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FOCUSED REVIEW

Navigating the challenges of engineering composite specialized metabolite pathways in plants

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SUMMARY

Plants are a valuable source of diverse specialized metabolites with numerous applications. However, these compounds are often produced in limited quantities, particularly under unfavorable ecological conditions. To achieve sufficient levels of target metabolites, alternative strategies such as pathway engineering in heterologous systems like microbes (e.g., bacteria and fungi) or cell-free systems can be employed. Another approach is plant engineering, which aims to either enhance the native production in the original plant or reconstruct the target pathway in a model plant system. Although increasing metabolite production in the native plant is a promising strategy, these source plants are often exotic and pose significant challenges for genetic manipulation. Effective pathway engineering requires comprehensive prior knowledge of the genes and enzymes involved, as well as the precursor, intermediate, branching, and final metabolites. Thus, a thorough elucidation of the biosynthetic pathway is closely linked to successful metabolic engineering in host or model systems. In this review, we highlight recent advances in strategies for biosynthetic pathway elucidation and metabolic engineering. We focus on efforts to engineer complex, multi-step pathways that require the expression of at least eight genes for transient and three genes for stable transformation. Reports on the engineering of complex pathways in stably transformed plants remain relatively scarce. We discuss the major hurdles in pathway elucidation and strategies for overcoming them, followed by an overview of achievements, challenges, and solutions in pathway reconstitution through metabolic engineering. Recent advances including computer-based predictions offer valuable platforms for the sustainable production of specialized metabolites in plants.

Keywords: metabolites, biosynthetic pathway, gene expression, metabolic engineering, enzyme activity.

INTRODUCTION

Plants, as sessile organisms, have evolved to produce an extensive and diverse array of specialized metabolites that play pivotal roles in their survival. These metabolites regulate endogenous metabolism, defend against threats, communicate within cells and with the external environment, and help plants adapt to diverse environmental challenges (Huang & Dudareva, 2023; Kessler & Kalske, 2018; Maeda & Fernie, 2021). These metabolites, often structurally complex and synthesized in specific plant tissues, cells, or developmental stages, are tightly controlled by complex networks of biosynthetic enzymes, transporters, and regulatory factors (Li et al., 2024). Historically recognized for their diverse properties, plant specialized metabolites have gained significant interest across various industries, including pharmaceuticals, agriculture, nutraceuticals, cosmetics, and environmental sciences due to their precise bioactive and structural (i.e., specific chiral forms) properties (Howes et al., 2020).

The complexity of these metabolites often lies in their intricate biosynthetic pathways, which involve multiple enzymes and regulatory elements, making them species- and tissue-specific as well as temporally and spatially regulated. Despite their potential, the practical applications of

many plant metabolites are constrained by challenges such as limited abundance in natural sources, difficulties in complete chemical synthesis, and the complexity of their extraction from plant tissues (Bai, Liu, & Baldwin, 2024; Owen et al., 2017; Pyne et al., 2019). Therefore, researchers genetically engineer microbial and plant model systems to express genes involved in the biosynthesis of such metabolites (Zhu et al., 2021). Biotechnological approaches have been developed to elucidate and reconstruct the biosynthetic pathways of these valuable metabolites in suitable heterologous plant systems. These strategies include the use of advanced techniques in genomics, transcriptomics, metabolomics, and synthetic biology to identify, characterize, and engineer the enzymes involved. *Nicotiana benthamiana* has emerged as a prominent model system for plant synthetic biology due to the efficiency of transient expression and the capacity to functionally validate complex biosynthetic pathways. As plant synthetic biology develops further innovative prospects arise to generate specialized metabolites with commercial value and 'new to nature' ones in plants (Joshi & Hanson, 2024; Li et al., 2021; Perrot et al., 2024). This review will focus on the major successes, challenges, and future directions in the elucidation and synthetic reconstruction of complex metabolite biosynthesis pathways in plants.

ROADMAP FOR PLANT METABOLIC PATHWAY ENGINEERING

Elucidating the biosynthetic pathways of plant metabolites requires the integration of chemistry, molecular biology, genomics, transcriptomics, bioinformatics, and genetic engineering. In-depth transcriptomics analysis of plant tissues where metabolite synthesis or storage occurs, followed by co-expression and differential expression analyses, often guides the identification of potentially associated individual genes. Genome sequencing can also reveal metabolic gene clusters involved in biosynthetic pathways and facilitate gene discovery. In silico tools and methods, such as GeNeCK (Zhang et al., 2019), CoExpNetViz (Tzfadia et al., 2016), and MapMan (Schwacke et al., 2019), are vital for candidate gene selection. Functional analysis frequently involving gene overexpression, knockdown, knockout, enzyme assays, and metabolic profiling is then used to characterize individual genes. *N. benthamiana* is widely utilized as a platform for pathway reconstruction. Its efficiency in transient expression, scalability, ability to co-express multiple genes simultaneously, high product levels, and relative reproducibility make it an ideal system (Golubova et al., 2024; Vollheyde et al., 2023). Several examples of complex plant metabolic pathways reconstructed in *N. benthamiana* are presented in Table 1. For stable expression, other plants such as *Arabidopsis*, *Nicotiana tabacum*, tomato, and rice are commonly used, particularly when the expression of three or more genes is involved (Table 2).

KEY BREAKTHROUGHS IN THE ELUCIDATION AND ENGINEERING OF COMPLEX PLANT METABOLIC PATHWAYS

Advancements in metabolite analysis and multi-omics integration support plant specialized metabolite pathway elucidation

The integration of omics data has revolutionized metabolic pathway discovery and gene function elucidation (Figure 1). Researchers are driving advances in metabolomics through the use of high-end analytical tools such as liquid chromatography-high resolution mass spectrometry (LC-HRMS), gas chromatography–mass spectrometry (GC–MS), and nuclear magnetic resonance (NMR) spectroscopy, which have accelerated the discovery and structural elucidation of plant metabolites (Table 3). Annotation of metabolites remains challenging, even with advanced mass spectrometry (MS) techniques. High-confidence metabolite identification is achieved using MS technologies, following significant developments in mass analyzers that display enhanced scanning speed, resolution, sensitivity, tandem MS (MSn) fragmentation, coupling to ion mobility separation (IMS) and throughput. Improvements in liquid chromatography separation, Fourier transform ion cyclotron resonance (FT-ICR), and quadrupole time-of-flight (QTOF) MS further advance targeted metabolite analysis. Significant progress in NMR hardware, such as cryoprobes and high-field magnets (1–1.2 GHz), has enhanced sensitivity and resolution in NMR-based metabolomics (Kupče et al., 2021; Palmer, 2014). Techniques like high-resolution magic-angle spinning (HRMAS) NMR, X-ray crystallography using crystalline sponge methods, and electron cryo-microscopy for small-molecule structure determination have refined metabolite structure prediction (Beckonert et al., 2010; Inokuma et al., 2013; Nannenga & Gonen, 2018) (Table 3). The combination of MS imaging and metabolomics has expanded spatial metabolite analysis, enabling precise determination of specialized metabolites' dynamics *in vivo* (Peng et al., 2021).

Computational fragment analysis tools such as SIRIUS (Ludwig et al., 2020) enhance the accuracy of molecular formula determination from MS data by improving spectral fragment interpretation. The availability of public metabolomics spectral databases like METLIN, GNPS, and MassBank has facilitated metabolite identification and analysis through data sharing and comparison. Recent updates to the GNPS platform, including tools like Qemistree and Feature-Based Molecular Networking (FBMN), advance metabolomics research by enabling sophisticated spectral analysis and structure elucidation. Machine learning tools, such as CANOPUS (Dührkop et al., 2021) and WEIZMASS (Shahaf et al., 2016), leverage MS/MS spectral data to predict compound classes even without reference spectra (Table 3). Approaches using support vector machines and

Table 1 Examples of complex and transient metabolic pathway engineering (≥8 genes) in *Nicotiana benthamiana* (past 5 years)

Type of product	Final product	Host plant	Main methods used	Number of expressed genes	Yield	References
Terpenoid	Momilactones	*Oryza sativa*	NMR, Confocal microscopy, GC–MS, transcriptome data mining and analysis	8	167 μg g^{-1} dry weight	de la Peña and Sattely (2021)
Indole glucosinolate	Crucifalexin	*Brassica rapa*	NMR, LC–MS	8	nr	Calgaro-Kozina et al. (2020)
Tropane alkaloid	Cocaine	*Erythroxylum novogranatense*	Transcriptome analysis, *in vitro* assays, yeast expression, NMR, LC–MS	8	398.3 ± 132.0 ng mg^{-1} dry weight	Wang et al. (2022)
Flavonol glycoside	Montbretin	Montbretia (*Crocosmia* X *crocosmiiflora*)	Transcriptome sequencing, *in vitro* assays, LC–MS	9	nr	Irmisch et al. (2020)
Monoterpene Indole Alkaloids	Brucine	*Strychnos nux-vomica*	In vitro assays, LC–MS, transcriptomics, co-expression, confocal microscopy, NMR	9	nr	Hong et al. (2022)
Triterpenoid Saponin	Medicagenic acid and its (poly)glycosylated derivatives	*Spinacia oleracea*	RNA-Seq, Co-expression, LC–MS, NMR, *in vitro* assays	9	nr	Jozwiak et al. (2020)
Terpenoid	Azadirone	*Melia azedarach*	Genome sequencing, NMR, LC–MS	10	nr	de la Peña et al. (2023)
Terpenoid	Kihadalactone A	*Citrus sinensis*	Transcriptome data mining and analysis, NMR, co-expression, LC–MS	11	nr	de la Peña et al. (2023)
Anthocyanin	Cyanidin 3-*O*-glucoside	*Arabidopsis thaliana*	CRISPR, LC–MS	13	nr	Grützner et al. (2024)
Monoterpene Indole Alkaloids	Strictosidine	*Catharantus roseus*	LC–MS, crispr-cas9, transcriptomics	14	nr	Dudley et al. (2022)
Triterpenoid Saponin	Saponarioside B	*Saponaria officinalis*	Genome sequencing, RNA-Seq, RNA interference, NMR, LC–MS	14	nr	Jo et al. (2024)
Terpenoid	N-Formyldemecolcine	*Gloriosa superba*	Transcriptomics, co-expression, *in vitro* assays, yeast expression, NMR, LC–MS	16	6.3 ± 1.3 μg g^{-1} dry weight	Nett et al. (2020)
Phenolic compounds	(−)-deoxy-podophyllotoxin	*Sinopodophyllum hexandrum*	Transcriptome data analysis, LC–MS, NMR	16	4300 μg g^{-1} dry weight	Schultz et al. (2019)
Terpenoid	Baccatin III	*Taxus media var. hicksii*	Single-cell transcriptomic, Co-expression, GC–MS, immunoblot, NMR	17	10–30 μg g^{-1} dry weight	McClune et al. (2024)
Triterpene glycoside	QS-7	*Quillaja saponaria*	Genome sequencing, transcriptomics, LC–MS, GC–MS	19	nr	Reed et al. (2023)

(continued)

Table 1. (continued)

Type of product	Final product	Host plant	Main methods used	Number of expressed genes	Yield	References
Steroidal saponin	Diosgenin	*Paris polyphylla*	Co-expression analysis, GC–MS, NMR, VIGS	19	2120 μg g^{-1} dry weight	Yin et al. (2023)
Triterpene glycoside	QS-21	*Quillaja saponaria*	Genome mining, transcriptome data analysis, yeast expression, LC–MS, *in vitro* assays, NMR	23	nr	Martin et al. (2024)

nr, not recorded.

deep neural networks trained on numerous compound structures have improved prediction efficiency (Chen et al., 2018; Poltorak et al., 2024).

Early research on plant specialized metabolites relied on classical methods such as radiolabel tracing and enzyme purification, but advancements in next-generation sequencing, integrated omics approaches, and synthetic biology have transformed the field, enabling rapid pathway discovery (Figure 1A). The combination of techniques such as single-molecule real-time sequencing (PacBio) (Eid et al., 2009; Flusberg et al., 2010), nano-pore sequencing, Hi-C, and linked-read technologies (10X genomics) has facilitated the identification of genes involved in metabolite biosynthesis (Cheng et al., 2021) (Table 3). The third-generation sequencing technology from PacBio provides long reads and accurate genomic and transcriptomic data for genes and pathway discovery (Liu et al., 2022; van Dijk et al., 2018). Emerging methods, including single-cell RNA sequencing, spatial transcriptomics, and single-cell spatial resolution omics sequencing (Stereo-seq), further enhance pathway elucidation by providing spatial gene expression data (Belchikov et al., 2024; Shen et al., 2024; Xia et al., 2022; Yu et al., 2023). These approaches also help to identify regulatory proteins and metabolite transporters linked to metabolic pathways.

Chemical biology approaches, including isotope labeling, affinity probes, and biomimetic reactions, accelerate pathway elucidation by guiding researchers in selecting enzyme isoforms and reaction intermediates (Seligmann et al., 2024). Machine learning tools like DeepEC, PRISM, PathPred, deep transfer learning (GTC), and GeneNet have advanced the prediction of enzyme functions and pathway dynamics, aiding gene identification and pathway engineering optimization (Bao et al., 2023; Ryu et al., 2019; Skinnider et al., 2020) (Figure 1B). Additionally, genome-wide association studies (GWAS) and the use of hybrid and mutant populations have linked genomic polymorphisms with variations in specialized metabolite abundance, uncovering biosynthetic genes (Fang et al., 2019; Szymański et al., 2020).

Advanced synthetic biology platforms for complex metabolic pathway engineering

Metabolic engineering often involves the overexpression of multiple genes and, less commonly, the suppression or knockout of genes to reduce their activity. Until about half a decade ago, RNA interference (RNAi) was the preferred method for silencing genes. However, advancements in genome editing tools, such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9) and related systems, now enable precise modification of genes and their regulatory elements (Figure 1C, Table 3). CRISPR technology directs the Cas9 protein to target and cleave

Table 2 Examples of stable metabolic pathway engineering (≥3 genes) in plant systems

Type of product	Final product	Host plant	Number of expressed genes	Yield	References
Vitamin E	Tocopherol	*Nicotiana tabacum*, *Solanum lycopersicum*	3	nr	Lu et al. (2013)
Glycosidic food dye	Betanin	*Nicotiana tabacum*, *Solanum lycopersicum*, *Solanum tuberosum*, *Solanum melongena*, *Nicotiana glauca*, *Solanum nigrum*, *Petunia*, *Nicotiana benthamiana*	3	nr	Polturak et al. (2016)
Thiamin	Vitamin B1	*Oryza sativa*	3	nr	Strobbe et al. (2021)
Amino acid	Lysine	*Oryza sativa*	3	437.8 μg g^{-1} dry weight	Yang et al. (2016)
Polyphenols	Stilbenes	*Nicotiana tabacum*	3	40 μg L^{-1}	Hidalgo et al. (2017)
Carotenoid	β-carotene	*Brassica napus*	3	nr	Ravanello et al. (2003)
Carotenoid	4-keto-lutein	*Nicotiana tabacum*	3	nr	Tanwar et al. (2023)
Sesquiterpene	Artemisinin	*Nicotiana tabacum*	4	120 mg kg^{-1} fresh weight	Fuentes et al. (2016)
Carotenoid	β-carotene	*Oryza sativa*	4	37 μg g^{-1} dry weight	Paine et al. (2005)
Carotenoid	β-carotene	*Oryza sativa*	4	1.6 μg g^{-1} dry weight	Ye et al. (2000)
Carotenoid	β-carotene	*Zea mays*	5	26.33 ± 0.15 μg g^{-1} dry weight	Farré et al. (2013)
Sterol	Cholesterol	*Arabidopsis thaliana*	11	nr	Sonawane et al. (2016)
Monocarboxylic acid	Glycolate	*Nicotiana tabacum*	7	nr	South et al. (2019)

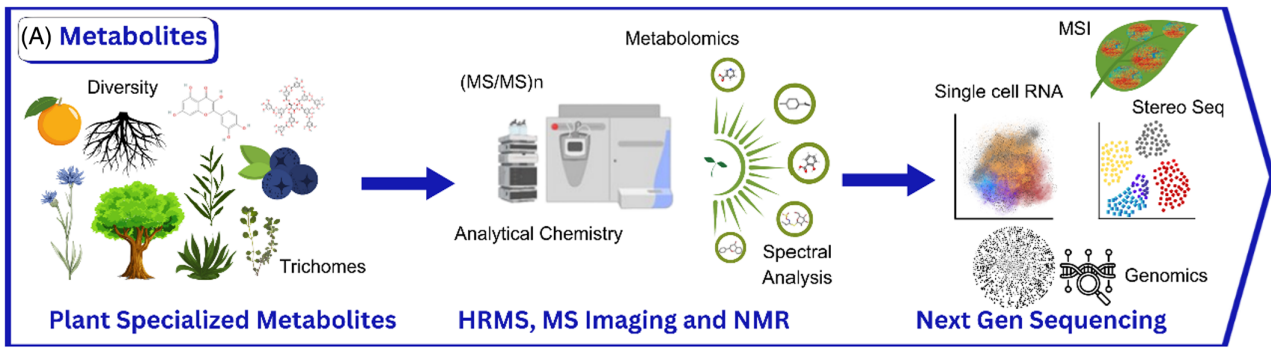
nr, not recorded.

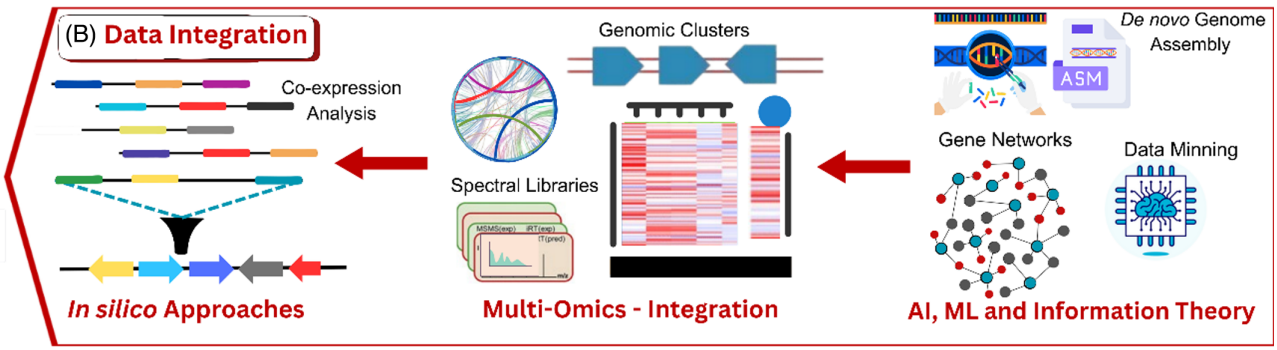
double-stranded DNA for introducing precise mutations (Pickar-Oliver & Gersbach, 2019). Although genome editing is primarily used to generate gene knockouts, it can also be used to activate endogenous genes and perform various other genome manipulations. Recent developments include new Cas systems (e.g., Cascade, Cas12, Cas13) for efficient DNA base editing and the use of nuclease-deactivated Cas proteins fused with effectors for post-transcriptional RNA regulation (Bhambhani et al., 2022; Das et al., 2024; Pickar-Oliver & Gersbach, 2019).

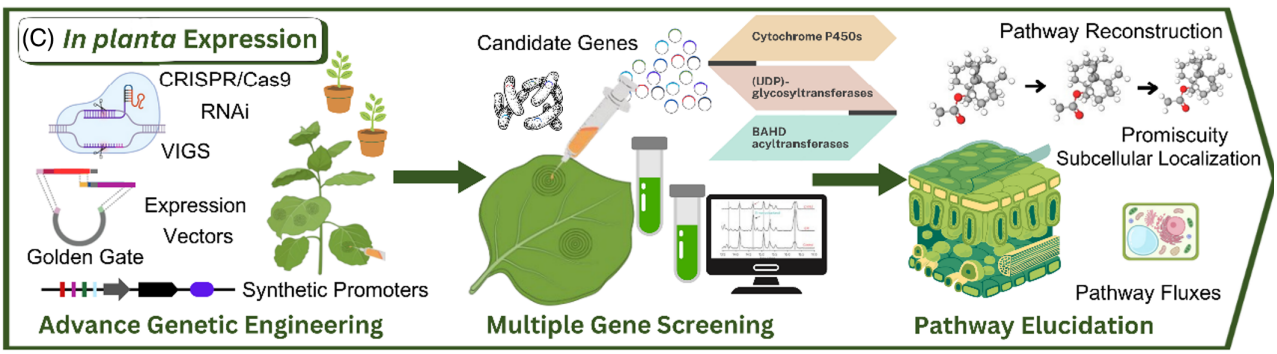
Advanced gene assembly techniques and vector systems, such as Golden Gate cloning (using type IIS restriction enzymes) and Gibson assembly, facilitate seamless integration of large, multi-gene constructs, making them ideal for reconstructing complex metabolic pathways in plants (Sonawane et al., 2016; Sorida & Bonasio, 2023). Recent advancements in sequencing technologies have reduced synthesis costs and enhanced sequence fidelity, offering new alternatives for producing large gene constructs in synthetic biology (Hughes & Ellington, 2017). Self-replicating viral vectors, like geminiviral replicons, have been used to enhance transient expression in *N. benthamiana*, boosting the production of target metabolites. The adoption of synthetic biology and metabolic engineering tools, such as synthetic promoter design and optimized expression vectors, allows for precise regulation of gene expression, adjusting pathway fluxes (Ali & Kim, 2019; Brooks et al., 2023; Cai et al., 2020; Jores et al., 2021; Selma et al., 2023; Tian et al., 2022).

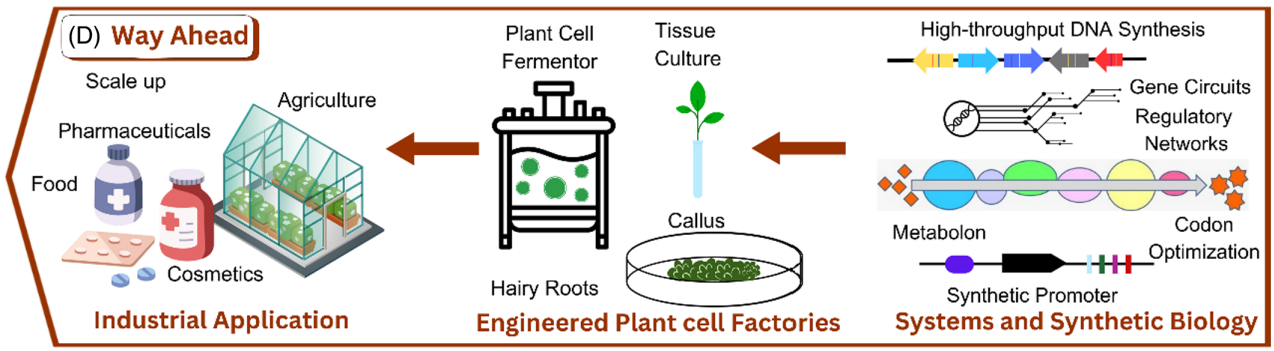
Researchers combine synthetic and systems biology to advance the development of synthetic genetic circuits and chimeric pathways with enzymes from different species. Despite the challenges, this approach holds significant potential to re-engineer complex metabolic pathways in plants (Vazquez-Vilar et al., 2023). Artificial intelligence and machine learning models are employed in plant systems to predict gene combinations for high-yield metabolite production, for the identification of transcriptional activation domains, for integrating the multi-omics data, to optimize metabolic fluxes, for plant genome engineering, pathway reconstruction, plant metabolic modeling, and augmented recombinant protein expression in planta (Bai, Li, et al., 2024; Hummel et al., 2024; Parthiban et al., 2023; Sampaio et al., 2022; Shah et al., 2021).

Compartmentalization strategies at the multicellular and subcellular levels, such as targeting enzymes to specific organelles (e.g., chloroplasts, peroxisomes), can significantly enhance metabolite production efficiency by minimizing side reactions and overcoming metabolic bottlenecks (Shen et al., 2024; Yao et al., 2023). Advances in synthetic transcription factors and transcriptional machinery engineering enable precise regulation of multiple genes within a metabolic pathway (Deng et al., 2022). Optimizing promoter elements to have a precise control on its activity and expression levels will also have a significant









(E) Metabolites

- Mass Spectrometry Imaging
- Computational Metabolomics
- De Novo Omics
- Hi-C Technology
- Spatial Omics
- WEIZMASS

Data Integration

- CANOPUS
- Co-expressing Genes-PathPred, GeneNet
- Metabolite Enzyme Genomic Clusters
- Gene Expression Atlas
- DeepEC, PRISM

***In planta* Expression**

- CRISPR/Cas9
- Engineered and gene edited *N. benthamiana*
- Pooled and rapid pathway testing
- Pathway Compartmentalization

Way Ahead

- Gene Regulatory Network
- GWAS
- Synthetic Promoters and Transcription Factors
- Chimeric Pathways
- Cell-free Platforms

Figure 1. Roadmap and major breakthroughs in complex plant metabolic pathway engineering.
(A) Metabolite discovery from plants using analytical tools and identifying the enzymes through next-generation sequencing approaches.
(B) The high-throughput omics data are analyzed using artificial intelligence-based machine learning to discover networks and interactions.
(C) Genetic engineering tools and multiple gene screening approaches are used for pathway elucidation using *Nicotiana benthamiana*.
(D) Systems and synthetic biology approaches are transforming the field assisting in the generation of gene circuits for transient and stable expression of complex specialized metabolites in plant cell factories for industrial applications.
(E) The key breakthroughs and techniques which transformed this field are listed. Pictures were generated in BioRender (https://www.biorender.com/) and Canva (https://www.canva.com/).

impact on synthetic biology and pathway engineering efforts. Jores et al. (2021) studied nearly the complete set of promoters from the dicot plant *Arabidopsis* and the monocot plant *Sorghum*, using a massively parallel reporter assay (MPRA) system, namely STARR-Seq, and identified the TATA box, GC content, and promoter-proximal transcription factor binding sites, enhancer elements as key influencers of promoter strength. Furthermore, the computational models they developed predict promoter strength in plants, designing highly active synthetic and tailored promoters crucial for precise gene regulation in plants. MPRAs were effectively used to elucidate the function of signals downstream of the transcription start site (TSS) as a unique mechanism to regulate gene expression in plants (unlike animals) and led to the identification of a conserved DNA sequence motif (downstream of TSS) capable of imparting dose-dependent enhancement in gene expression in vascular plants (Voichek et al., 2024). Plant terminator sequence and strength analysis as reported by Gorjifard et al. (2024) resulted in the identification of strong naturally occurring terminator sequences and synthetic ones for enhanced gene expression. These recent plant genome-wide studies will serve as a base for future predictive engineering of plant traits, including specialized metabolite pathways. Advances in designing orthogonal regulatory sequences, such as synthetic transcription factors and minimal promoters functional in plant systems, have facilitated the modulation of plant development (Belcher et al., 2020). Additionally, the creation of synthetic gene circuits using synthetic promoters, engineered transcription factors, reporter genes, and signal modules has enabled fine-tuning of gene expression and complex genetic engineering in plants (Brophy et al., 2022).

Moreover, the creation of synthetic promoters tailored for specific tissues, environmental conditions, or inducible factors has improved the spatial and temporal control of pathway expression in plants, leading to more efficient metabolite production (Cazier & Blazeck, 2021).

Plant cell-free systems and chloroplast cell-free systems offer a robust and flexible platform for the rapid prototyping, optimization of plant enzymes, and metabolic pathways and enable enhanced metabolite production by providing a controlled and manipulable biosynthesis machinery (Bogart et al., 2020; Böhm et al., 2024), as demonstrated for lycopene, indigoidine, betanin, and betaxanthins *in vitro* synthesis in BY-2 cell lysates (Buntru et al., 2022; Karim & Jewett, 2018).

The primary technical hurdles in identifying and characterizing complex natural products typically involve a combination of challenges, such as detecting complex natural products, expressing genes successfully, and identifying missing steps (Atanasov et al., 2021). Detecting complex natural products is perhaps the most prominent initial hurdle. Once the product is detected and identified, expressing the genes in heterologous hosts and determining the full biosynthetic pathway (including missing steps) become the next challenges. Advances in genome sequencing, synthetic biology, and high-throughput screening are gradually alleviating these difficulties, but they remain significant obstacles in the field.

Plant expression systems for metabolic engineering of complex pathways

Over the past decade, *N. benthamiana* has emerged as a leading model for reconstituting complex plant pathways. Its advantages include straightforward leaf or whole-plant vacuum infiltration, which provides rapid results within 3–7 days. Compared to microbial or other plant systems, *N. benthamiana* has proven highly effective for quickly testing various gene combinations, from single genes to complex constructs involving dozens of genes, for the production of known and novel specialized metabolites (Bally et al., 2018; Golubova et al., 2024). The plant's cellular environment facilitates proper protein folding, targets proteins to the correct subcellular locations, and supplies a range of precursors and cofactors necessary for successful enzyme activity (Grützner et al., 2024; Li et al., 2019; Reed & Osbourn, 2018; van Herpen et al., 2010; Yao et al., 2023). One of the key benefits of this system is the ability to mix *Agrobacterium* cultures carrying different genes prior to infiltration, thus bypassing the need for time-consuming construction of individual transformation vectors. Carlson et al. (2023) established experimental systems that can deliver and co-express over 20 genes in *N. benthamiana* leaves. Recently, Golubova et al. (2024) reviewed *N. benthamiana* development strategies such as overexpression, transplastomic expression, and loss-of-function mutations to enhance the availability of precursor substrates, boost the production of target metabolites, and

Table 3 Advanced techniques and tools for research on plant specialized metabolism

Sr. no.	Name of the technique	Application	Relevance to research on plant specialized metabolism	Limitations	Remarks and references
Metabolomics					
1	LC–MS/MS GC–MS/MS	High-throughput, targeted and untargeted metabolite identification	Profiling metabolites in medicinal plants to discover novel compounds	Challenging to resolve the identify of unknown metabolites	Aksenov et al. (2017)
2	Quadrupole time-of-flight (Q-TOF) MS	Powerful tool for identifying metabolites in complex mixtures	Suitable for accurate mass measurements and identification of unknowns	Complex to operate, maintain, calibrate, and high costs	Allen and McWhinney (2019)
3	Ion mobility separation (IMS)	IMS can distinguish between ions with identical mass-to-charge ratios (*m*/*z*) but different shapes or conformations (structural isomers)	IMS-MS provides better resolution, identification, and quantification of molecules from complex metabolite mixtures from plants	Limited resolution of high molecular mass biomolecules	Rivera et al. (2020) Dodds and Baker (2019) Christofi and Barran (2023)
4	MS imaging	Visualizes the spatial distribution of metabolites, lipids, proteins, and other biomolecules directly within tissue sections	Important for label free, multiplexed and spatial analysis of plant metabolites and the changes therein in response to stresses	It is challenging to have efficient ionization, retaining sample integrity apart from complex data interpretations	Vats et al. (2024) Yin et al. (2024) Dong and Aharoni (2022)
5	Single cell metabolomics	Elucidates metabolite profiles of individual cells, offering insights into cellular heterogeneity and metabolic dynamics	Exploring plant cell type specific metabolic pathways	Separation of cell types is challenging and detection of small metabolite in low concentrations needs sensitive detection methods	Petrova and Guler (2024) Pandian et al. (2023)
Metabolite structure elucidation					
6	Fourier transform ion cyclotron resonance (FT-ICR)	High-resolution analytical technique that offers unparalleled mass accuracy and resolving power for metabolite identification	Accurately resolving plant metabolites with similar *m*/*z* values, and discovering novel metabolites	High cost and complexity of the instrumentation	Maia et al. (2023)
7	High-resolution magical angle spinning (HR-MAS) nuclear magnetic resonance (NMR)-based metabolomics	Determining structures of newly identified plant metabolites. HRMAS—high-resolution than the liquid state-NMR	Technique is relevant for plant metabolites which have enormous uncharacterized diversity	Relative to LC–MS-based techniques, NMR requires a large quantity of samples. Structure elucidation of complex plant metabolites is challenged due to signal overlap and also database limitations	Ocampos et al. (2024) Mascellani Bergo et al. (2024) Kim et al. (2010) Joseph et al. (2021) Augustijn et al. (2021)
8	X-ray crystallography using crystalline sponge methods	Small-molecule structure determination without the need to have them in crystal form	Structure elucidation of plant metabolites, agrochemicals and volatile compounds as well	Stability of the sponge material in different solvents, chemical and physical compatibility of the molecules to be elucidated with the sponge is necessary, and data interpretation is challenging	Habib et al. (2022)

(continued)

Table 3. (continued)

Sr. no.	Name of the technique	Application	Relevance to research on plant specialized metabolism	Limitations	Remarks and references
9	Electron cryo-microscopy	Imaging technique that enables the visualization of biological macromolecules in their near-native states at high resolution	Revealing the structures of protein complexes involved in metabolic pathways, thereby enhancing the understanding of metabolic processes at a molecular level	Sample sensitivity to electron beam, technical complexity, costs and sample preparation challenges	Benjin and Ling (2020) Fukuda et al. (2023) Danev et al. (2019)
Metabolite identification					
10	SIRIUS	Java-based software framework for identification of metabolites and biomolecules to enhance molecular structure elucidation	Enables untargeted structural elucidation of unknown compounds, aids discovery of novel plant metabolites	Needs intensive computational infrastructure for efficient processing	Dührkop et al. (2019)
11	METLIN, GNPS, MassBank	Mass spectrometry databases widely used in metabolomics research	Accurate identification of metabolites	Several limitations with respect to plant-specific data including incomplete coverage, unavailability of spectral data for many rare metabolites	METLIN Database: https://metlin.scripps.edu/ GNPS Platform: https://gnps.ucsd.edu/ MassBank Repository: https://massbank.eu/MassBank/
12	The Plant Metabolite Hub (PmHub), Plant Metabolic Network (PMN), KNApSAcK, and Phytometasyn	Plant-specific metabolomics databases	Effective tools in identifying candidate genes, pathway classes for constructing metabolic networks and further metabolomic studies	Needs to be elaborated to include more plant species and metabolite classes	Tian et al. (2024) https://pmhub.org.cn/#/ PMN Database: https://plantcyc.org KNApSAcK: Nakamura et al. (2014) Phytometasyn: Dugé de Bernonville et al. (2015)
13	WEIZMASS, CANOPUS	Identification and classification of plant metabolites	Applies high-resolution mass spectrometry data (Weizmass) and predict the structural classes of metabolites based on their mass spectrometry fragmentation spectra (Canopus)	Need to increase coverage, limit redundant entries, high quality spectral fragmentation data may be limited	Shahaf et al. (2016) Dührkop et al. (2021)
Genomics and transcriptomics					
14	Single-molecule real-time sequencing Nano-pore sequencing, PacBio	These are third-generation sequencing technologies which can sequence long DNA fragments and applied to genome, transcriptome, epigenome and small RNA	Facilitates genomics and transcriptomics, characterizing alternative splicing events in genes	Higher error rates, higher costs, and limited accessibility (for PacBio) and high error rates, technical issues with the instrument and high computational demand (Nanopore)	Nanopore: Wang et al. (2021) PacBio: van Dijk et al. (2018) and Liu et al. (2022)

(continued)

Table 3. (continued)

Sr. no.	Name of the technique	Application	Relevance to research on plant specialized metabolism	Limitations	Remarks and references
15	Hi-C Sequencing	Captures three-dimensional chromatin architecture, helps in scaffolding plant genomes and identifying gene regulatory networks	Plant metabolite biosynthesis gene cluster identification from genomes	Need of high sequencing depth and high computational power	Šimková et al. (2024)
16	Single-cell RNA sequencing	High-resolution single cell level gene expression profiling	Mapping biosynthetic pathway genes and their regulation from specialized plant cells	Difficulties in sample preparation, low yield of RNA from single cells, and high costs	Aldridge and Teichmann (2020)
17	Stereo-seq	Provides single cell level RNA sequencing information in tissue sections retaining spatial context	Spatially resolves biosynthetic pathways and facilitates integration of transcriptomics and metabolomics	Challenging tissue and sample preparation, high costs and computationally demanding	Bawa et al. (2024)
18	Synteny analysis	Helps in identifying conserved genetic regions that may serve similar functions across species	Aids in improving genome assemblies, gene discovery, cross species comparison, and understanding evolution	Complexity in polyploid species, incomplete genomic data, and species-specific differences, making it difficult to pinpoint true gene orthologs	Hakim et al. (2024) and Naake et al. (2024)
19	Co-transcriptional analysis	Predicts gene interactions within a biological context	Helps in understanding gene regulations, and predicting gene interactions	Incomplete or low-quality sequencing data, complexity of gene regulation, limits the application of co-transcriptional analysis	Uygun et al. (2016)
Genetic engineering					
20	CRISPR-Cas genome editing	A transformative tool to manipulate the genetic makeup of plants with unprecedented precision	Allows targeted modification of specific genes with greater precision than traditional breeding techniques, making it possible to alter plant metabolism with minimal unintended effects	Challenges such as off-target effects, regulatory hurdles, and incomplete knowledge of complex regulatory networks involved in plant metabolism	Das et al. (2024), Pickar-Oliver and Gersbach (2019) and Bhambhani et al. (2022)
21	Golden Gate cloning (using type IIS restriction enzymes) and Gibson assembly	A versatile and efficient molecular cloning technique that enables simultaneous assembly of multiple DNA fragments into a single vector	Simplifies the construction of multigene expression cassettes. System is adaptable for different cloning needs, such as gene knock-ins, overexpression, or gene knockouts	Large constructs can affect vector stability and transformation efficiency	Bird et al. (2022), Biró et al. (2024) and Sorida and Bonasio (2023)
22	DNA synthesis	Crucial for designing and constructing DNA sequences	Enabling the precise design, construction, and optimization of metabolic pathways	Challenges in pathway assembly, transformation efficiency, regulatory hurdles, high costs	Hughes and Ellington (2017)

increase plant biomass. Additionally, genome editing enabled the creation of nicotine-free *N. benthamiana* lines that produce desired metabolites (Schachtsiek & Stehle, 2019). The plant has also been engineered to increase lipid body formation, which aids in the storage, stability, and accumulation of engineered metabolites (Bibik et al., 2022; Delatte et al., 2018). The research findings of Chen et al. (2024) completed the first genome assembly of the allotetraploid model plant *N. benthamiana*. This work enhanced our understanding of the genome evolution and centromere development processes providing valuable resources for biotechnological advancements. Furthermore, multiplexed genome engineering holds promise for accelerating the use of *N. benthamiana* in industrial applications.

Developments in biosynthetic pathway elucidation coupled with advances in plant cell transformation technology have accelerated work with transgenic plant cell cultures for metabolite production, though challenges in tuning complex pathways in cells need to be resolved (Arya et al., 2021; Marchev et al., 2020). Suspension cultures of plant cells have been engineered by transforming them with host or heterologous metabolite biosynthesis pathway genes and transcription factors to enhance the production of specific metabolites. For example, in *Capsicum frutescens*, these cultures have been developed to produce vanilla (Chee et al., 2017); in rice cell suspensions and *Vitis* species, to produce resveratrol (Baek et al., 2013; Chu et al., 2017; Kiselev & Dubrovina, 2021; Martínez-Márquez et al., 2025); in *Taxus chinensis* cells, to produce taxol (Zhang et al., 2011); and in *N. tabacum* cell cultures, to produce geraniol (Vasilev et al., 2014) and artemisinin (Malhotra et al., 2016). Furthermore, secondary metabolite biosynthesis using multiple genes has been demonstrated in *Nicotiana* species for anthocyanins (Appelhagen et al., 2018) and various terpenoids as reviewed in Ikram et al. (2015). Although transgenic plant cell cultures are more commonly implemented for protein biosynthesis, advancements in understanding of plant metabolic pathways and synthetic biology they hold significant promise as efficient bio-factories (Wu et al., 2021) using a range of small and industrial scale bioreactors (Baranski & Lukasiewicz, 2019; Verdú-Navarro et al., 2023).

Hairy root cultures represent another plant-based expression system. It is developed by infecting plants with the bacterium *Rhizobium rhizogenes*, which transfers part of its DNA into the plant genome, inducing prolific root formation, capable of growth on hormone-free plant cell culture media. They are valued for their potential to synthesize a variety of bioactives, proteins, or metabolites from a range of plants (Gutierrez-Valdes et al., 2020). Hairy root cultures could generate high metabolite production due to the possibility of precursor feeding, elicitation, excretion of the metabolite and scale-up (Biswas et al., 2023; Gutierrez-Valdes et al., 2020). Enhanced metabolite biosynthesis using transgenic hairy roots or cell suspensions of model (*N. tabacum*) or non-model plant systems (*Vinca minor, Senna obtusifolia, Centauries maritimum*) expressing multiple genes from a particular biosynthetic pathway has been demonstrated and even scaled up to a bioreactor (Hidalgo et al., 2017; Kowalczyk et al., 2022). Taxol a widely used chemotherapeutic drug which has been transgenically produced in different species of *Taxus* root cell cultures and hairy roots through multigene assembly consisting of taxol biosynthesis genes and transcription factors (Perez-Matas et al., 2023). Non-model species are often difficult to transform, and their complex metabolite biosynthetic pathways are not fully understood, resulting in low biosynthetic output.

MULTI-STEP, TRANSIENT RECONSTRUCTION OF PLANT BIOSYNTHETIC PATHWAYS IN *N. BENTHAMIANA*

Agrobacterium-mediated transient expression in *N. benthamiana* has been widely used for engineering complex pathways of plant specialized metabolism. Several impressive examples of complex metabolic pathways reconstituted in *N. benthamiana* are presented in Table 1. Through genome sequencing of soapbark tree *Quillaja saponaria*, genome mining and combinatorial expression in *N. benthamiana*, a biosynthetic pathway of vaccine adjuvants, QS-7 (compromising a total of 14 enzymes, Reed et al., 2023) and QS-21 (23 enzymes, Martin et al., 2024), were discovered and reconstructed. The biosynthetic pathway of the saponarioside B, structurally like QS, was uncovered using combinatorial expression of 14 enzymes from soapwort (*Saponaria officinalis*; Reed et al., 2023). Hong et al. (2022) identified nine enzymes responsible for brucine biosynthesis from the poison fruit *Strychnos nux-vomica*, using omics datasets, *in vitro* assays and the *N. benthamiana* platform. Genes involved in specialized metabolites biosynthesis are often clustered together for a coordinated expression. By comparing genomic (synteny) or transcriptomic (co-transcriptional) context in one species, we can predict the presence of similar gene clusters in another species (Hakim et al., 2024; Naake et al., 2024; Uygun et al., 2016) (Table 3). Recently, Jozwiak et al. (2024) identified *GLYCOALKALOID METABOLISM 15* (*GAME15*) in tomato and potato using steroidal glycoalkaloid gene cluster analysis and successfully engineered the tomatidine (8 enzymes) biosynthetic pathway in *N. benthamiana*. In another study, co-expression of *Solanum nigrum* 6 enzymes (GAME6, GAME8, GAME11, GAME4, GAME12, and GAME15) with 10 UDP-glycosyltransferases (UGTs) in stepwise-pooled pathway engineering approach led to identification of four UGT enzymes (UGT73L14, UGT73DU3, UGT93M3, and UGT93N4) catalyzing α-solasonine and α-solamargine biosynthesis (Lucier et al., 2024). Nett et al. (2020) provide a potential of

"correlation and clustering studies" for the prediction of candidate genes for methylation. The engineering of N-formyldemecolcine, a colchicine precursor (16 enzymes), starting from the amino acids phenylalanine and tyrosine, has also been reported. The biosynthetic pathway of two limonoids, azadirone (10 enzymes) and kihadalactone A (11 enzymes), was discovered from *Citrus* and *Melia* species, respectively, using combinatorial expression in *N. benthamiana* (de la Peña et al., 2023). Schultz et al. (2019) showed accumulation of up to 4.3 mg g^{-1} dry (-)-deoxypodophyllotoxin in *N. benthamiana*, by transient expression of 16 genes, encoding both coniferyl alcohol and main etoposide aglycone pathway enzymes from mayapple. Strictosidine biosynthesis was successfully reconstituted by the co-expression of 14 enzymes (Dudley et al., 2022), showing its future potential for biomanufacturing. Irmisch et al. (2020) demonstrated the reconstruction of the plant specialized metabolite montbretia (9 enzymes) in *N. benthamiana*. Using a step-by-step screening approach, Yin et al. (2023) established the biosynthetic route of cholesterol from cycloartenol and reconstructed the diosgenin pathway (compromising a total of 19 enzymes). Wang et al. (2022) reconstructed cocaine biosynthesis using eight enzymes in *N. benthamiana*. In another example, the diterpenoid momilactone biosynthetic pathway (7-steps) of rice was reconstituted in *N. benthamiana* (de la Peña & Sattely, 2021). Calgaro-Kozina et al. (2020) exploited the substrate promiscuity of enzymes to express the crucifalexin biosynthetic pathway (8 genes) and produce bioactive halogenated variants in planta.

Several research groups attempted the metabolic engineering of taxol precursors, especially taxadiene, taxadiene-5α-ol, and baccatin III in *N. benthamiana* (Jiang et al., 2024; Li et al., 2019; Zhang et al., 2023). High-level production of taxadiene (56.6 μg g^{-1} FW) and taxadiene-5α-ol (1.3 μg g^{-1} FW) was achieved using enhancement of isoprenoid precursor levels, combined with a chloroplast compartmentalized engineering strategy (Li et al., 2019). Several studies reported the promiscuous activity of taxadiene 5α-hydroxylase (T5αH) in generating multiple oxidized taxadiene products, considering it a bottleneck in heterologous taxol (paclitaxel) production (Gou et al., 2024; Liu et al., 2024). By tuning the promoter strength of *T5αH* expression in *N. benthamiana*, Liu et al. (2024) reduced the level of oxidized by-products with a concomitant increase in the accumulation of taxadiene-5α-ol by threefold. Through heterologous expression, Zhang et al. (2023) identified missing paclitaxel biosynthetic enzymes and produced a taxol key intermediate (baccatin III) and taxol (paclitaxel). In other recent studies, Jiang et al. (2024) and McClune et al. (2024) successfully reconstituted taxol precursor baccatin III production in *N. benthamiana*. These achievements demonstrated the potential of *N. benthamiana* as a host in complex pathways characterization and engineering via efficient co-expression of multiple enzymes.

ENGINEERING COMPLEX PATHWAYS IN STABLE TRANSGENIC PLANTS

Stable expression of multiple transgenes was reported early on to be a promising approach to produce nutrient-rich crops (Farré et al., 2013; Lu et al., 2013; Paine et al., 2005; Ye et al., 2000) or pharmaceutically active compounds (Fuentes et al., 2016; Jung & Maeda, 2024; Polturak et al., 2016). Both nuclear and plastid (chloroplast) genomes can be engineered with multiple transgenes separately (Molina-Hidalgo et al., 2021). However, many challenges exist for creating a stable multigene plant expression system. These include a lengthy, iterative process required for optimization of pathway and gene sets, lack of suitable genetic parts (promoters, terminators, etc.), development of rapid methods for integrating multiple genes into the genome, and analysis of a large population of stable lines. Herein, we discuss a few examples of pyramidizing (≥3) genes in stable transgenic plants mostly for producing the high levels of metabolites in plants (Table 2).

Biofortification via genetic engineering can enable an increase in multiple micronutrients such as vitamins, amino acids, and minerals (van der Straeten et al., 2020; Wang & Galili, 2016; Yang et al., 2016). Transgenic and first-generation golden rice (GR1) was developed by *Agrobacterium*-mediated co-transformation of three β-carotene biosynthetic pathway genes, phytoene synthase (*NpPSY*, from *Narcissus pseudonarcissus*), bacterial phytoene desaturase (*ErCrtI*, from *Erwinia uredovora*), and lycopene β-cyclase (*NpLCY*, from *N. pseudonarcissus*) along with the selectable marker hygromycin phosphotransferase (*aphIV*) gene and expression in the rice endosperm (Ye et al., 2000). The levels of provitamin A carotenoids (1.6 μg g^{-1} dry weight) in GR1 were too low to make a sufficient contribution to alleviate provitamin A deficiency. Since PSY is a major rate-limiting enzyme in the carotenoid biosynthesis pathway, Paine et al. (2005) generated new Golden Rice (GR2) lines using variants of PSY from maize (*Zea mays*, ZmPSY) and rice (*Oryza sativa*, OsPSY) and achieved increased carotenoid levels up to 37 μg g^{-1} dry weight. In another example, transgenic maize lines expressing a combination of five transgenes, namely *Zmpsy1*, *Pacrtl* (*Pantoea ananatis* phytoene desaturase), *Gllycb* (*Gentiana lutea* lycopene β-cyclase), *Glbch* (*G. lutea* β-carotene hydroxylase), and *ParacrtW* (*Paracoccus* spp. β-carotene ketolase) accumulated β-carotene (26.33 ± 0.15 μg g^{-1} dry weight) in the maize endosperm (Farré et al., 2013). Metabolic engineering of rice endosperm was achieved by expressing three major thiamin (Vitamin B1) biosynthesis genes, *HMP-P synthase (THIC)*, *HET-P synthase* (*THI1*), and *HMP-P kinase/TMP*

pyrophosphorylase (*TH1*), either separate or in combination, to alleviate vitamin B1 malnutrition (Strobbe et al., 2021). Increasing the level of essential amino acids (e.g., lysine, leucine, tryptophan) in a heterologous plant host is still difficult since (i) the biosynthetic pathways of essential amino acids are strongly regulated by negative feedback loops and (ii) some targeted essential amino acids are catabolized, for example, lysine is degraded in the tricarboxylic acid cycle (Wang & Galili, 2016; Yang et al., 2016). Transgenic rice lines were developed by expressing bacterial lysine feedback-insensitive *aspartate kinase* (*AK*) and *dihydrodipicolinate synthase* (*DHPS*) genes and downregulating *lysine ketoglutarate reductase/-saccharopine dehydrogenase* (*LKR/SDH*) genes, leading to an elevation of free lysine content to 25-fold as compared to wild type without changing the plant phenotype (Yang et al., 2016).

Several metabolites have been produced by expressing synthetic operons in plastids (Fuentes et al., 2018), such as keto-carotenoids (Tanwar et al., 2023), tocopherol (Lu et al., 2013), and the antimalarial drug precursor artemisinic acid (Fuentes et al., 2016) (Table 2). Metabolic engineering of "high-cholesterol" *Arabidopsis* plants was achieved by transforming a binary vector (pCHOLESTEROL), harboring 11 genes of the tomato cholesterogenesis pathway (Sonawane et al., 2016). In an approach termed "combinatorial supertransformation of transplastomic recipient lines" (COSTREL), a plastid operon was designed and combined with combinatorial nuclear transformation to enhance the production of artemisinic acid in plastids (Fuentes et al., 2016). This approach not only ensures strong and stable expression of pathway enzymes but also fine tunes the metabolic flux through the pathway as well as the metabolic output. Tanwar et al. (2023) assembled three carotenogenic genes, *lycopene cyclase* (*lcy*), *β-carotene hydroxylase* (*bhy*), and *β-carotene ketolase* (*bkt*) as a synthetic operon, expressed with intercistronic expression elements for effective mRNA splicing and stably transformed them to tobacco chloroplasts, resulting in the production of 4-keto-lutein. In another example of plastid engineering, South et al. (2019) transformed tobacco chloroplasts with 17 different constructs of three photorespiratory alternative pathway designs, to improve photosynthetic efficiency.

Transient expression takes only a few days to produce a sufficient amount of target products. In contrast, stable transgenics take considerably more time, and the yields of target products are lower, as observed in lines producing human milk oligosaccharides (Barnum et al., 2024). High yields can be achieved by using optimized constructs for creating stable transgenics, finding optimal species for production, and optimizing growth conditions (Golubova et al., 2024). However, regulatory processes for growing stable transgenics in the natural field remain challenging. A further challenge of stable transgenics is controlling gene expression. Constitutive strong expression of transgenes may affect plant growth and development, as shown in lines producing insect sex pheromones (Mateos-Fernández et al., 2021). This can be overcome by using an inducible promoter, but it is still challenging to maintain high target product yields. Overall, complex metabolic pathways that are highly regulated require the generation of large populations of transgenic lines and necessitate high-throughput screening methods to identify the desired (metabolic) phenotypes.

STRATEGIES TO ENHANCE DESIRED TARGET METABOLITE(S) YIELD IN PLANT METABOLIC ENGINEERING

In general, engineering strategies to enhance metabolite levels can be achieved through several approaches: (i) increasing the activity of rate-limiting enzymes that create tailbacks for enhancing the pool of precursor supply; (ii) engineering regulatory elements (e.g., activator/promoter) or transcription factors (TFs) to upregulate the expression of target genes; (iii) reducing the activity of competing pathway enzymes to avoid modification of intermediates and accumulation of undesired products; (iv) enhancing enzyme activity, substrate specificity, stability, removing regulatory sequences in enzymes, and adaptability to the host by amino acid site mutagenesis; and (v) engineering transporters to uptake or efflux pathway intermediates or products through membranes (Figure 2).

Increasing the supply of precursors or pathway intermediates

One of the important parameters to enhance product yield in metabolic engineering is to increase and balance the supply of precursors (Figure 2A). Overexpression of genes encoding rate-limiting steps that compete for the same precursor has been shown to increase the levels of target products. In terpenoid production, overexpression of rate-limiting enzymes, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMGR), 1-deoxy-D-xylulose 5-phosphate synthase (DXS), and geranylgeranyl pyrophosphate synthase (GGPPS) in heterologous hosts has been well established (de la Peña & Sattely, 2021). For example, Yin et al. (2023) expressed a truncated feedback-insensitive version of HMGR (tHMGR) with 2,3-oxidosqualene cyclase (OSC) from *Paris polyphylla* to obtain a sufficient supply of cycloartenol (28.79 mg g^{-1} dry weight), a precursor for cholesterol biosynthesis in the leaves of *N. benthamiana*. An increase in the yield of the triterpenes β-amyrin (4-fold) and its oxidized β-amyrin scaffold (~10-fold) was achieved by overexpressing tHMGR (Reed et al., 2017). Similarly, the production of the diterpenoid momilactone in *N. benthamiana* was enhanced over 10 times higher than that observed in rice (de la Peña & Sattely, 2021). In another example, the

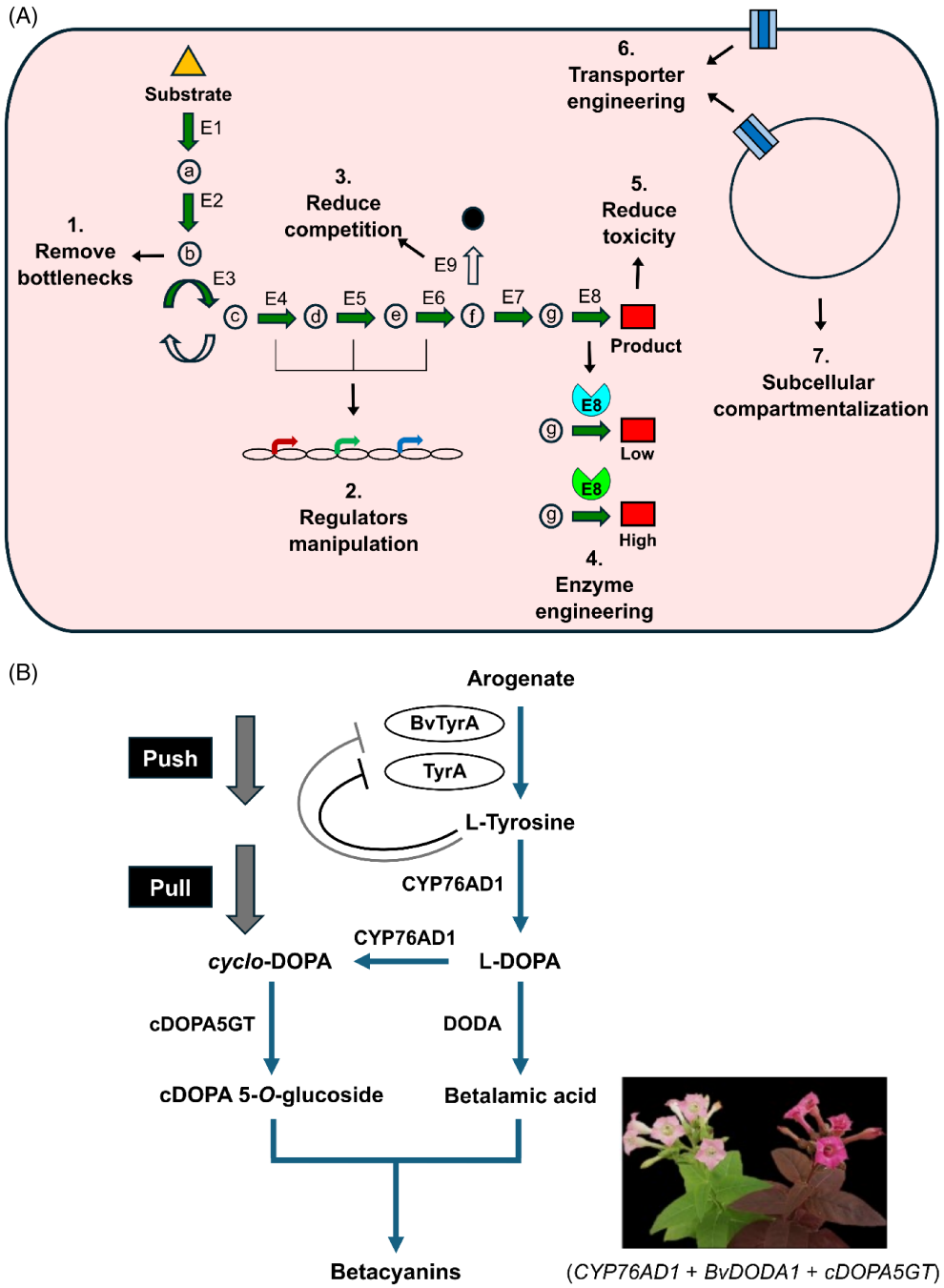


Figure 2. Schematic representation of plant engineering strategies applied to increase plant products.
(A) Substrate (yellow triangle) undergoes sequential enzyme-catalyzed reactions (E1–E8) to yield a final product (red rectangle) through pathway intermediates (a–g). Strategies to increase product yield include: (1) Eliminating pathway bottlenecks by enhancing substrate availability and expression of rate-limiting enzymes. (2) Increasing enzyme efficiency by controlling the level of transcription factors and regulatory elements (e.g., promoters/enhancers). (3) Suppressing competing pathways (enzyme E9) to avoid the production of undesired products (black-filled circle). (4) Enhancing enzyme activity, substrate specificity, stability, and adaptability to the host by amino acid site mutagenesis (native enzyme E8, light teal blue; engineered enzyme E8, pale green). (5) Controlling the toxicity of products and/or intermediates to reduce the burden on host cells or toxic effects. (6) Engineering transporters to uptake or efflux pathway intermediates or products through membranes. (7) Storing pathway intermediates or target products in subcellular compartments.
(B) "Push-and-pull" strategy applied by Jung and Maeda (2024) to efficiently produce betacyanins from arogenate in *Nicotiana benthamiana*. In most plant species, arogenate dehydrogenase (TyrA) is feedback inhibited by L-tyrosine. BvTyrAα from beet showing relaxed feedback inhibition by L-tyrosine was used to "push" the supply of tyrosine and co-expression of further genes (*cytochrome P450 CYP76AD1*, *DODA*, *cDOPA5GT*) were used to "pull" the L-tyrosine supply for producing purple betacyanins. Pigmentation phenotypes were observed in transgenic tobacco (*Nicotiana tabacum*) plants expressing betacyanin biosynthetic pathway genes (*CYP76AD1*, *BvDODA*, *cDOPA5GT*) (Polturak et al., 2016). Left, wild-type tobacco; right, transgenic tobacco. Bv, *Beta vulgaris*; cDOPA5GT, cyclo-dopa 5-O-glucosyltransferase; DODA, DOPA 4,5-dioxygenase; DOPA, 3,4-dihydroxy-L-phenylalanine; TyrA, arogenate dehydrogenase.

production of linalool and costunolide in *N. benthamiana* was enhanced following the expression of tHMGR and DXS (Park et al., 2022). Moreover, co-expression of tomato *DXS* and a tobacco *GGPPS* resulted in an increased production of the diterpenoid cembratrienol in *N. benthamiana* (Brückner & Tissier, 2013).

The activity of some endogenous enzymes (represented as Enzyme number 9 "E9" in Figure 2A) in the pathway can compete for the same precursors, resulting in reduced yield of the target product. Suppressing the activity of competing enzymes can effectively divert the flux of intermediates toward target metabolite production. In strictosidine production, knocking down the endogenous *N. benthamiana* UDP-dependent glycosyltransferases (UGTs) reduced the derivatization of pathway intermediates (Dudley et al., 2022). In another example, nonspecific glycosylation of montbretin A was observed in *N. benthamiana*, but coexpressing three genes from the shikimate shunt of the phenylpropanoid pathway enhanced the production of the caffeoyl-CoA group required for montbretin A biosynthesis (Golubova et al., 2024). Jung and Maeda (2024) used a "push-and-pull" strategy to increase the production of betalains in *N. benthamiana* (Figure 2B). The deregulated arogenate dehydrogenase (BvTyrAα) from *Beta vulgaris* was expressed for "pushing" the supply of the L-tyrosine precursor, and simultaneously, betalain biosynthetic genes (*CYP76AD1*, *cytochrome P450 CYP76AD1*; *cDOPA5GT*, *cyclo-3,4-dihydroxy-L-phenylalanine 5-O-glucosyltransferase*; *DODA*, *DOPA 4,5-dioxygenase*) were co-expressed to "pull" the increased levels of L-tyrosine. Overall, these reports suggested that balancing between precursor supply and effective utilization of precursors is an important strategy to produce the high levels of target compounds.

Altering subcellular compartmentalization and manipulating the expression of regulators

Plant specialized metabolites are synthesized and accumulate at multiple subcellular locations, such as peroxisomes, vacuoles, chloroplasts, or transport vesicles. Several reports revealed the benefits of altering the compartmentalization of engineered proteins. For example, the production of *N*-formyldemecolcine was enhanced transiently by removing the N-terminal mitochondrial localization signal of the *Gloriosa superba N*-methyl transferase enzyme (Nett et al., 2020). The monoterpene indole alkaloid (MIA) pathway in *Catharanthus roseus* is highly compartmentalized, and the first committed step in MIA is geraniol synthesis (GES), which is localized in chloroplast. To increase GES substrate availability, Dudley et al. (2022) transiently expressed a chloroplast-localized *GPPS* from *Picea abies* and ultimately improved strictosidine yield. In another example, higher levels of artemisinin (800 $\mu g\ g^{-1}$ dry weight) were achieved by localizing the last steps of the artemisinin biosynthetic pathway in transgenic *N. tabacum* chloroplasts (Malhotra et al., 2016). Additionally, the production of halogenated indican (Fräbel et al., 2018) and vanillin (Gallage et al., 2018) also benefited from chloroplast localization when transiently expressed in *N. benthamiana*. The study of Aharoni et al. (2003) revealed that transgenic *Arabidopsis* and potato plants overexpressing the strawberry *FaNES1* (*Fragaria ananassa Nerolidol Synthase 1*) gene produced the high levels of the monoterpene linalool in the cytosol. Altering the localization of the FaNES1 protein by fusing the plastid targeting region of the wild strawberry FvNES1 (*Fragaria vesca* Nerolidol Synthase 1) to the N terminus of FaNES1 resulted in the release of 7.2–13.3 $\mu g\ day^{-1}\ plant^{-1}$ linalool from the highest producing transgenic lines. However, Kappers et al. (2005) generated higher levels of the sesquiterpene nerolidol in transgenic *Arabidopsis* plants by targeting the strawberry FaNES1 protein to the mitochondria. Thus, the change in FaNES1 protein localization enabled it to form both linalool and nerolidol.

As transporters localized on plasma membranes or tonoplasts are involved in the uptake or efflux of various metabolites and substrates across cellular membranes, transporter engineering is another advantageous strategy to increase metabolite production (Figure 2A). For example, transient co-expression of *Crocus sativus* ATP-binding cassette C (CsABCC4a) type of transporter and carotenoid cleavage dioxygenase 2 (CsCCD2) the first enzyme in the crocin biosynthetic pathway resulted in enhanced crocin production in *N. benthamiana* (Demurtas et al., 2019). In *Artemisia annua*, a pleiotropic drug resistance (PDR) transporter *AaPDR3* is involved in the transport of β-caryophyllene. Silencing of *AaPDR3* led to increased artemisinin in transgenic plants (Fu et al., 2017). The excessive accumulation of products and intermediates could be toxic to the host cells and affect the yield of the target product. Therefore, enhancing the secretion of toxic metabolites and compartmentalization by regulating the transporter proteins is of significant importance in plant metabolic engineering (Miao et al., 2021; Nogia & Pati, 2021) (Figure 2A). In another example, expression of the *Coptis japonica* multidrug resistance transporter (CjMDR) increased the intracellular content of ajmalicine and tetrahydroalstonine in *Catharanthus roseus* suspension cell lines (Pomahačová et al., 2009); the heterologous expression of *Tripterygium wilfordii* PDR1 transporter enhanced the extracellular secretion of the sesquiterpene pyridine alkaloids and triptolide (Miao et al., 2017, 2021); and the co-expression of squalene biosynthetic enzymes (farnesyl pyrophosphate synthase and squalene synthase) together with the droplet-forming protein (oleosin) resulted in a record yield of squalene (2.6 mg g^{-1} fresh weight) without affecting plant growth (Zhao et al., 2018). Yet, our currently limited information on specialized metabolite transporters

activity is a major hurdle for more extensive use of these proteins in metabolic engineering efforts.

Changes in endogenous metabolic pathways can also be achieved through manipulating the expression of regulators (Figure 2, steps c–f). For example, recent work demonstrated the use of transcription factors for enhancing phenylpropanoids (transient co-expression of MYB4; Irmisch et al., 2020), etoposides (transient overexpression of a MYB85; Kim et al., 2022), isoflavones (transgenic *N. tabacum* coexpressing *At*MYB12 and *Gm*IFS1; Pandey et al., 2014), and isoprenoids (silencing of the MsYABBY5 repressor; Wang et al., 2016).

Enhancing the efficiency of pathway enzymes

Natural product biosynthesis enzymes unlike those of core metabolism typically exhibit slow reaction rates and high promiscuity, and often assembled in a pathway by "mix and match" of an existing enzymatic machinery (Tawfik, 2020). Multistep biosynthetic pathway engineering in heterologous hosts often faces difficulties due to low stability and weak activities of enzymes, resulting in the reduced production of target metabolites and/or accumulation of intermediates (Figure 2A). One common strategy to overcome this issue is to improve enzyme efficiency via protein engineering. Mutants for enzyme engineering are typically selected through a combination of random mutagenesis and directed evolution, enabling the identification of improved functionalities or desirable traits in enzymes (Packer & Liu, 2015). Sites that are important for substrate binding, catalysis, stability, or the formation of reaction intermediates are frequently targeted in mutagenesis. Computational methods, such as molecular docking, molecular dynamics simulations, and quantum mechanical calculations, are commonly used to predict which residues or regions of the enzyme might have a significant impact on the desired properties (Krishna et al., 2024). Mutant enzymes generated for enzyme engineering are typically validated through *in vitro* and *in vivo* studies. Recently, Orsi et al. (2024) proposed an integrated solution to enzyme engineering challenges, combining ML-guided automated workflows and *in vivo* growth-coupled selection to accelerate the development of superior biocatalysts. Measuring enzyme kinetic parameters like the Michaelis–Menten constant (Km) and the turnover number (kcat) help to assess how the mutations have altered enzyme's affinity for substrates and the speed at which it catalyzes reactions (Bar-Even & Tawfik, 2013).

In enzyme engineering, researchers have used site-directed mutagenesis and the CRISPR-Cas9 system for creating mutant enzymes with tailored properties (Chen et al., 2023; Dudley et al., 2022; Ito et al., 2016). Modification in the active site of β-amyrin synthase and cytochrome P450 monooxygenases (CYPs) has been shown to enhance triterpenoids (e.g., β-amyrin, oleanolic acid, dammarane) product yield. Site-directed mutagenesis in the *Astragalus membranaceus* β-amyrin synthase resulted in an increase in β-amyrin production by 12.8-fold in *N. benthamiana* and 4.4-fold in yeast (Chen et al., 2023). Moreover, the F416A variant of *Euphorbia tirucalli* β-amyrin synthase increased the yield of the dammarane triterpenoid, but decreased β-amyrin levels (Ito et al., 2016). Romsuk et al. (2022) enhanced the production of oleanolic acid by 13.1-fold in *N. benthamiana* leaves by expressing a mutant of CYP716A12 (D122Q). Mutating the nine residues in CYP90Bs of *Paris polyphylla* (PpCYP90B52) and *Trigonella foenum–graecum* (TfCYP90B51) resulted in a significant increase in the cholesterol 16,22-dihydroxylase activity (Christ et al., 2019). Transient expression of feedback-insensitive *Q. saponaria* threonine deaminases mutant (QsTD-P540L) with the gene set for QS21 enhanced product levels in *N. benthamiana* (Martin et al., 2024).

Different artificial intelligence (AI)-based machine learning (ML) algorithms have been recently used in protein engineering for optimizing stability, localization, solubility, protein–protein interaction, and catalytic activity, which increase the functionality and production of recombinant proteins (Jiang et al., 2021; Khersonsky et al., 2018; Listov et al., 2024; Sahu et al., 2021). FuncLib is an automated method for designing multipoint mutations in enzyme active site design to generate diverse catalytic repertoires, starting from a natural or engineered enzyme (Khersonsky et al., 2018; Listov et al., 2024). These approaches have not yet been used to plant metabolic pathway enzymes but are expected to be widely adopted in the near future to enhance their specificity and activity, thereby improving the availability of precursors and intermediates, which will lead to higher levels of metabolite biosynthesis. Sahu et al. (2021) developed a Plant-mSubP tool based on integrated ML approaches to predict the localization of proteins to single and dual organelle targets. This is anticipated to aid in the design and optimization of metabolic pathways, enhancing the effectiveness of pathway engineering. In another example, the MULocDeep program was developed to predict protein localization in different plant organelles such as the nucleus, mitochondria, plastid, thylakoid, and extracellular matrix (Jiang et al., 2021).

Plant tissue heterogeneity at the level of cell morphology, pathway gene expression, cell–cell interactions, and effector–ligand networks has profound effects on metabolite biosynthesis, and these need to be deciphered for speeding up the process of metabolic pathway engineering. Several AI-based tools have been specifically designed to uncover plant molecules and pathways, including the Plant Cell Marker Database (PCMDB), PlantPhone DB, and Plant Cell Atlas. Incorporating existing biomedical applications like BIAS, DIABLO, and MESSI into plant metabolite pathway engineering can further accelerate discoveries (Sears et al., 2024).

FUTURE DIRECTIONS

Compared to transient expression, the number of successfully reconstructed complex pathways in stably transformed plants remains relatively limited. This is partly due to fewer overall attempts, but also to challenges such as multi-gene vector construction and the scarcity of diverse promoter options for constitutive, cell-type-specific, or inducible transgene expression. While methodologies for multi-gene expression in plants continue to improve, they still lag behind the efficiency and routine practices seen in microbial metabolic engineering. One major obstacle in stable transformation is phytotoxicity arising from intermediates and end products of engineered pathways. Addressing this issue requires the development of a broader range of cell- and organ-specific, as well as inducible promoters. Another significant challenge is the activity of endogenous enzymes that compete for pathway precursors, intermediates, and end products, thus reducing target metabolite levels. Silencing or knocking out genes encoding these multi-substrate enzymes is an obvious solution, yet often difficult due to the large, complex enzyme families in plants, necessitating extensive time and effort for discovery and manipulation. Future strategies should consider testing multiple host plants or focusing on specific organs and cell types where such promiscuous enzymes exhibit reduced activity. The localization of engineered proteins and metabolites is another crucial factor for successful plant metabolic engineering. Recent advances in plastid genome engineering offer potential solutions, though well-characterized transporter proteins are still needed to direct pathway intermediates to specific cell compartments. The limited knowledge about plant metabolite subcellular localization will continue to hinder efforts to engineer precise metabolic compartmentalization. Beyond experimental approaches, computational, deep learning-based strategies could revolutionize plant metabolic engineering. Comprehensive metabolic network models including flux maps, enzyme kinetics data, knowledge of multi-substrate specificity, and metabolite subcellular localization linked to transporter activity could enable accurate predictive strategies. While such models have been used in microbial engineering for decades, they remain rare in plants, where they have mainly addressed core metabolic pathways. The use of AI to design "new-to-nature" enzymes and synthetic metabolic pathways may soon significantly reduce the need for pathway elucidation from exotic plants, speed up the generation of gene-pyramidized transgenics, and help to circumvent feedback inhibition, phytotoxicity, and other current challenges. In the coming years, these advancements could help close the considerable gap between the metabolic engineering capabilities in whole plants and microbial systems (Boxes 1 and 2).

Box 1. A bullet point summary of the main points covered in the review

- Roadmap for attempting complex plant metabolic pathway engineering.
- Integrated approaches for predicting pathway-specific genes/enzymes.
- Challenges and progress in developing tools for pathway elucidation and engineering.
- Recent developments in the strategies to improve desired product yields.
- Prospects and challenges need to be addressed in the future for pathway engineering.

Box 2. Open questions related to the topic reviewed in the article

- Can we limit flux diversion and prevent the accumulation of undesirable products?
- How to identify and engineer cell and organelle-specific steps of metabolic pathways?
- How can we fine-tune the level of the engineering target end product?
- Can we use epigenetic control in metabolic pathway engineering?
- Can we develop a technology to more efficiently purify target end product?

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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