022, **5**:949.
This manuscript describes the reconstruction of strictosidine biosynthesis from primary metabolism by co-expression of 14 genes, as well as the identification and gene-editing of *Nicotiana benthamiana* glycosyltransferases that modify pathway intermediates

40. Cankar K, Jongedijk E, Klompmaker M, Majdic T, Mumm R, Bouwmeester H, Bosch D, Beekwilder J: **(+)-Valencene production in *Nicotiana benthamiana* is increased by down-regulation of competing pathways**. *Biotechnol J* 2015, **10**: 180–189.

41. Grützner R, König K, Horn C, Engler C, Laub A, Vogt T, Marillonnet S: **A transient expression tool box for anthocyanin biosynthesis in *Nicotiana benthamiana***. *Plant Biotechnol J* 2024, **22**:1238–1250.

42. Park S, Mani V, Kim JA, Lee SI, Lee K: **Combinatorial transient gene expression strategies to enhance terpenoid production in plants**. *Front Plant Sci* 2022, **13**, 1034893.

43. van Herpen TWJM, Cankar K, Nogueira M, Bosch D, Bouwmeester HJ, Beekwilder J: **Nicotiana benthamiana as a production platform for artemisinin precursors**. *PLoS One* 2010, **5**, e14222.

44. Selma S, Sanmartín N, Espinosa-Ruiz A, Gianoglio S, Lopez-Gresa MP, Vázquez-Vilar M, Flors V, Granell A, Orzaez D: **Custom-made design of metabolite composition in N. benthamiana leaves using CRISPR activators**. *Plant Biotechnol J* 2022, **20**:1578–1590.

45. Henriques de Jesus MPR, Zygadlo Nielsen A, Busck Mellor S, Matthes A, Burow M, Robinson C, Erik Jensen P: **Tat proteins as novel thylakoid membrane anchors organize a biosynthetic pathway in chloroplasts and increase product yield 5-fold**. *Metab Eng* 2017, **44**:108–116.

46. Forestier ECF, Brown GD, Harvey D, Larson TR, Graham IA: **Engineering production of a novel diterpene synthase precursor in *Nicotiana benthamiana***. *Front Plant Sci* 2021, **12**, 757186.

47. Liu Q, Majdi M, Cankar K, Goedbloed M, Charnikhova T, Verstappen FWA, de Vos RCH, Beekwilder J, van der Krol S, Bouwmeester HJ: **Reconstitution of the costunolide biosynthetic pathway in yeast and *Nicotiana benthamiana***. *PLoS One* 2011, **6**, e23255.

48. Brückner K, Tissier A: **High-level diterpene production by transient expression in *Nicotiana benthamiana***. *Plant Methods* 2013, **9**:46.

49. Miettinen K, Dong L, Navrot N, Schneider T, Burlat V, Pollier J, Woittiez L, van der Krol S, Lugan R, Ilc T, *et al.*: **The seco-iridoid pathway from *Catharanthus roseus***. *Nat Commun* 2014, **5**:3606.

50. Dong L, Jongedijk E, Bouwmeester H, Van Der Krol A: **Monoterpene biosynthesis potential of plant subcellular compartments**. *New Phytol* 2016, **209**:679–690.

51. Lu X, Huang L, Scheller HV, Keasling JD: **Medicinal terpenoid UDP-glycosyltransferases in plants: recent advances and research strategies**. *J Exp Bot* 2023, **74**:1343–1357.

52. **GRAS notice 910 thaumatin II produced in Nicotiana benthamiana**. *Food and Drug Administration* 2020. https://fda.report/media/139609/GRAS-Notice-GRN-910-Thaumatin-II-produced-in-Nicotiana-benthamiana.pdf; 2020.

53. Lewis RS, Drake-Stowe KE, Heim C, Steede T, Smith W, Dewey RE: **Genetic and agronomic analysis of tobacco genotypes exhibiting reduced nicotine accumulation due to induced mutations in berberine bridge like (BBL) genes**. *Front Plant Sci* 2020, **11**.

54. Vollheyde K, Dudley QM, Yang T, Oz MT, Mancinotti D, Fedi MO, Heavens D, Linsmith G, Chhetry M, Smedley MA, *et al.*: **An improved *Nicotiana benthamiana* bioproduction chassis provides novel insights into nicotine biosynthesis**. *New Phytol* 2023, **240**:302–317.

55. Delatte TL, Scaiola G, Molenaar J, de Sousa Farias K, Alves Gomes Albertti L, Busscher J, Verstappen F, Carollo C, Bouwmeester H, Beekwilder J: **Engineering storage capacity for volatile sesquiterpenes in *Nicotiana benthamiana* leaves**. *Plant Biotechnol J* 2018, **16**:1997–2006.

56. ** Bibik JD, Weraduwage SM, Banerjee A, Robertson K, Espinoza-Corral R, Sharkey TD, Lundquist PK, Hamberger BR: **Pathway engineering, re-targeting, and synthetic scaffolding improve the production of squalene in plants**. *ACS Synth Biol* 2022, **11**:2121–2133.

The engineering of biosynthetic scaffolds on lipid droplets to improve yields of squalene. These scaffolds were also re-targeted to plastids where they ameliorated the negative impact of squalene biosynthesis on photosynthesis.

57. Frusciante S, Demurtas OC, Sulli M, Mini P, Aprea G, Diretto G, Karcher D, Bock R, Giuliano G: **Heterologous expression of *Bixa orellana* cleavage dioxygenase 4-3 drives crocin but not bixin biosynthesis**. *Plant Physiol* 2022, **188**:1469–1482.

58. Kim S, Lee K-R, Suh MC: **Ectopic expression of *Perilla frutescens* WRI1 enhanced storage oil accumulation in *Nicotiana benthamiana* Leaves**. *Plants* 2023, **12**.

59. Bowman JL, Arteaga-Vazquez M, Berger F, Briginshaw LN, Carella P, Aguilar-Cruz A, Davies KM, Dierschke T, Dolan L, Dorantes-Acosta AE, *et al.*: **The renaissance and enlightenment of Marchantia as a model system**. *Plant Cell* 2022, **34**:3512–3542.

60. Reski R, Bae H, Simonsen HT: ***Physcomitrella patens*, a versatile synthetic biology chassis**. *Plant Cell Rep* 2018, **37**:1409–1417.

61. ** Ranawaka B, An J, Lorenc MT, Jung H, Sulli M, Aprea G, Roden S, Llaca V, Hayashi S, Asadyar L, *et al.*: **A multi-omic *Nicotiana benthamiana* resource for fundamental research and biotechnology**. *Nat Plants* 2023, **9**:1558–1571.

An exceptional multiomic resource for *Nicotiana benthamiana*. This manuscript also compares the widely used *N. benthamiana* LAB with a wild ecotype (QLD).