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Synthetic biology of plant natural products: From pathway elucidation to engineered biosynthesis in plant cells

Xiaoxi Zhu[1,2,4,6], Xiaonan Liu[1,4,6], Tian Liu[1,3,4], Yina Wang[1,4,5], Nida Ahmed[1,2,4], Zhichao Li[1,4,*] and Huifeng Jiang[1,4,*]

[1]Key Laboratory of Systems Microbial Biotechnology, Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China

[2]University of Chinese Academy of Sciences, Beijing 100049, China

[3]Life Science and Technology College, Guangxi University, Nanning, Guangxi 530004, China

[4]National Technology Innovation Center of Synthetic Biology, Tianjin 300308, China

[5]Yunnan Agricultural University, Kunming, Yunnan 650201, China

[6]These authors contributed equally to this article.

***Correspondence: Zhichao Li (jiang_hf@tib.cas.cn), Huifeng Jiang (lizhch@tib.cas.cn)**

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ABSTRACT

Plant natural products (PNPs) are the main sources of drugs, food additives, and new biofuels and have become a hotspot in synthetic biology. In the past two decades, the engineered biosynthesis of many PNPs has been achieved through the construction of microbial cell factories. Alongside the rapid development of plant physiology, genetics, and plant genetic modification techniques, hosts have now expanded from single-celled microbes to complex plant systems. Plant synthetic biology is an emerging field that combines engineering principles with plant biology. In this review, we introduce recent advances in the biosynthetic pathway elucidation of PNPs and summarize the progress of engineered PNP biosynthesis in plant cells. Furthermore, a future vision of plant synthetic biology is proposed. Although we are still a long way from overcoming all the bottlenecks in plant synthetic biology, the ascent of this field is expected to provide a huge opportunity for future agriculture and industry.

Key words: plant natural products, plant synthetic biology, plant transgenic technology

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INTRODUCTION

Plants produce numerous natural products with diverse structures and bioactive properties, especially secondary metabolites involved in biological and environmental responses. These plant natural products (PNPs) are widely used in the food, pharmaceutical, and cosmeceutical industries and have a huge commercial value in human society (Liu et al., 2017; Maeda, 2019). However, traditional extraction methods and excessive exploitation could lead to the exhaustion of plant resources and to environmental pollution because of the low abundance of PNPs. The structural complexity of some PNPs often makes total chemical synthesis inefficient (Morrison and Hergenrother, 2014). Therefore, with the boom in synthetic biology and omics-based strategies, great success has been achieved in the production of PNPs in engineered hosts.

Because of their well-defined metabolic background and ease of further genetic manipulation, microbial cells such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Corynebacterium glutamicum* have been widely used as chassis cells to produce PNPs (Ajikumar et al., 2010; Paddon et al., 2013; Galanie et al., 2015; Li et al., 2018e; Liu et al., 2018; Luo et al., 2019). Standard microbial synthesis strategies include selecting and engineering hosts, planning and reconstructing metabolic pathways, regulating metabolic networks, and protein engineering (Cravens et al., 2019; Pyne et al., 2019; Birchfield and Mcintosh, 2020; Yang et al., 2020). Efficient production of PNPs requires significant effort to artificially modify and engineer microbes, owing to their specific metabolic pathways with very strict regulatory mechanisms. However, reconstruction in heterologous microorganisms is almost impossible for extremely complex or unclear pathways,

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such as that of paclitaxel, which contains multiple P450 reactions (McElroy and Jennewein, 2018).

In recent years, increasing progress in the biosynthesis of PNPs in plants has been reported (Table 1). The complex multicellular eukaryotic system has become a topic of significant research interest in synthetic biology (Liu and Stewart, 2015). Engineering of host plants for suspension cell culture/hairy root cultures or heterologous plants with the accumulation of intermediate products seems to be a complementary way to achieve the high-yield production of complex PNPs (Nagegowda and Gupta, 2020). Compared with single-celled microorganisms, plants are better systems for synthesizing PNPs, and their better performance can be attributed to three plant homology aspects (Figure 1): (1) high expression and activity of plant-derived enzymes, (2) richness in various endomembrane systems and organelles that provide the most suitable reaction environment, and (3) high tolerance to the toxicity of PNPs and their intermediates.

Even in this emerging research field, many specialized metabolic pathways have been introduced into heterologous plants, and advances in enabling technologies such as omics analysis techniques, plant genetic engineering technology, and analytical techniques have accelerated the development of this field (Naqvi et al., 2010; Bock, 2014; Mortimer, 2019). Even so, whether in microorganisms or plants, the identification of biosynthetic pathways remains the most significant bottleneck in PNP biosynthesis. Accordingly, it is very important to prioritize the reactions and identify the genes involved in the biosynthesis of natural products, especially the genes involved in modification.

In this review, we outline strategies and techniques for the elucidation of PNP biosynthetic pathways and the engineered biosynthesis of PNPs in plant cells. Further progress in plant synthetic biology will not only greatly relieve the pressure on ecological systems but will also facilitate the synthesis of more complex and toxic PNPs. The use of plant cells has become another powerful system for PNP biosynthesis to supplement the deficiencies of microbial chassis.

ELUCIDATION OF PNP BIOSYNTHETIC PATHWAYS

Discovery of key catalytic elements in the biosynthetic pathway is the basis of heterologous production of valuable PNPs. To date, only portions of PNP biosynthetic pathways have been completely elucidated. The lack of the key catalytic element is becoming the greatest obstacle to the elucidation of complex PNP biosynthesis, as the complex metabolism of PNPs and limited analytical techniques hinder the identification of metabolic pathways. With advances in next-generation sequencing technology, computational biology, and synthetic biology, numerous gene-mining techniques have been developed.

Next-generation sequencing-guided pathway discovery

Rapid development of high-throughput sequencing makes it possible to unveil the genetic blueprint of many important plant species, as well as the gene variation among individuals at the population level; it has become the basis for in-depth gene mining and investigation of PNP biosynthesis mechanisms (Shang et al., 2014; Guo et al., 2018; Liu et al., 2018; Kang et al., 2020; Tu et al., 2020; Rai et al., 2021). For the analysis of specific metabolic pathways, the transcriptome data of species are often more helpful and easier to obtain. Screening candidate genes that may participate in the synthesis of specific natural products from tens of thousands of genes is always challenging. Hence, it is possible to identify candidate genes by comparative analysis of samples with different metabolite compositions in target pathways; these samples may be from different plant species, accessions, or mutants, or from plants induced by a target-pathway stimulus. Differential transcriptional analysis can be performed on different samples to obtain candidate genes and enable further functional analysis (Winzer et al., 2012; Galanie et al., 2015; Lau and Sattely, 2015; Polturak et al., 2016; Farrow et al., 2018; Kim et al., 2018; Christ et al., 2019; Yang et al., 2019; Nett et al., 2020; Stander et al., 2020; Xie et al., 2020).

Secondary metabolic pathways in microorganisms often exist in the form of gene clusters on chromosomes, which are very helpful for the analysis of metabolite biosynthetic pathways, excavation of biological components, or subsequent engineering design. In recent years, with the in-depth study of plant genomes, biosynthetic genes of some specific plant secondary metabolites have also been found to be arranged in clusters on plant chromosomes. Plant gene clusters are composed of at least three non-homologous genes involved in a distinct chemical pathway (Nutzmann and Osbourn, 2014). Lately, many computational tools have been developed and have facilitated the identification of increasing numbers of predicted plant gene clusters, thereby accelerating the discovery of PNP biosynthetic pathways and the design of synthetic clusters in heterologous hosts (Medema and Osbourn, 2016; Chavali and Rhee, 2018; Arya et al., 2020). These tools include plantiSMASH, PhytoClust, PlantClusterFinder, and MIBiG (Medema et al., 2015; Kautsar et al., 2017; Schlapfer et al., 2017; Topfer et al., 2017). They also provide abundant alternative and modified biological elements for synthetic biology research. The biosynthetic pathway for noscapine, a potential anti-cancer compound, was shown to consist of a 10-gene cluster in opium poppy (Winzer et al., 2012). Guo et al. (2018) reported the draft genome of opium poppy, which enabled them to identify a large 15-gene cluster and obtain information on its evolution. Homology analysis showed that cytochrome P450 and oxidoreductase genes fused to form a STORR gene fusion, which plays a key role in the biosynthesis of morphine in poppy. Cheng et al. (2021) presented a chromosome-level genome assembly for Himalayan yew and revealed that tandem duplication was the driving force for gene family evolution in the genome. This reference genome will serve as a platform for decoding the complete biosynthetic pathway of paclitaxel and for understanding the chemodiversity of taxoids in gymnosperms.

Gene knockdown/knockout-guided pathway verification

At present, the main approach for functional verification of target pathways in their original plants is to inhibit the function of all or part of the enzymes by gene deletion or expression silencing

Classification	Secondary metabolites	Plant host	Strategy and method	References
Isoprenoids	Isoprenoids	*N. tabacum*	Overexpression	(Kumar et al., 2012)
Monoterpenoids	Geraniol	*N. tabacum*	Overexpression	(Vasilev et al., 2014)
	Geraniol	*N. tabacum*	Overexpression	(Ritala et al., 2014)
	Limonene	*M. piperita*	Suppression	(Mahmoud et al., 2004)
	Mentha oil	*M. piperita*	Suppression	(Mahmoud and Croteau, 2001)
Sesquiterpenoids	Artemisinin	*N. tabacum*	Overexpression	(Farhi et al., 2011)
	Artemisinin	*N. tabacum*	Compartmentalized metabolic engineering	(Malhotra et al., 2016)
	Artemisinin	*A. annua*	Over expression and Global regulation	(Shen et al., 2018)
	Artemisinin	*A. annua*	Suppression	(Lv et al., 2016b)
	Artemisinin	*A. annua*	Global regulation	(Han et al., 2014b; Lv et al., 2016a; Shen et al., 2016; Tan et al., 2015; Yan et al., 2017)
	Artemisinin	*P. patens*	Overexpression	(Ikram et al., 2017; Ikram et al., 2019)
	Artemisinin acid	*N. tabacum*	COSTREL	(Fuentes et al., 2016)
	Patchoulol/santalene	*P. patens*	Compartmentalized metabolic engineering	(Zhan et al., 2014)
	Sesquiterpenoids	*N. tabacum*	Compartmentalized metabolic engineering	(Wu et al., 2006)
Diterpenoids	Diterpenes	*P. patens*	Overexpression	(Banerjee et al., 2019)
	Sclareol	*P. patens*	Overexpression	(Pan et al., 2015)
	Tanshinone	*S. miltiorrhiza*	Elicitors	(Yang et al., 2018)
	Taxadiene	*A. thaliana*	Compartmentalized metabolic engineering	(Besumbes et al., 2004)
	Taxadiene/taxadiene-5-ol	*N. benthamiana*	Compartmentalized metabolic engineering	(Li et al., 2019)
	Taxadiene	*P. patens*	Overexpression	(Anterola et al., 2009)
	Taxol	*Taxus* spp. cell suspension culture	Plant Cell Fermentation (PCF) Technology	https://phytonbiotech.com/apis/paclitaxel/
	Taxol	*Taxus* spp. cell suspension culture	Samyang's plant cell culture technology	https://www.samyangbiopharm.com/eng/
Triterpenoids	Astragaloside	*A. membranaceus* hairy root	Elicitors	(Jiao et al., 2016)
	Diosgenin	*N. benthamiana*	Pooled screens and Overexpression	(Christ et al., 2019)
	Dammarenediol-II	*N. tabacum*	Overexpression	(Han et al., 2014a)
	Ginsenoside	*P. ginseng*		www.nitto.com
	Protopanaxatriol/ protopanaxadiol	*N. tabacum*	Overexpression	(Chun et al., 2015; Gwak et al., 2019)
	Squalene	*N. tabacum*	Compartmentalized metabolic engineering	(Pasoreck et al., 2016)
	Triterpene	*N. benthamiana*	Combinatorial transformation	(Reed et al., 2017)
	Triterpenoids	*C. asiatica* hairy root	Elicitors	(Baek et al., 2020)

Table 1. List of representative examples of engineered biosynthesis of plant natural products in plant cells

(Continued on next page)

Classification	Secondary metabolites	Plant host	Strategy and method	References
Tetraterpenoids	β-Carotene	Rice	Overexpression	(Ye et al., 2000)
	β-Carotene	Tomato	Overexpression	(Apel and Bock, 2009)
	β-Carotene/carotenoids	Maize	Combinatorial transformation	(Zhu et al., 2008)
	Carotenoid	Potato	Overexpression	(Diretto et al., 2007)
	Canthaxanthin/astaxanthin	Rice	Overexpression	(Zhu et al., 2018)
	GABA	Tomato	CRISPR/Cas9-mediated metabolic engineering	(Nonaka et al., 2017)
	Lycopene	Tomato	Suppression	(Li et al., 2018d)
	Provitamin A	Tomato	Overexpression	(Wurbs et al., 2007)
Phenylpropanoids	Anthocyanin	*A. thaliana*	Global regulation	(Xu et al., 2017)
	Anthocyanin	*L. ruthenicum*/*L. barbarum*	Global regulation	(Zong et al., 2019)
	Anthocyanin	*N. tabacum*/*A. thaliana*	Global regulation	(Appelhagen et al., 2018)
	Anthocyanin	Rice	Global regulation	(Zhu et al., 2017)
	Anthocyanin	Tomato	CRISPR/Cas9-mediated metabolic engineering	(Cermak et al., 2015)
	Anthocyanin	Tomato	Global regulation	(Meng et al., 2014)
	Anthocyanin/phenylpropanoid flavonoid derivatives	Tomato	Global regulation	(Tohge et al., 2015)
	Anthocyanin	Tomato	Global regulation	(Li et al., 2018a)
	Chrysin/wogonin/baicalein	*Scutellaria bornmuelleri*	Elicitors	(Zg et al., 2020)
	Coumarin/phenolic compounds	*P. sidoides* hairy root	Elicitors	(Yousefian et al., 2021)
	Cyanidin 3-*O*-rutinoside	*N. tabacum*	Global regulation	(He et al., 2017)
	Delphinidin	*Chrysanthemum morifolium*	Overexpression	(Noda et al., 2017)
	Delphinidin	*Rosa hybrida*	Overexpression	(Katsumoto et al., 2007)
	Delphinidin/petunidin	Tomato	Global regulation	(Outchkourov et al., 2018)
	Flavonoids	Tomato	Global regulation	(Zhang et al., 2015)
	Flavonols	Tomato	Global regulation	(Brown et al., 2015; Zhang et al., 2015)
	Isoflavone	Soya bean	CRISPR/Cas9-mediated metabolic engineering	(Zhang et al., 2020a)
	Pterostilbene/piceatannol	*V. vinifera*	Elicitors	(Martinez-Marquez et al., 2018; Martinez-Marquez et al., 2016)
	Resveratrol	*V. vinifera*	Elicitors	(Chu et al., 2017)
	Resveratrol/dihydroquercetin	*V. vinifera*	Global regulation	(Manela et al., 2015)
	Silymarin	*Silybum marianum*	Elicitors	(Gabr et al., 2016)

Table 1. ***Continued***

(Continued on next page)

Classification	Secondary metabolites	Plant host	Strategy and method	References
Alkaloids	Berberine	*Coptis japonica*/*Thalictrum minus* cell suspension culture		(Matsubara et al., 1989)
	Catharanthine/tabersonine	*N. benthamiana*	Overexpression	(Caputi et al., 2018)
	Etoposide aglycone	*N. benthamiana*	Overexpression	(Lau and Sattely, 2015)
	Hyoscyamine	*A. belladonna*	CRISPR/Cas9-mediated metabolic engineering	(Zeng et al., 2021)
	N-Formyldemecolcine	*N. benthamiana*	Overexpression	(Nett et al., 2020)
	Strictosidine	*N. benthamiana*	Overexpression	(Miettinen et al., 2014)
	Tropane alkaloids	*A. belladonna*	Global regulation	(Bai et al., 2019)
	Tropane alkaloids	*A. belladonna*	Overexpression	(Zhao et al., 2020)
	Tropane alkaloids	*Hyoscyamus* hairy root	Elicitors	(Hedayati et al., 2020)
	Vincristine/vinblastine	*C. roseus*	Elicitors	(Mekky et al., 2018)
Other PNPs	Dhurrin	*N. benthamiana*	Compartmentalized metabolic engineering	(de Jesus et al., 2017; Gnanasekaran et al., 2016)
	Vanillin	*C. frutescens*	Overexpression	(Chee et al., 2017)
	Vitamins	Maize	Combinatorial transformation	(Naqvi et al., 2009)
	Vitamin E	Tomato	Overexpression	(Lu et al., 2013)
	Vitamin E	Lettuce	Overexpression	(Yabuta et al., 2013)

Table 1. *Continued*

Abbreviations: *A. belladonna* (*Atropa belladonna*); *A. membranaceus* (*Astragalus membranaceus*); *C. asiatica* (*Centella asiatica*); *C. frutescens* (*Capsicum frutescens*); *C. roseus* (*Catharanthus roseus*); *L. barbarum* (*Lycium barbarum*); *L. ruthenicum* (*Lycium ruthenicum*); *M. piperita* (*Mentha*×*piperita* L.); *P. ginseng* (*Panax ginseng* L.); *P. sidoides* (*Pelargonium sidoides*); *S. miltiorrhiza* (*Salvia miltiorrhiza*); *V. vinifera* (*Vitis vinifera*).

and then to detect changes in secondary metabolites in order to identify the functions of candidate pathway genes. Gene expression silencing technology is a widely used reverse genetics method that can verify the function of plant genes (Winzer et al., 2012; Kamthan et al., 2015; Qua et al., 2015; Zhao et al., 2015, 2016; Ma et al., 2016; Fu et al., 2021). Qua et al. (2015) used virus-induced gene silencing technology to rapidly identify a cytochrome P450 and an alcohol dehydrogenase that completed the pathway from tabersonine to the anti-cancer drug precursor vindoline. Fu et al. (2021) completed chicoric acid biosynthesis in purple coneflower through RNA interference technology for the identification of acyltransferase.

In recent years, with the rise of the CRISPR/Cas9 system, this gene-editing technology has been widely used for functional verification of key enzymes and regulatory elements because of its potency, versatility, and ability to knock out multiple genes simultaneously (Mercx et al., 2017; Montecillo et al., 2020). Compared with previous genome-editing methods, CRISPR/Cas9 enables convenient design with high accuracy and precision, and homozygous mutations at the desired site can be stably inherited by the subsequent generation. Editing plant genomes for transcriptional regulation and enzyme manipulation using CRISPR/Cas9 has been successful in many plants (Lowder et al., 2015; Alagoz et al., 2016; Liu et al., 2017; Feng et al., 2018; Zhu et al., 2018). A gene cluster and two key genes involved in medium-chain acyl sugar accumulation in Solanaceae were discovered by making loss-of-function mutations using the CRISPR/Cas9 system (Fan et al., 2020). This gene-editing technology has been established in rice, corn, and other crops and used to identify the role of various genes (Duan et al., 2016; Ma et al., 2017; Shen et al., 2017; Wang et al., 2017, 2019; Yasui et al., 2017; Huang et al., 2018; Li et al., 2018c; Zhao et al., 2019; Zuo et al., 2019).

Synthetic biology platform-guided pathway reconstruction

Using synthetic biology platforms is a practical approach for pathway elucidation (Ignea et al., 2016; Wang et al., 2020). With the decreasing costs of high-throughput DNA synthesis, an increasing number of alternative enzymes have been codon-optimized, synthesized, and used to complete metabolic pathways. Microbes and model plants are widely used to reconstruct pathways of many important PNPs. *E. coli* and *S. cerevisiae* are two kinds of microorganisms with strong biosynthetic ability, and their genetic operation and metabolic modifications have been studied in detail (Borodina and Nielsen, 2014; Blount, 2015). Yeast is a more suitable host as a synthetic biology platform for pathway discovery of complex PNP metabolic pathways because of its eukaryotic properties (Li et al., 2018e; Luo et al., 2019; Srinivasan and Smolke, 2020). Wang et al. (2020) established a platform to elucidate the UGT-mediated glycosylation process of the main triterpene in *Panax notoginseng*. The creation of such a synthetic biology platform bypassed the difficulties in obtaining intermediates and expressing enzymes *in vitro* (Wang et al., 2020). Longer PNP biosynthetic pathways are often divided into several biosynthetic modules.

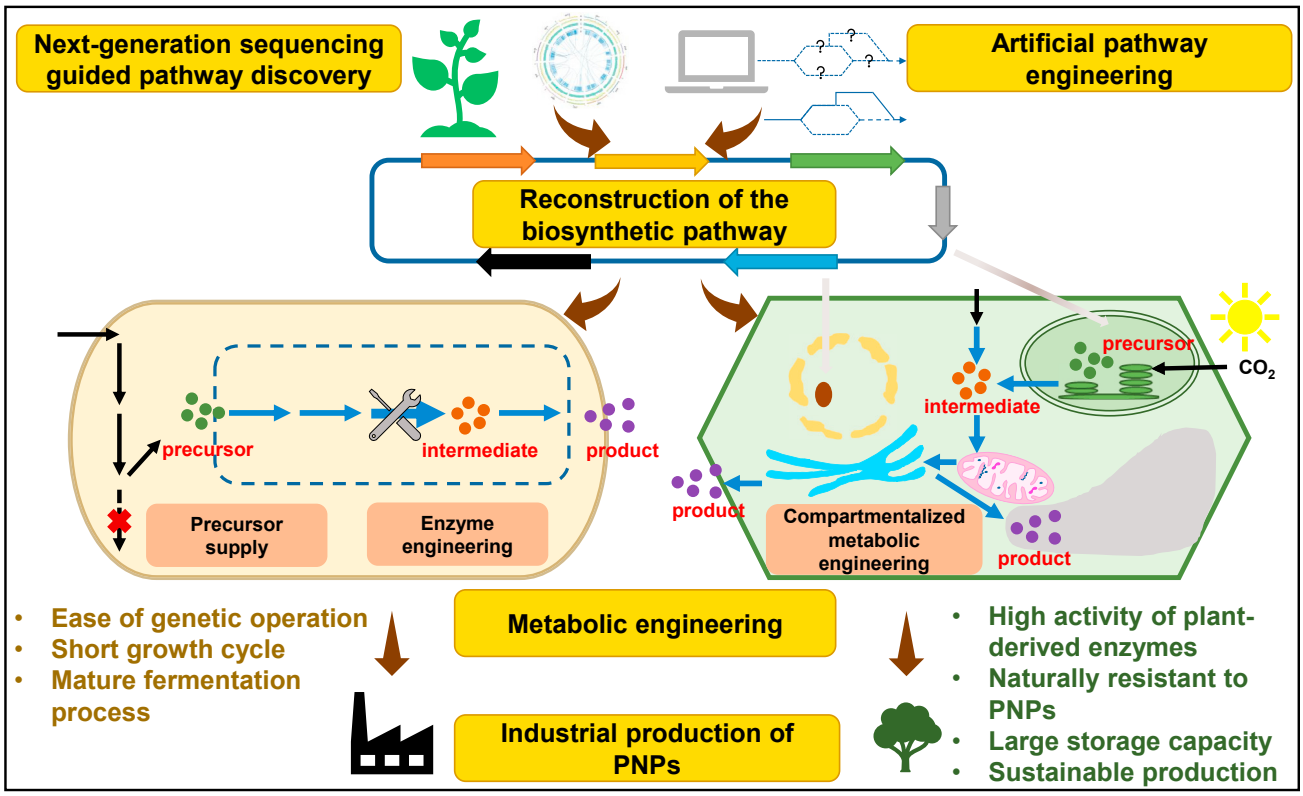

Figure 1. Biosynthesis of plant natural products in microbial and plant cells.

The substrates and products of these modules are generally available, which facilitates the identification of unknown enzyme reactions in the module and the detection of target products. One successful case was the construction of a breviscapine synthetic factory in yeast through the use of three modules involving eight genes (Liu et al., 2018). Similarly, candidate pathways of different PNPs such as dihydrosanguinarine, opioids, strictosidine, noscapine, and scopolamine have been successfully elucidated and constructed in microbes using a modular strategy (Fossati et al., 2014; Brown et al., 2015; Galanie et al., 2015; Trenchard and Smolke, 2015; Li and Smolke, 2016; Srinivasan and Smolke, 2020). It is worth noting that in the case of scopolamine biosynthesis, the final strain comprises 34 chromosomal modifications (26 genes, 8 gene disruptions) in six diverse subcellular locations (Srinivasan and Smolke, 2020).

Expression of enzymes in microbial systems often has issues of post-translational modification and requires multigene engineering. Genetic elements in plants, such as cytochrome P450 (Liu et al., 2020), are inefficient when transferred to heterogeneous microorganisms. Therefore, it has become a powerful measure to identify the key enzymes and determine the reaction sequence using plant hosts. Transient expression refers to the transfer of a target gene into cells for a relatively short time. Exogenous DNA introduced into the cells does not integrate with the host DNA but temporarily exists in the cells for transcription and translation, enabling the rapid functional characterization of unknown genes by analysis of the final metabolites (Lau and Sattely, 2015; Caputi et al., 2018; Christ et al., 2019; Nett et al., 2020). Christ et al. (2019) used a pooled-screen approach to identify CYPs that were necessary to catalyze the 5,6-spiroketalization of cholesterol in the biosynthesis of diosgenin in *Paris polyphylla* and *Trigonella foenum-graecum*. Combinatorial expression of 29 full-length candidate *P. polyphylla* CYPs and 33 full-length candidate *T. foenum-graecum* CYPs in *Nicotiana benthamiana* led to the construction of a screening pool. The key enzymes were screened by removing one candidate gene at a time from the pool. In the end, several active pairs of CYPs were identified. Recently, an automated high-throughput screening platform was established on the basis of transient expression for screening recombinant protein production, showing that the innovation of various technologies will greatly accelerate the elucidation of PNP biosynthetic pathways (Gengenbach et al., 2020).

Overall, the elucidation of PNP biosynthetic pathways is a laborious process that often requires a combination of various strategies, including but not limited to those mentioned above, such as sequencing technology, bioinformatics analysis, high-throughput screening, and other synthetic biology technologies. For example, Galanie et al. (2015) combined gene mining, enzyme engineering, and metabolic pathway and strain optimization to achieve opiate biosynthesis. A combination of transcriptomics and metabolomics enabled the elucidation of the nearly complete colchicine alkaloid synthesis pathway and the synthesis of a colchicine alkaloid precursor in *N. benthamiana* (Nett et al., 2020). Gao et al. (2020) reported a biosynthetic intermediate probe-based target identification strategy. Combined with traditional activity-based protein

purification and transcriptome analysis, the first flavin adenine dinucleotide-dependent enzyme-catalysed intermolecular [4 + 2] cycloaddition in natural product biosynthesis was discovered, providing a new way to study PNP biosynthetic pathways.

ENGINEERED BIOSYNTHESIS OF PNPs IN PLANT CELLS

Secondary metabolic pathways of plants are very complex and can be linear, cyclical, or arranged in a three-dimensional grid with many branches (Verpoorte et al., 2000; Farre et al., 2014). Because of the limitations of microbial chassis, such as poor expression of P450 enzymes from plants and poor tolerance to active products, many microbial cell factories cannot produce the final active products or have low yields. Therefore it is very advantageous to perform metabolic engineering transformation in plants, because the whole or partial biosynthetic pathway of many target PNPs already exists. Production of target metabolites can be implemented by overexpression of missing or rate-limiting enzyme genes in the biosynthesis pathway or by blocking competing reactions (Ikram and Simonsen, 2017; Badshah et al., 2018; Nagegowda and Gupta, 2020). It is worth noting that plants have three major genetic systems: nuclear, plastid, and mitochondrial. Some plastids (chloroplasts) are exclusive to plants and produce some specific metabolites, such as terpenoids. Owing to the advantage of plant homology, plant cells are very suitable for the synthesis of some PNPs. Here we introduce recent progress in the engineered biosynthesis of PNPs in plant cells, mainly based on the engineering of different genetic systems (Table 1).

Nuclear genome engineering

Multiple gene co-transformation: Reconstruction of metabolic pathways

Integrating multiple transgenes into the nuclear genome of plants and ensuring stable expression of these genes in future generations have always been the focus of plant synthetic biology. Golden rice is a successful case of plant synthetic biology (Ye et al., 2000). Through *Agrobacterium*-mediated multigene co-transformation, the entire biosynthetic pathway of β-carotene was introduced into rice endosperm. In subsequent studies, canthaxanthin rice and astaxanthin rice appeared one after another, making rice a dietary supplement to meet daily nutritional needs (Zhu et al., 2018). In addition, rice cell suspension culture is becoming a model for the production of specialized plant metabolites (Arya et al., 2021).

As a model organism, tobacco has been widely used in synthetic biology research. Farhi et al. (2011) achieved heterologous synthesis of artemisinin in *Nicotiana tabacum* for the first time through co-transformation of several artemisinin synthases and rate-limiting enzymes, which increased the supply of precursors greatly and generated yields of artemisinin as high as 6.8 μg/g dry weight. Forestier et al. (2021) developed an *N. benthamiana* platform for the production of high-value diterpenoids. The synthesis of plant secondary metabolites is closely related to the regulation of endogenous transcription factors (TFs). There are broad prospects for improving the levels of plant cell secondary metabolites by activating the expression of multiple synthetase genes in plant secondary metabolite synthesis pathways. Appelhagen et al. (2018) achieved stable production of anthocyanin by constitutively expressing the TFs MYB Rosea1 (AmRos1) and bHLH Delila (AmDel) from *Antirrhinum majus* in *N. tabacum* suspension cells.

Besides tobacco, other plants also have the potential to serve as excellent hosts. Because of its short life cycle, small genome size, and efficient homologous recombination, *Physcomitrella patens* has become a promising model organism (Reski et al., 2018; Campos et al., 2020; Decker and Reski, 2020). *P. patens* was previously used to synthesize taxadiene derived from *Taxus*, and the yield of taxadiene reached 5 μg/g (Anterola et al., 2009). Since then, owing to its natural tolerance to terpenoids and simple metabolic profile of endogenous terpenoids, *P. patens* has been widely used for the expression of terpenoid biosynthesis genes and the reconstruction of pathways (Bach et al., 2014; Banerjee et al., 2019). For example, artemisinin was synthesized by directly engineering DNA fragments of five enzymes of the artemisinin synthesis pathway into the moss *P. patens* without any other modification (Ikram et al., 2017, 2019). The first active ingredient from biotechnologically produced moss is MossCellTec No. 1, which is a highly innovative active compound based on a novel anti-aging concept (https://mibellebiochemistry.com/mosscelltectm-no-1). In addition, the low-scent background characteristics of *P. patens* make it very suitable for the production of perfume material, such as α/β-santalol, patchoulol, and sclareol (Zhan et al., 2014; Pan et al., 2015). Mosspiration Biotech launched Orbella Moss, a fragrance-producing moss and the first genetically modified fragrant plant (https://www.mosspirationbiotech.com/fragrant-moss/).

The CRISPR/Cas9 system is gradually becoming a powerful tool for plant metabolic engineering (Arya et al., 2020; Montecillo et al., 2020; Sabzehzari et al., 2020; Dey, 2021). For example, anthocyanin content has been shown to accumulate through the introduction of double-strand breaks mediated by CRISPR/Cas9 for a powerful promoter inserted in tomato (Cermak et al., 2015). Also in tomato, the autoinhibitory domain of glutamate decarboxylase, a key enzyme in γ-aminobutyric acid (GABA) biosynthesis, was deleted using CRISPR/Cas9, resulting in 7- to 15-fold increases in GABA yield (Nonaka et al., 2017). A multiplex gene-editing system was used to metabolically engineer the isoflavone content of soya bean. Three genes were targeted simultaneously, and the triple gene mutation efficiency reached 44.44%, confirming the editing efficiency of CRISPR/Cas9 technology (Zhang et al., 2020a). Inhibiting competitive pathway genes can produce more metabolic flow to the target products to improve yield. Lv et al. (2016b) introduced antisense cDNA into *Artemisia annua* to downregulate branch pathways, resulting in artemisinin accumulation. Li et al. (2018d) used CRISPR/Cas9 multiplexing to simultaneously regulate multiple genes involved in the carotenoid biosynthesis pathway, thus increasing the content of lycopene 5.1-fold in tomato fruit. In the same way, GABA-enriched tomato was developed by manipulating the GABA shunt (Li et al., 2018b). Zeng et al. (2021) generated *Atropa belladonna* plant lines with high yields of hyoscyamine and without anisodamine and scopolamine via CRISPR/Cas9-based disruption of hyoscyamine-6β-hydroxylase.

Combinatorial transformation: Screening high-yield transgenic plants

With the deepening of research, more and more complex pathways need to be reconstructed in plants. This requires the generation of many transformants with combinations of different candidate genes and expression levels to ensure proper pathway reconstruction and high yields of target products. However, this is difficult to achieve because of the long cycles and demanding operations involved in traditional plant transformation. To overcome these problems, Zhu et al. (2008) proposed a combinatorial nuclear transformation method to dissect and modify the carotenoid biosynthetic pathway in maize to produce multiplex-transgenic plants in a short time. In this process, five carotenogenic genes were constructed in separate plasmids and integrated into the genome at the same locus using biolistic transformation to introduce all plasmids simultaneously. Transgenic plants with distinct metabolic phenotypes that expressed different enzyme combinations were generated to identify the rate-limiting enzymes in the metabolic pathway, providing a unique strategy for the analysis of PNP metabolic pathways (Zhu et al., 2008). The team then used this method to generate transgenic maize plants that could simultaneously increase the production of three vitamins (Naqvi et al., 2009). Results showed that the contents of carotene, ascorbic acid, and folic acid were significantly increased in the endosperm by expression of four transgenes, indicating that multigene transformations could efficiently regulate multiple metabolic pathways at the same time.

Combinatorial transformation is a multifunctional method that can be used to engineer metabolic pathways and control other biochemical, physiological, or developmental processes. Many transformations are performed to create a combinatorial library of transformants, each with a different combination of transgenes with varying copy number and genomic arrangement, which result in diverse expression levels. To date, there is no sign of a limit to the number of transgenes that can be introduced into the plant in one transformation, making this method a powerful form of co-transformation.

Transient expression: Rapid production of target compounds

Stable transformation with good reproducibility has the potential advantage of large-scale production, but transgenic plants are usually the second generation, so their creation is often very laborious (Tyurin et al., 2020). Transient expression can be used to express recombinant protein in batches within a few days and then used to co-transfer candidate genes into plants to rapidly produce target compounds (Lau and Sattely, 2015; Christ et al., 2019; Nett et al., 2020). Many transient expression systems are based on plant viruses. High levels of recombinant protein expression depend on rapid replication and transmission and high expressionyield of these viruses in the plant host (Gleba et al., 2014). The pEAQ vectors have been used to produce a wide variety of proteins in plants. Besides viral proteins and virus-like particles, other types of proteins including active enzymes have also been expressed. Recently, the pEAQ system has been frequently used for PNP production in tobacco. Taxane biosynthesis was carried out in *N. benthamiana* using *Agrobacterium*-mediated transient transformation (Li et al., 2019). Transient expression of 16 genes from mayapple in *N. benthamiana* resulted in the accumulation of the hemotherapeutic drug etoposide at the milligram scale (Schultz et al., 2019). Miettinen et al. (2014) successfully reconstructed the upstream pathway of terpenoid indole alkaloid biosynthesis in *N. benthamiana*, providing a basis for the development of a variety of therapeutic compounds. Co-infiltration of *Agrobacterium* strains with different expression constructs is a powerful method of rapidly expressing numerous enzyme combinations (Reed and Osbourn, 2018). Transient combinatorial transformation of multiple P450s generated over 40 oxygenated triterpene analogs in *N. benthamiana*, many of which had not been reported previously (Reed et al., 2017). Kashkooli et al. (2019) used a combinatorial metabolic engineering approach to generate costunolide and parthenolide derivatives. Calgaro-Kozina et al. (2020) rationally broadened the set of primary metabolites by expressing diverse enzymes, leading to the production of crucifalexins, a novel class of compounds with potent antifungal activity.

These works illustrate the ability to produce high-quality PNPs by transient plant expression technology. They highlight the potential of rapid combinatorial transformation for the synthesis of new PNP derivatives and provide novel ideas for the discovery of new drugs. Moreover, owing to rapid and high-level expression, some transient expression systems have already been used for industrial-scale production of target proteins (Kopertekh and Schiemann, 2019; Huebbers and Buyel, 2021). Recently, a highly efficient *Agrobacterium*-mediated strategy for transient transformation was created, making this technology a promising application for a variety of plant species (Zhang et al., 2020b).

Plastid engineering

Plastid genetic engineering has attracted much attention due to the plastid's high expression of genes, accuracy of homologous recombination, and pattern of gene stacking in operons. In addition, its use can avoid environmental safety problems caused by pollen transmission to some extent because of the maternal mode of plastid inheritance in most angiosperm species (Bock, 2014; Yu et al., 2020). However, conventional chloroplast transformation is mainly limited to the Solanaceae (Yu et al., 2020). A recent study reported an efficient system for plastid genetic transformation in *Arabidopsis* root callus (Ruf et al., 2019). A novel method of plastid transformation using nanoparticles was reported, accelerating the development of plastid genetic transformation technology for other species (Kwak et al., 2019). For plants that cannot undergo chloroplast transformation, horizontal gene transfer mediated by grafting is a promising way to obtain foreign chloroplast genes (Stegemann et al., 2012; Thyssen et al., 2012; Lu et al., 2017). This strategy is helpful for expanding the range of species suitable for plastid engineering.

Plastid engineering for high yield of PNPs

Because there are many copies of the chloroplast genome in each cell, higher levels of protein accumulation can often be achieved from transgenes inserted into the chloroplast genome compared with the nuclear genome, and the operon pattern of chloroplast genes makes expression regulation of the transgene much easier (Jensen and Scharff, 2019). The methylerythritol phosphate (MEP) pathway is unique to plants and takes place in the chloroplast; chloroplast transformation is therefore

usually used to increase the yield of isoprenoid compounds (Saxena et al., 2014; Pasoreck et al., 2016). By inserting the whole mevalonate (MVA) pathway into the *N. tabacum* chloroplast genome, metabolic flux in the isoprenoid biosynthesis pathway was remolded, resulting in the higher accumulation of mevalonate, carotenoids, squalene, sterols, and triacylglycerols (Kumar et al., 2012). Moreover, redirecting the reaction into the plastid has always led to production improvement. One of the successful examples described in tobacco was the targeting of sesquiterpenoid synthase to the plastids. This afforded a 40 000-fold increase in yield (Wu et al., 2006). Through chloroplast-compartmentalized engineering and overexpression of rate-limiting enzymes of isoprenoid pathways, the yield of taxadiene and taxadiene-5α-ol were increased to 56.6 μg/g and 1.3 μg/g fresh weight, respectively, in tobacco (Li et al., 2019).

Plant cytochrome P450s are the largest family of enzymes involved in PNP biosynthesis, and they mainly play the role of monooxygenases in plants (Liu et al., 2020). A new green production plant has been built by coupling P450 enzyme with an *in vitro* light system or by transforming the P450-dependent pathway into chloroplasts using photosynthetic reducing power to drive the catalytic activity of P450s (Jensen et al., 2011; Nielsen et al., 2013; Lassen et al., 2014; Mellor et al., 2016). Gnanasekaran et al. (2016) expressed two P450 enzymes and one glycosyltransferase in tobacco chloroplasts to construct the biosynthetic pathway of dhurrin. On this basis, three enzymes involved in the pathway were fused with Tat protein to carry out co-localization in plastids, which increased titers of dhurrin 5-fold (de Jesus et al., 2017). Mellor et al. (2019) further investigated the efficacy of four electron carrier proteins fused to CYP79A1 in *N. benthamiana*. A flavodoxin-like carrier showed the greatest improvement in sequestration of photosynthetic reducing power (nearly 25-fold on a per-protein basis). These studies provide a new strategy for the metabolic engineering of PNPs in plants.

Combined application of chloroplast and nuclear transformation

Under natural conditions, PNPs are synthesized in different compartments of plant cells, and the enzymes of catalytic reactions are located in different organelles, reducing the toxic effects of intermediates on cells (Espinosa-Leal et al., 2018). The strategy of combining nuclear transformation with chloroplast transformation to reorient different reactions in the metabolic pathway often has excellent effects. Artemisinin was successfully biosynthesized in tobacco by engineering the MVA pathway and artemisinin biosynthesis pathway targeted to three different cellular compartments using chloroplast and nuclear transformation. With this approach, the yield of artemisinin reached 0.8 mg/g dry weight (Malhotra et al., 2016). A new synthetic biology strategy called combinatorial supertransformation of transplastomic recipient lines (COSTREL) was developed to achieve the *de novo* synthesis of artemisinic acid (Fuentes et al., 2016). The core pathway of artemisinic acid biosynthesis was transferred into the chloroplast genome, after which the transplastomic plants were combinatorially supertransformed with cassettes for all additional enzymes known to affect flux through the artemisinin pathway. By screening large populations of COSTREL lines, a plant that could produce more than 120 mg of artemisinic acid per kg of biomass was isolated.

Development of non-green plastid transformation

Although we have acquired adequate knowledge about the gene expression mechanisms of chloroplasts in the past few decades, little is known about non-green plastids. Increased β-carotene production in tomato showed the possibility of using chloroplast transformation to construct biochemical pathways for important nutrients in non-green plastids, potentially impacting many metabolic processes in plants (Wurbs et al., 2007; Apel and Bock, 2009; Ahmad et al., 2016). Chromoplasts in mature tomatoes are differentiated from chloroplasts, which are very active in gene expression. This makes plastid transgenic expression in tomato more successful than in other plants (Sidorov et al., 1999; Ruf et al., 2001; Wurbs et al., 2007). Non-green plastids are ideal hosts for recombinant protein expression; they are more suitable for the production and storage of PNPs than chloroplasts without affecting plant adaptability (Mellor et al., 2018). Recent advances have further promoted the efficient expression of transgenes in non-green plastids (Valkov et al., 2011; Zhang et al., 2012; Caroca et al., 2013; Fuentes et al., 2018; Schaub et al., 2018; Yu et al., 2019; Cortese et al., 2021). This improvement in non-green plastid transformation technology accelerates the development of PNP production in edible fruits and also solves the problem of toxic by-product accumulation caused by metabolic engineering transformation in tobacco (Fuentes et al., 2018).

CONCLUDING REMARKS AND PERSPECTIVES

Secondary metabolites from plants have always been an important source of drugs. Owing to limited resources, we need to establish sustainable routes to supplement the sources of these secondary metabolites. It is important to transform cellular genetic material and metabolic processes into an ideal host and to synthesize PNPs in cells. Although the number of known natural products has exceeded 500 000, the biosynthetic pathways of most natural products have not been identified (Zhang and Schrader, 2017). Therefore, the analysis and biosynthesis of these prodrug compounds have profound significance and considerable prospects.

To date, some synthetic cell factories for PNPs have been successfully established and have reached the level of industrial production. Microbial production of artemisinin, resveratrol, lycopene, and astaxanthin has been successfully commercialized (Mehta et al., 2003; Schmidt et al., 2011; Paddon et al., 2013). Although industrial production of terpenoids in microorganisms has made great advances, microbial production of some specific PNPs still suffers from strong resistance due to pathway complexity, low activity of plant-derived enzymes in the microbial host, and product toxicity (Liu et al., 2017; Maeda, 2019). Use of plants for the synthesis of valuable PNPs has natural advantages. First, plants can better express plant-derived enzymes. Second, some compounds that are toxic to microorganisms, such as alkaloids and phenols, can be stored in plants. Third, the complex organelles and membrane structures

of plants allow fine artificial design because plant metabolic pathways are highly localized. Finally, plants synthesize all kinds of high-value natural products through photosynthesis without the need for supplementation with other raw materials, making them low in cost and sustainable. The development of energy crops reduces the competition of land and food crops to the greatest extent, allowing further development of plant cell factories. Using model plants such as tobacco, tomato, rice, and *P. patens* as platforms for PNP production is becoming very attractive because of their publicly available genome sequences and simplicity of use in biotechnological techniques (Fu et al., 2018).

Even so, it remains challenging to use plants as a chassis for PNP biosynthesis (Sweetlove et al., 2017; Maeda, 2019). First, the genetic and metabolic backgrounds of plants are very complex, with many details still not very clear compared with microorganisms. Second, some enabling technologies for plants are still lacking or inefficient. In addition, the long growth cycle of plants and the difficulty in scaling up to industrial production are always obstacles to plant synthetic biology. Unlike whole plants, the culture cycle of suspension cells is only several days. PNPs could be produced by a process similar to microorganism fermentation using plant suspension cells. In addition, after dedifferentiation of plant cells, a large number of metabolic processes, including secondary metabolism, are silent. This reduces metabolic complexity and may facilitate the systematic regulation of target pathways by genetic engineering, diverse plant endogenous metabolic elicitors, and culture techniques. Some PNPs have already been produced commercially through suspension cell culture and have achieved high yields even without transgenic modification (www.phytonbiotech.com/) (Fujita, 1988; Hibino and Ushiyama, 1999; Wilson and Roberts, 2012; Lange, 2018; Arya et al., 2020). Metabolic engineering of a suspension cell system is expected to be an efficient approach for PNP production in the future. Plant cell culture technology has been widely used for industrial production of active cosmetic ingredients and food additives, such as the novel authorized food ingredients TEUPOL (10P or 50P), ECHINAN 4P, and ACTEOS 10P and the skin care ingredients PhytoCellTec Symphytum and PhytoCellTec nunatak (Georgiev et al., 2018; Krasteva et al., 2021). Some other emerging technologies, such as plant living-root exudation technology–plant milking (https://www.plantadvanced.com/expertises-2/pat-plant-milking), also promote the development of plant cells for PNP biosynthesis. We believe that with the maturity of next-generation sequencing technology, bioinformatics analysis, genetic engineering technology, and other synthetic biology technologies, more and more biosynthetic pathways will be deciphered in medicinal plants. Although there is still a long way to go to overcome the bottlenecks in the development of plant synthetic biology, plant cells will be an excellent alternative candidate for PNP production (Arya et al., 2020; Eljounaidi and Lichman, 2020) and provide a huge opportunity for future agriculture and industry.

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AUTHOR CONTRIBUTIONS

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