Article type: Invited Expert Review

Received Apr. 29, 2022; Accepted July 18, 2022

Edited by Zhizhong Gong，China Agricultural University, China

Running title: Making small molecules in plants

Making small molecules in plants: A chassis for synthetic biology-based production of plant natural products

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ABSTRACT

Plant natural products have been extensively exploited in food, medicine, flavor, cosmetic, renewable fuel and other industrial sectors. Synthetic biology has recently emerged as a promising means for cost-effective and sustainable production of natural products. Comparing to engineering microbes for the production of plant natural products, the potential of plants as chassis for producing these compounds is underestimated largely due to challenges encountered in engineering plants. Knowledge in plant engineering is instrumental for enabling the effective and efficient production of valuable phytochemicals in plants, which also paves ways for a more sustainable future agriculture.

In this manuscript, we briefly recap the biosynthesis of plant natural products, focusing primarily on industrially important terpenoids, alkaloids and phenylpropanoids. We further summarize the plant hosts and strategies that have been employed to engineer the production of natural products. The challenges and opportunities of using plant synthetic biology to achieve rapid and scalable production of high-value plant natural products are also discussed.

Keywords: Biosynthesis, Natural products, Plant engineering, Synthetic biology

INTRODUCTION

Plants produce a wide variety of organic compounds, which can be classified into primary or secondary metabolites, with the latter known also as natural products or specialized metabolites. Unlike primary metabolites (such as phytosterols, acyl lipids, nucleotides, amino acids, and organic acids), which are ubiquitous and essential for the life of every plant, secondary metabolites usually occur in a limited number of taxonomic groups within the plant kingdom and play important roles in the adaptation of plants to their environment, for instance, defense against herbivores and interactions with other species (Stamp, 2003; Samuni-Blank et al., 2012). Furthermore, plant secondary metabolites are also a rich source of medicines, flavors, and recreational drugs (Tiwari and Rana, 2015).

With our ever-increasing demands for food, medicines, flavors, cosmetics and renewable fuels, plants become attractive natural sources for high-value products. However, challenges exist when sourcing secondary metabolites from plants, for reasons as follow: a) The abundance of secondary metabolites is generally low in plants, thus impeding their isolation in large amount from natural plant sources; b) Some plants, especially medicinal plants which contain high-value metabolites, grow slowly and require demanding growth conditions; c) Owing to excessive harvesting and difficulty in domestication, some wild plant species are endangered of extinction, which also makes it unrealistic to massively harvest plants for isolation of natural products; d) Some compounds are difficult to be purified from chemically complex plant extracts; e) Chemical synthesis of structurally complex natural products is difficult, costly and not

environmentally friendly (Owen et al., 2017; Reed and Osbourn, 2018). Therefore, alternative production systems and approaches are warranted for the production of high-value plant natural products.

Within the recent decades, synthetic biology has emerged as a promising means to cost-effective and sustainable production of natural products. Synthetic biology is about building and re-designing biological devices or systems (e.g., bacteria, yeasts, cell cultures or whole plants) for realizing goals such as producing high-value natural products. Synthetic biology has many advantages over conventional approaches such as direct isolation of molecules from plant resources and chemical synthesis as: a) it provides a sustainable and green way for compound production, b) the production cycle is relatively short and facilitates the large-scale industrial production, c) compounds can be produced without excessive harvesting of wild medicinal plant resources, therefore protecting the endangered plant species from extinction. For example, *Saccharomyces cerevisiae* was used as the chassis for the industrial-scale production of 25 g per/L of artemisinic acid by fermentation, which was followed by a chemical conversion process to synthesize artemisinin. Semi-synthetic artemisinin can be produced at industrial scale and has been approved by the WHO for the preparation of approved pharmaceutical compounds for incorporation into Artemisinin-based combination therapies (Paddon et al., 2013).

Microbes are great platforms for engineering the production of plant natural products and have been extensively reviewed (Luo et al., 2015; Van Wyk et al., 2018; Vavitsas et al., 2018; Wang et al., 2018; Choi et al., 2019; Moser and Pichler, 2019; Pham et al., 2019). In contrast, plants as chassis are more or less underestimated potentially due to challenges encountered in engineering plants. In this review, we first introduce the biosynthesis of plant natural products, focusing primarily on industrially important terpenoids, alkaloids and phenylpropanoids. Knowing how these compounds are made in nature is the prerequisite for developing appropriate engineering strategies. Next, we compared and discussed the advantages and disadvantages between plant chassis and micro-organisms as production platforms. We further introduced strategies that have been applied to engineer the production of natural products in plant hosts. These engineering

strategies are introduced in the order according to their target molecules (i.e., DNA, RNA, or protein) to be engineered in the central dogma of biology. Methods used to regulate or alter different stages of the central dogma, including DNA replication, transcription, and translation are discussed in detail. Finally, we discussed the challenges and opportunities of using plant synthetic biology to achieve rapid and scalable production of valuable phytochemicals.

CLASSIFICATION AND BIOSYNTHESIS OF PLANT NATURAL PRODUCTS

Terpenoids

Terpenoids are the largest class of natural products, with more than 80,000 known compounds (Christianson, 2017). Terpenoids have various physiological and ecological functions, protecting plants from different stress conditions (Tholl, 2015) and serving as important source of fragrances, cosmetics, perfumes, food additives, flavors, chemical feedstocks and biofuels (Holstein and Hohl, 2004; Tetali, 2019).

Terpenoids can be grouped into different sub-classes based on the number of five-carbon isoprene units, including hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30), and polyterpenes (>C30). All terpenoids are derived from the two universal five-carbon building blocks: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Figure 1A). These five-carbon prenyl phosphate precursors are synthesized by two non-homologous metabolic routes including the cytosolic mevalonate (MVA) and the plastidial 2-C-methyl-D-erythritol4-phosphate (MEP) pathways (Lange et al., 2000; Hemmerlin et al., 2012; Vranová et al., 2013). IPP and DMAPP can be condensed to form a series of linear intermediates by *trans*-prenyltransferases (isoprenyl diphosphate synthases), providing precursors for different classes of terpene synthases (TPSs) which convert linear prenyl diphosphate-derived into miscellaneous terpene scaffolds (Chen et al., 2011; Sumit, 2016). Subsequently, the terpene backbones can be further modified by tailoring enzymes including cytochrome P450 monooxygenases (CYP450s), methyltransferases, acyltransferases, glucosyltransferases and dehydrogenases to furnish terpenoids with diverse structures (Aharoni et al., 2003; Nes, 2011; Hamberger and Bak, 2013).

Alkaloids

Alkaloids are either directly or indirectly derived from amino acids, such as phenylalanine, ornithine, arginine, tyrosine, and tryptophan (Staniek et al., 2013). Alkaloids provide important pharmacological activities in the human therapeutic arsenal, such as antitumor, analgesics, and anti-inflammatory activities, or being stimulants (Matsuura and Fett-Neto, 2015).

Alkaloids consist of four major families, incuding monoterpene indole alkaloids (MIAs), benzylisoquinoline alkaloids (BIAs), tropane and nicotine alkaloids (TNAs), purine alkaloids, and others (Figure 1B) (Ziegler and Facchini, 2008). The common precursor to all MIAs is strictosidine, which is formed by the condensation of indole precursor tryptamine and terpenoid precursor secologanin, catalyzed by strictosidine synthase (STR) (McKnight et al., 1990). Tryptamine is produced by tryptophan decarboxylase (TDC) from tryptophan, which is derived from the shikimate pathway, whereas secologanin is synthesiezed from terpenoid precursor geranyl diphosphate (GPP) by continuous catalysis of nine enzymes (Chang et al., 2014). BIAs are tyrosine-derived alkaloids synthesized from the central intermediate (*S*)-reticuline, which is generated from (*S*)-norcoclaurine via the catalysis of a few different enzymes (Ziegler and Facchini, 2008). TNAs are derived from the amino acids ornithine and arginine, both can be converted to putrescine. TNAs are widely used in medicine as anticholinergic compounds, such as hyoscyamine and scopolamine (Dräger, 2002; Oksman-Caldentey, 2007).Purine alkaloids are derived from purine nucleotides. The main purine alkaloids are caffeine and theobromine known as neurostimulants.

Phenylpropanoids

Phenylpropanoids are derived from phenylalanine, a key product of the shikimate pathway which also gives rise to other aromatic amino acids, i.e., tyrosine and tryptophan (Herrmann and Weaver, 1999; Tzin and Galili, 2010). Phenylpropanoids contribute to plant responses towards biotic and abiotic stresses, and are involved in plant defense, structural support, and survival (Vogt, 2010).

The phenylalanine-derived metabolites comprise multiple biosynthetic branches, including stilbenes, coumarins, lignins, lignans and neolignans and flavonoids (Figure

1C). Stilbenes are derived from *p*-coumaroyl-CoA but with the other half of the structure furnished via polyketide cyclization, and possess the 1,2-diphenylethylene structure (Valletta et al., 2021). Coumarins are produced from C2-hydroxylation of the hydroxycinnamoyl-CoA esters and lactonization of the side-chain (Vanholme et al., 2012). The monolignol specific pathway, branched from *p*-coumaric acid and *p*-coumaroyl-CoA, leads to the biosynthesis of lignins, lignans and neolignans. Flavonoids are also derived from *p*-coumaroyl-CoA in the phenylpropanoid pathway and share a common diphenylpropane (C6–C3–C6) backbone in which two aromatic rings are linked via a three carbon chain (Tohge et al., 2013). According to the heterocyclic ring, flavonoids can be divided into six major subclasses including flavones, flavonols, flavanones, flavanols, anthocyanidins (anthocyanins and proanthocyadins), and isoflavonoids (Tohge et al., 2013).

PLANTS AS HOST FOR THE PRODUCTION OF NATURAL PRODUCTS

Microorganisms, especially *Escherichia coli* and yeast (*S. cerevisiae*), are the two best established hosts for engineering plant specialized metabolites. Until now, the highest terpenoid titers reported, 30 g/L amorphadiene in *E. coli* and more than 130 g/L β-farnesene in *S. cerevisiae*, were achieved using these two hosts (Meadows et al., 2016; Shukal et al., 2019). The advantages of using microorganisms are that they grow fast in controlled cultivation conditions, have the capacity to grow on different carbon sources and well-established methods for genetic and metabolic manipulations. Besides *E. coli* and yeast, photosynthetic microorganisms like cyanobacteria have also been explored for engineering the production of different classes of terpenoids (Beekwilder et al., 2014; Krieg et al., 2018). Although many trials have been done on the production of targeted metabolites in microorganisms, the expression of plant-derived genes (enzymes) in microbial systems has its own limitations. Bacterial hosts, such as *E. coli*, lack essential membrane compartments, making the effective expression of membrane bound enzymes such as cytochrome P450 monooxygenases a challenge. In addition, protein misfolding and aggregation into insoluble inclusion bodies are commonly observed during recombinant expression in microbes (Sabate et al., 2010). Although yeast has endogenous membrane compartments and is more suitable for expressing plant-derived enzymes, it

also relies on costly carbon sources such as sucrose or glucose, and takes longer time to engineer the correct functioning of enzymes and optimize fermentation titer.

Plants represent unique and powerful chassis for synthetic biology, because they only rely on atmospheric carbon (as opposed to exogenous carbohydrates for microbes), water, essential nutrients, and sunlight for efficient growth, thus representing a more sustainable and green platform of synthetic biology (Table 1). In addition, plant platforms harbor common enzyme cofactors and metabolic precursors, and provide ideal environment for correct mRNA and protein processing, protein localization and subcellular compartmentalization, many of which are absent in microbes. Plant chassis can be engineered either at cells (suspension cell culture), tissues (hairy root culture) or the whole plant levels. Plant cell suspension cultures have several advantages including shorter life cycles, not affected by environmental effects (e.g., climate, soil quality, diseases and insect pests), less of biosafety issues, such as gene flow through pollen (Sun et al., 2011). Plant cell culture and hairy root culture are well-established technology platforms for the production of natural products, which have been applied in cosmetic, food and pharmaceutical industries (Ochoa-Villarreal et al., 2016). Plant cell culture has been proved to be excellent plant-based expression platform for production of geraniol and related natural products (Vasilev et al., 2014), whilst hairy root culture has exhibited potential in the production of a variety of secondary metabolites, including nicotine, ginsenoside, camptothecin, tropane and pyrrorisidine alkaloids (Wink et al., 2005; Guillon et al., 2006). The advantage of metabolic engineering at the whole plant level is that combinatorial engineering strategies can be employed simultaneously to improve metabolite production, including global regulation, overexpression of key enzymes, and tissue-specific production of metabolites, which can facilitate isolation of targeted metabolites.

The most widely used plant chassis for natural product production is *Nicotiana benthamiana*, since it has large biomass, relatively short growth cycle, and is amenable to scaled-up transient co-expression of many different genes of interest (Figure 2A). Vacuum agroinfiltration is the key scaled-up process to enable gram-scale production of phytochemicals (Reed et al., 2017). So far, a variety of natural products have been

successfully produced via transient co-expression in *N. benthamiana*, including alkaloids (Miettinen et al., 2014; Wang et al., 2016; Delatte et al., 2018; Lee et al., 2019; Li et al., 2019; Sadre et al., 2019; De La Peña and Sattely, 2021; Forestier et al., 2021), terpenoids (Reed and Osbourn, 2018; Li et al., 2019), ketocarotenoids (Nogueira et al., 2019), glucosinolates (Geu-Flores et al., 2009; Crocoll et al., 2016), tyrosine-derived phenolics (Polturak et al., 2017; Torrens-Spence et al., 2018), cyanogens (Rajniak et al., 2015), polyketides (Andersen-Ranberg et al., 2017), etoposide aglycone (Lau and Sattely, 2015; Schultz et al., 2019) and also new-to-nature compounds (Beyraghdar Kashkooli et al., 2019; Calgaro-Kozina et al., 2020). Besides tobacco, other plant species have been applied for transient pathway reconstitution. Recently, the biosynthetic pathway of CPH, a caffeoylputrescine–green leaf volatile compound, was transiently reconstituted in *Solanum chilense* and *Vicia faba* (a bean crop) (Bai et al., 2022). Another potential plant platform for metabolic engineering is tomato, which has a robust plant transformation system and excellent germplasm resources. In one study, the *Arabidopsis thaliana* transcription factor *AtMYB12* was introduced to tomatoes and induced at the final stage of fruit growth and ripening, leading to higher accumulation of polyphenol in transgenic tomatoes (Butelli et al., 2008; Zhang et al., 2015b) (Figure 2B). Rice has also been applied as a chassis to enhance phenylpropanoids and terpenoids production. For example, 'Golden Rice' was generated by introducing two genes encoding phytoene synthase (*psy*) and the *Erwinia uredovora* carotene desaturase (*crtI*). The Golden Rice accumulate more β-carotene (pro-vitamin A), which can help alleviate vitamin A deficiency in human diet (Paine et al., 2005). Eight genes related to anthocyanin biosynthesis were introduced and expressed in rice endosperm, resulting in high contents and antioxidant activity in transgenic rice (Zhu et al., 2017) (Figure 2C). In another study, bioengineering of astaxanthin biosynthesis in rice endosperm was achieved by introducing four synthetic genes (Zhu et al., 2018). Grains of transformed rice were enriched with astaxanthin in the endosperm and had higher antioxidant activity (Zhu et al., 2018). Additionally, metabolic engineering for betalain production was conducted in three major food crops: tomato, potato, and eggplant, as well as tobacco flowers. The betalain production in tobacco resulted in significantly enhanced resistance toward gray mold (*Botrytis cinerea*) (Figure

2D) (Polturak et al., 2017).

Maize has been engineered to accumulate astaxanthin in seeds, which was further used to feed laying hens, and the results showed that hens could take up astaxanthin from the maize leading to accumulation of astaxanthin in egg yolks without affecting egg production and quality (Liu et al., 2021) (Figure 2E). *Arabidopsis* is also a promising plant chassis for natural product production due to the easy transformation and extraordinarily comprehensive resources. Different classes of metabolites have been produced in *Arabidopsis*, including isoprenoids (Lange et al., 2015), lignin (Vanholme et al., 2013; Oyarce et al., 2019), lignin-related product syringin (Chu et al., 2014), vitamin B1 (Dong et al., 2015) and long-chain polyunsaturated fatty acids (Napier et al., 2014) (Figure 2F). *Artemisia annua* has been engineered to enhance the production of artemisinin, which is the active ingredient to treat the disease malaria (Han et al., 2006; Jiang et al., 2016; Shi et al., 2017) (Figure 2G). Furthermore, the possibility of developing other plant chassis for metabolic engineering have been discussed and reported, including, rapeseeds (i.e., *Camelina sativa*) (Nour-Eldin et al., 2017; Malik et al., 2018), *Atropa belladonna* (Zhang et al., 2022) and some relatively simple systems, such as *Physcomitrella patens* (Reski et al., 2018), *Marchantia paleacea* (Zhang et al., 2020) and wolffian (Lam and Michael, 2022) (Figure 2H).Despite the aforementioned advantages of plants as chassis for metabolic engineering, several major challenges remain to be overcome to unleash the full potential of plants as production platforms. Unlike some single-celled microbes, plants have differentiated cells and tissues. Their complex structures and tight gene regulation could affect the transformation and function of genes to be introduced. Moreover, plants have tight metabolic regulations and intricate feedback regulation mechanisms which can interfere with targeted pathways to be engineered. Therefore, before proceeding with plant engineering, a thorough survey of the endogenous metabolism of plants is required to formulate practical plans. A good design requires extensive fundamental knowledge, including knowledge of the native metabolic pathways in the organism that will be re-engineered, updated molecular tools to build customized pathways, and strategies that can be used to achieve efficient production of desired products.

PLANT SYNTHETIC BIOLOGY CONCEPTS

Synthetic biology is an interdisciplinary branch of biology and engineering, which incorporates the design and development of new biological elements, equipment, and systems, as well as the re-design of existing natural biological systems for useful functions (Liu and Stewart, 2015). The concept of synthetic biology is fully applicable to engineer plants for the production of plant natural products. Execution of the synthetic biology concept generally follows the Design–Build-Test–Learn (DBTL) cycles (Mortimer, 2019) (Figure 3). In the Design phase, a customized engineering strategy is formulated based on the targeted compounds to be produced and the plants to be engineered. The basic elements of genetic circuits include but not limited to *cis*-regulatory elements (e.g., promoters, enhancers and silencers), *trans*-regulatory elements (e.g., transcription factors, activators and repressors), protein domains, protein-coding open reading frames (ORFs) and terminators. Manipulating any of these components in the genetic circuit may influence gene expressions, protein translations, and have effects on the final production of desired metabolites. The effects of genetic circuits applied in plants can be assessed and optimized to some extent by computer-aided design (CAD) tools, such as GeneDesign (Richardson et al., 2006), Gene Designer 2.0 (Villalobos et al., 2006), BiopartsBuilder (Yang et al., 2016), GenoCAD (Coll et al., 2015), GoldenBraid (Vazquez-Vilar et al., 2017) and CellModeller (Dupuy et al., 2008; Federici et al., 2012).

During the Build phase, genetic parts are synthesized and constructs are assembled. The genetic elements with known DNA sequence can be cloned and amplified by traditional polymerase chain reaction (PCR) from specific sources, or obtained directly via DNA synthesis if the DNA sequences do not exist or DNA sources are not available. The improved *de novo* DNA synthesis methods have significantly reduced the cost and shortened the “Build” cycle of synthetic biology (Redding-Johanson et al., 2011; Rai et al., 2019). Next, the synthesized DNA fragments need to be further assembled by various assembly methods. The *in vitro* DNA assembly methods can be classified into three major categories: a) sequence homology-based methods, such as In-Fusion (Sleight et al., 2010) and Gibson assembly (Gibson et al., 2010), b) phage site-specific recombination system,

such as Gateway cloning (Hartley et al., 2000), and c) restriction enzymes-based methods. Restriction digestion and ligation using type II restriction enzymes and DNA ligase has been exploited as the standard cloning technique, including BioBrick (Phillips and Silver, 2006), Golden Gate (Engler et al., 2008), MoClo (Weber et al., 2011), GoldenBraid (Sarrion-Perdigones et al., 2013), Golden Mutagenesis (Püllmann et al., 2019) and multi-kingdom Golden Gate cloning platform (Chiasson et al., 2019). The *in vivo* DNA assembly approaches have also been applied in plant metabolic engineering, including Cre/*loxP* system (Li et al., 2007; Zhu et al., 2018), which is a recombinase mediated assembly method, and multiple-round *in vivo* site-specific assembly (MISSA) method is mediated by inducible Cre recombinase and phage λ site-specific recombination (Chen et al., 2010; Zhang et al., 2017).

The development of genome editing tools facilitates efficient genome editing and make it much easier to discover and characterize gene functions and optimize the activity of enzymes in metabolic pathways. Several genome editing tools have been applied to edit plant genomes, including meganucleases (or called homologous recombination (HR)), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), pentatricopeptide repeat proteins (PPRs), Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein (CRISPR/CAS9 and CRISPR/Cpf1), RNA interference (RNAi) (Mohanta et al., 2017). Recently, the most commonly used genome editing tool in plants is the CRISPR/Cas9 system, followed by the CRISPR/Cpf1 system, owing to its low cost, ease of execution and high efficiency. The endonucleases Cas9 and Cpf1 are guided to specific genomic sites by single-guide RNAs (sgRNAs) that recognize the target DNA sequences (Jinek et al., 2012; Endo et al., 2016). Then double-stranded breaks are generated by these sequence-specific nucleases at targeted genome sites, and generally repaired by either non-homologous end joining (NHEJ) or HR, resulting in gene knockout or replacement. Multiple genes in the biosynthesis or regulation of the same pathway can be edited simultaneously by designing several gRNAs for different target genes, which can be assembled into binary expression vectors using Golden Gate or Gibson assembly methods (Xie et al., 2015; Wang et al., 2017). In addition, the expression of Cas9 driven by inducible or tissue-specific promoters can be

used to control gene expression in a specific cell type, tissue, development stage and different environmental conditions (Decaestecker et al., 2019; Wang et al., 2020). This technique is very powerful when the edited genes showed pleiotropic developmental defects of system-wide losses of gene function. Furthermore, DNA-free CRISPR/Cas9 delivery methods now provide great opportunities for plant genome engineering, and facilitate the commercialization of edited plants in the future. The CRISPR/Cas9 protein and gRNA are assembled to pre-assembled ribonucleoproteins (RNPs), which can be further delivered to plant by particle bombardment (Malnoy et al., 2016; Svitashev et al., 2016; Andersson et al., 2018). What's more, dead Cas9 variant (dCas9) proteins fused to DNA deaminases have been developed as base editors that can perform precise single-base editing of the target genes without inducing double strand breaks (Piatek et al., 2015). Adenine deaminase-base editors (ABEs) have been developed to convert nucleotide A to G, whereas cytidine deaminase-incorporating DNA base editors (CBEs) can facilitate the C to T conversion. Both base editors have been successfully applied for base editing of plant genomes (Komor et al., 2016; Nishida et al., 2016).

In the Test phase, the constructs are transformed into the selected host organisms, and modified organism is analyzed and data collected. At this stage, the synthesized genetic circuit can be transformed to suitable plants by different gene transfer approaches, including stable nuclear transformation, stable plastid transformation and transient expression systems. The most commonly used nuclear transformation method in plants is *Agrobacterium*-mediated transformation, which is based on the finding that Ti plasmid of *Agrobacterium tumefaciens* can be partially integrated into the plant genome. The DNA flanked by defined left- and right-borders of the Ti plasmid can be integrated into plant genomes. *Agrobacterium*-mediated gene transfer is a simple, highly efficient and cost-effective method, but its efficiency varies depending on factors, such as plant genotypes, explant, vector plasmid, bacterial strain, composition of culture medium and degree of tissue damage (Mehrotra and Goyal, 2012). Nanotechnology and carrier proteins have recently been developed for delivering genes into plants. Nanoparticles and carrier proteins complexed with DNA or RNA can be delivered into plant tissues or plant cells by biolistics, electroporation, magnetofection or microinjection. The

nanoparticle-mediated gene transformation has various advantages, including large loading capacity, strong penetration ability, protection of foreign genes from degradation and a short genetic transformation cycle (Lv et al., 2020). The advantages of the protein or peptide-based gene delivery system lie in that it is not limited by the transformed plant types or sizes of transgenes (Lakshmanan et al., 2013).

Plastids are 'semi-autonomous organelles', since they contain their own genome (the plastome) and reproduce independently of the nucleus. Recently, plastid transformation was successfully developed and its application has been achieved in many species. Compared to nuclear genome transformation, plastid transformation has several advantages: 1) high precision of genetic engineering enabled by efficient homologous recombination; 2) multiple genes can be connected in series and placed in the same operon; 3) absence of epigenetic effects, gene silencing or positional effects; 4) potential for high-level expression of gene products; 5) avoidance of unwanted foreign genes due to the maternal genetic characteristics (Bock, 2015; Boehm and Bock, 2019). Plastid transformation methods have been successfully applied in the production of pharmaceuticals, vaccine and other synthetic fusion proteins (Cardi et al., 2010). The transient transformation systems provide a platform to rapidly test the designed gene circuits and optimize engineering design before performing the time-consuming stable transformation. The most commonly used transient expression platform is the *Agrobacterium* mediated infiltration of leaves of *N. benthamiana* (Reed et al., 2017). An alternative approach for rapid testing of gene constructs is the protoplast-based systems. Protoplast transformation involves the direct delivery of DNA to individual plant cells using polyethylene glycol or electroporation, which has been used to successfully deliver genome-editing reagents in multiple crop plants, including potato, wheat, rice, and flax (Baltes et al., 2017).

In the Learn phase, the data from the Test phase are analyzed, and used to inform the next Design stage. Information from the previous three phases is collected, analyzed and integrated using artificial intelligence or machine learning-based approaches, with a view to extract rules and features underlying the performance from the Test phase. The outcomes from the Learn phase enable machine learning-based approaches to achieve a

wide range of purposes, such as sensible and reliable prediction of gene functions and, elucidation of compound structures, optimization of genome editing tools, design of synthetic biological products, assembly of metabolic pathways and optimization of metabolic flux. For example, in order to accurately identify genes involved in the secondary metabolism, a machine learning algorithm-based model was built to effectively distinguish *Arabidopsis* genes involved in primary and specialized metabolism (Moore et al., 2019). Apart from predicting single gene functions, algorithms have also been developed to identify and predict functions of biosynthetic gene clusters. For instance, several computational studies (Chae et al., 2014; Schläpfer et al., 2017), R package (Banf et al., 2019) and web tools have been implemented, including plantiSMASH (http://plantismash.secondarymetabolites.org/) (Kautsar et al., 2017) and PhytoClust (http://phytoclust.weizmann.ac.il/) (Töpfer et al., 2017) to identify plausible biosynthetic gene clusters based on the increasingly appreciated clustering phenomenon of plant biosynthetic genes in plant genomes (Chae et al., 2014; King et al., 2014; Nützmann et al., 2016; Franke et al., 2019). Furthermore, the optimization of genome editing tools, such as CRISPR/Cas, also benefit from machining learning. Numerous tools and websites can be used to predict the knock-out efficiency of sgRNA and the potential off-target effects, such as DeepCRISPR (Chuai et al., 2018), sgRNA Scorer 2.0 (Chari et al., 2017) and DeepCpf1 (Kim et al., 2018).

The rapid development of synthetic biology, including advances in DBTL technologies, have greatly broaden the toolbox of plant metabolic engineering, enabling the discovery and improved production of natural products. Most plant metabolic engieering studies follow the overall concept in the DBTL cycle. Until now, the DBTL cycle has been implemented to enhance the production of natural products, including geraniol and geraniol-derived products, momilactones, forskolin and taxadiene (Dong et al., 2016; Li et al., 2019; De La Peña and Sattely, 2021).

SYNTHETIC BIOLOGY-BASED STRATEGIES FOR PRODUCTION OF NATURAL PRODUCTS IN PLANTS

In general, engineering strategies to enhance the yield of desired plant natural products can be considered from the following aspects: 1) increasing or engineering the activity of

structural genes or rate-limiting enzymes that create bottlenecks and cause the buildup of inhibitory intermediates; 2) enhancing the pools of precursor supply, directing flux of substrates, energy, and reducing power to specialized metabolic pathways; 3) suppressing competing pathways that divert metabolic fluxes, or downstream degrading and catabolizing pathways to avoid desired products being degraded or converted into other molecules; 4) engineering transcription factors (TFs) or *cis*-regulatory elements (e.g., activators/repressors/promoters) to upregulate the expression of structural genes for improved metabolite synthesis; 5) creating sink compartments that store the target metabolites; 6) engineering transporters of key intermediates for improving metabolic flux. The specific strategies can be manipulated throughout the whole central dogma process, including gene replication, transcription and translation, and their corresponding target molecules are DNA, RNA and proteins, respectively (Figure 4). Structural genes function at the genomic level, whereas TFs and *cis*-regulatory elements function at the transcript level. TFs control the rate of gene transcription via recognizing and binding specific DNA sequences (*cis*-regulatory elements) and protein interactions, thereby regulating the expression of pathway enzymes. TFs can be used as regulatory tools in metabolic engineering by overexpressing positive TFs, knocking-down or knocking-out the expression of negative ones (repressors). Pathway compartmentalization works at the protein level, which aims to relocate biosynthetic enzymes to certain subcellular compartments by altering the subcellular localization of proteins, making protein fusions and creating artificial organelles by using scaffold proteins. Outside the Central Dogma process, metabolic engineering can be also employed systematically by introducing transporters to channel or improve metabolic flux.

GENERAL STRATEGIES TO ENHANCE METABOLITE PRODUCTION

Increasing supply of precursors

Secondary metabolism is evolved from primary metabolism, which provides the essential precursor pools for making plant secondary metabolites. Increasing precursor supply can ensure sufficient source compounds to be channeled to the pathway of interest for natural product biosynthesis without risking running out of synthetic building blocks (Figure 4A-2). These precursors can be early at the upstream of biosynthetic pathways or at the

branching nodes of pathways towards synthesis of target compounds. For instance, re-engineering the interface of primary and specialized metabolism by transient expression of a de-regulated arogenate dehydrogenase TyrA/ ADHα, the yield of Tyr-derived betalain pigments was lifted 7 folds (855 mg/kg ·FW) in *N. benthamiana* (Timoneda et al., 2018). To enhance the production of terpenoids, engineering efforts have been taken to balance the ratio of glyceraldehyde 3-P and pyruvate in the central carbon metabolism (Liu et al., 2013). In addition, introduction of an IPP/DMAPP isomerase (IDI) can be applied to enhance isoprenoid precursors by balancing IPP and DMAPP pools, which can further elevate terpenoid production (Malhotra et al., 2016). It should also be noted that drastic alterations in primary metabolism may cause severe growth defects or yield penalties, which negatively affect the target compound accumulation. For example, growth defect has been observed in tobacco expressing transcription factor *MYB12*, which activates the pentose phosphate, shikimate, and phenylpropanoid pathways, because of the conflicting demands of primary metabolism and enhanced secondary metabolism (Luo et al., 2008). Ectopic expression of bacterial arogenate dehydrogenase TyrA leads to hyper-accumulation of tyrosine in *Arabidopsis*, accompanying with severely compromised plant growth (de Oliveira et al., 2019). One effective way to overcome this bottleneck is to express genes under tissue-specific promoters. Strategies related to this will be explained extensively in the following section - "Engineering at the RNA level".

Suppression of competitive pathways

The branch and catabolizing pathways can compete with the target pathway for energy and intermediates, resulting in reduced yield of desired metabolites. Suppression of unwanted competitive metabolic pathways can effectively direct metabolic flux towards synthesis of compounds of interest, increasing the yield of desired plant natural products (Figure 4A-3). The sterol pathway is a competing pathway of sesquiterpene artemisinin biosynthesis as it competes for the sesquiterpene biosynthetic precursor farnesyl diphosphate. Suppressing the expression of squalene synthase (SQS) by RNAi silencing in *A. annua*. led to more than 3-fold increase of artemisinin content in transgenic plants (31.4 mg/g DW) (Zhang et al., 2009). Farnesyl diphosphate (FPP) is an important

intermediate in terpenoid biosynthesis. It can be converted to miscellaneous sesquiterpenes, geranylgeranyl diphosphate or squalene. RNAi silencing of both endogenous 5-epi-aristolochene synthase (EAS) and SQS resulted in a 2.8-fold increase of sesquiterpenoid (+)-valencene content in transgenic *N. benthamiana* expressing the sesquiterpene valencene synthase (Bouwmeester et al., 1999; Mercke et al., 1999; Cai et al., 2002; Picaud et al., 2005; Bertea et al., 2006; Cankar et al., 2015). Similarly, blocking the flavonol pathway by inhibiting the activity of flavanone 3-hydroxylase that directly competes for naringenin as substrate with isoflavone synthase (IFS) resulted in about 7-fold increase of isoflavone yield (78 mg/ g DW) in tomato (Zhang et al., 2015b).

Introduction of transporters to sequester metabolites or improve metabolic flux

In eukaryotes, plant secondary metabolites have characteristic spatial–temporal distributions, which means that many of these valuable natural compounds and their precursors are biosynthesized and accumulated at diverse subcellular compartments, like vacuoles, transport vesicles, or peroxisomes, and some are even transported from source organs to sink ones via long-distance transport (Figure 4A-4). For example, the biosynthesis of bisindole alkaloid morphine is divided across at least three cell types (Onoyovwe et al., 2013). Pathway enzymes are transcribed and translated in companion cells, and transported to sieve elements or laticifers, while pathway intermediates are synthesized in the sieve elements and transported to the laticifers, where the final biosynthetic steps are completed to achieve the biosynthesis of morphine. In another example, glucosinolates are produced in leaf and silique tissues and transported to accumulate in seeds (Brown et al., 2003). Being localized on tonoplast or plasma membrane, transporters play a crucial role in uptake or efflux of various secondary metabolites and their precursors across biomembranes. Major transporter families transporting plant specialized metabolites reported so far include ATP-binding cassette (ABC) family (Shitan et al., 2003; Shoji, 2014), multidrug and toxic compound extrusion (MATE) family (Shoji et al., 2009; Shitan et al., 2015; Takanashi et al., 2017), nitrate transporter 1/peptide transporter family (NPF) (Nour-Eldin et al., 2012; Payne et al., 2017) and purine permease (PUP) family (Jelesko, 2012; Dastmalchi et al., 2019).

Since transporters constitute an important component in molecular machinery

involved in the biosynthesis and accumulation of secondary metabolites, transporter engineering is useful to increase the production of commercially valuable secondary metabolites (Takanashi et al., 2017). In *A. annua*, a pleiotropic drug resistance (PDR) transporter *AaPDR3* was reported in the transport of β-caryophyllene, whose biosynthesis compete for the common substrate FPP with the biosynthesis of sesquiterpenoids. Silencing the expression of *AaPDR3* by RNAi strategy led to increased artemisinin and decreased β-caryophyllene contents in transgenic plants (Fu et al., 2017). An ABC type transporter *CsABCC4a* was identified to mediate transport of several crocins in *Crocus sativus* (Demurtas et al., 2019). Co-expression of *CsABCC4a* and *CsCCD2*, the first dedicated enzyme in the crocin biosynthetic pathway, resulted in enhanced crocin accumulation in *N. benthamiana*.

ENZYME ENGINEERING AT THE DNA LEVEL

Overexpression of rate-limiting enzymes in the pathway

Rate-limiting enzymes are key gate-keeping steps that determine the rate of metabolic flux (Figure 4A-5). The rate-limiting step can be defined as a reaction that is catalyzed by an enzyme at a conversion rate relatively lower than other enzymes in the same pathway (Zhao and Qu, 2009). Rate-limiting enzymes have relatively low overall catalytic activity resulting in substrates not being converted into products fast enough to be consumed by the next-step enzyme and hence accumulation of substrates. The total activity of an enzyme to convert the substrate to a product (or an intermediate) in the pathway can be increased via (heterologously) over-expressing the enzyme (Figure 4B). In the following part, commonly appreciated rate-limiting enzymes in three major metabolic pathways which can boost the yield of downstream pathway products have been described (Table 2).

For terpenoids production, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) is the key rate-limiting enzyme in MVA pathway, which can be overexpressed to enhance the accumulation of sesquiterpenes and triterpenes (Reed et al., 2017). For example, transient co-expression of a truncated feedback-insensitive version of HMG-CoA reductase (*tHMGR*) with β-amyrin synthase *SAD1* in tobacco leaves led to a 4-fold increase in β-amyrin production (Reed et al., 2017). Besides HMGR, *SQS* is another

limiting factor in triterpene production, and transient expression of an oat *SQS* elevated yields of β-amyrin by 2-3 folds (Reed et al., 2017). On the other hand, 1-deoxy-d-xylulose 5-phosphate synthase (DXS) in MEP pathway constitutes the bottleneck for terpenoids accumulation (Brückner and Tissier, 2013). The strategy of co-expressing *DXS* with the relevant prenyltransferase enzymes can enhance the production of MEP pathway dependent terpenoids, such as monoterpenoids, diterpenoids, sesterterpenoids and tetraterpenoids. The constitutive overexpression of an *Arabidopsis DXS* in spike lavender led to higher accumulation of essential oils (mainly monoterpenes) in both leaves and flowers, without negative effects on growth or oil composition (Muñoz-Bertomeu et al., 2006). In another example, co-expression of a tomato *DXS* and a tobacco *GGPPS* resulted in a 3.5-fold increase in the production of cembratrienol, which is a macrocyclic diterpenoid in many varieties of cultivated tobacco (*Nicotiana tabacum*) (Ennajdaoui et al., 2010; Brückner and Tissier, 2013).

The strategy of overexpressing rate-limiting enzymes has also been applied in phenylpropanoid engineering. Several key enzymes involved in the phenylpropanoid metabolism have been isolated and identified in various plant species (Hamberger et al., 2007; Zhao et al., 2013). A rate-limiting enzyme in flavonol biosynthesis is chalcone isomerase (CHI), which converts naringenin chalcone to naringenin. Ectopic overexpression of a *Petunia CHI* gene in tomato led to a 78-fold increase of total flavonols in fruit peels (Muir et al., 2001). Genetic engineering of key structural genes, such as isoflavonone synthase (IFS) and 2-hydroxyisoflavanone dehydratase (HID), is an effective way to enhance isoflavonoid accumulation. Overexpression of *IFS* and *HID* leads to substantial enhancement in isoflavonoid (*i.e.* genistein and daidzein) production in alfalfa (Deavours and Dixon, 2005) and legumes (Akashi et al., 2005), respectively. Overexpression of a gerbera dihydroflavonol 4-reductase (*DFR*) gene, as well as suppression of flavonol synthase (*FLS*) and flavonoid 3′-hydroxylase (*F3'H*) resulted in red coloration of tobacco flowers due to enhanced accumulation of pelargonidin-based anthocyanin (Nakatsuka et al., 2007). The production of *trans*-resveratrol was enhanced in tomato fruits by constitutive expression of the key enzyme stilbene synthase (*StSy*) (Giovinazzo et al., 2005). For alkaloids production, rice calli expressing tryptophan

decarboxylase (*OsTDC*) and anthranilate synthase (*OsSA1D*) showed accumulation of serotonin and serotonin-derived indole compounds (Dubouzet et al., 2013). In another study, the yields of BIA alkaloids could be enhanced by overexpression of berberine bridge enzyme (BBE) in *Eschscholzia californica* root cultures (Park et al., 2003).

ENZYME ENGINEERING AT THE RNA LEVEL

Overexpression of transcription factors

TFs are regulatory proteins that can recognize and bind to specific regulatory sequences (*cis*-regulatory elements) in the promoter region of target genes. TFs have important functions in controlling the transcription of biosynthetic genes, thereby holding great promise for improving the production of plant secondary metabolites, either in plant cell cultures or at the whole-plant level (Grotewold, 2008) (Figure 4C). Over the last several years, advances have been made in discovering TFs involved in the regulation of plant secondary metabolites (Table 3).

As for artemisinin biosynthesis, the AP2/ERF transcription factors, including *AaORA* (Lu et al., 2013), *AaERF1*, *AaERF2* (Yu et al., 2012) and *TAR1* (Tan et al., 2015), were reported to regulate artemisinin biosynthesis. The WRKY transcription factors, *AaWRKY1* (Han et al., 2014; Jiang et al., 2016) and *AaGSW1* (Chen et al., 2017), were reported to up-regulate *ADS*, *CYP71AV1* and *DBR2* expression, resulting in improved artemisinin biosynthesis. Besides, the basic helix–loop–helix (bHLH) transcription factors, *AabHLH1* (Ji et al., 2014), *bHLH112* (Xiang et al., 2019), *AaMYC2* (Shen et al., 2016) and *AaPIF3* (Zhang et al., 2019), are all able to promote artemisinin accumulation. In addition, ngineering the regulatory TFs has been extensively studied in phenylpropanoid biosynthesis. MYB proteins, one of the largest plant transcription factor families, have key functions in regulation of the biosynthesis of phenylpropanoids in plants. (Lloyd et al., 1992; Bovy et al., 2002). Many MYB transcription factors, such as *PAP1*, *PAP2*, *MYB11*, *MYB12* and *MYB111*, have been identified as key regulators in anthocyanin biosynthesis, and their overexpression results in elevated accumulation of anthocyanin in various plants (Stracke et al., 2007; Stracke et al., 2010; Gatica-Arias et al., 2012; Zvi et al., 2012; Maier et al., 2013; Liu et al., 2015). Additionally, artificially designing synthetic regulators, including activators, repressors, and promoters, offers

great potential for robust and accurate control of multiple transgenes, enabling tissue-specific expression, and circumventing the risk of gene silencing. Recently, an orthogonal regulatory system was developed by leveraging yeast TFs in conjunction with plant-specific regulatory DNA sequences. The designed regulatory elements could successfully modulate gene expression in *N. benthamiana* and *A. thaliana* (Belcher et al., 2020).

Engineering specific promoters

Promoter is a region of DNA at which transcription of particular gene initiates. Promoter engineering is a powerful technique in synthetic biology to fine-tune specific gene expression at the transcriptional level, in order to maximize the production of secondary metabolites (Figure 4C). Several promoter-engineering strategies, including construction of promoter library, replacement of endogenous promoters and rational design of inducible, hybrid or artificial promoters, have been extensively applied in many industrial microorganisms, such as *S. cerevisiae* and *E. coli* (Blazeck and Alper, 2013; Portela et al., 2017; Hwang et al., 2018). Comparing to microorganisms, application of promoter engineering in transgenic plants to improve metabolite production is largely unexplored. Current engineering focus is limited to the temporal and spatial expression via installing constitutive, tissue-specific or (synthetic) inducible promoters (Table 4).

Constitutive promoters, such as CaMV 35S and ubiquitin, are widely applied in introducing biosynthetic genes to elevate the production of plant natural products. However, many specialized metabolic pathways are partitioned to particular tissues, and metabolite flow between compartments are tightly regulated and orchestrated. In addition, compartmentalization reduces the effects of toxic compounds, which allows higher titers of toxic products with less impact on cell viability and growth (Jones et al., 2015). For example, the constitutive expression of a dual *linalool/nerolidol synthase* (*FaNES1*) from strawberry in *Arabidopsis* led to accumulation of linalool and its glycosylated and hydroxylated derivatives. However, the transgenic lines showed growth retardation due to the depletion of isoprenoid precursors in the plastids (Aharoni et al., 2003). Therefore, introducing genes driven by tissue-specific promoters can control the accumulation of metabolites within targeted tissues, which may facilitate isolation and production of

targeted metabolites and alleviate potential toxic effects. For example, trichome-specific promoters have been applied to produce terpenoids in trichomes, an ideal structure to synthesize, store and/or excrete hydrophobic and toxic metabolites (Huchelmann et al., 2017). Expression of *TAXADIENE SYNTHASE* (*TS*) or *CASBENE SYNTHASE* (*CS*) specifically in *N. tabacum* trichomes under the *CEMBRATRIENOL SYNTHASE* (CBTS) promoter led to the production of taxa-4(5),11(12)-diene (precursor of functionalized paclitaxel) and casbene at average 100 μg/g and 1 mg/g of fresh leave weight, respectively (Rontein et al., 2008; Tissier et al., 2013). In addition, some plant metabolites are synthesized under stress conditions to improve the tolerance of plants (Hong et al., 2021). Constitutive expression of these metabolites may cause growth defects and sterility, resulting in decreased yield of target compounds (Field and Osbourn, 2008). Under such scenario, inducible promoters can induce gene expression at particular developmental stages and places, providing more flexibility for metabolite accumulation.

ENZYME ENGINEERING AT THE PROTEIN LEVEL

Plant metabolic pathways are highly compartmentalized, which means biochemical steps of a single pathway can take place in multiple subcellular locations. Substrate channeling, a process of direct transfer of a reaction intermediate or product from one enzyme active site to another without dissociation into the medium (e.g., bulk solvent), can occur when two or more enzymes (or active domains) in close physical proximity (Geck and Kirsch, 1999). Conversion rates of multiple enzymes acting in sequential steps can be accelerated greatly if efficient substrate channeling can be realized, due to the improved substrate-product turn-over rate, minimizing diffusion, and avoidance of releasing toxic or unstable intermediates (Zhang, 2011). Plants have evolved many of such enzyme complexes (e.g., metabolons) in primary and secondary metabolisms, including polyamine metabolism (Panicot et al., 2002), lignin (Gou et al., 2018), flavonoids (Fujino et al., 2018), alkaloids (Stavrinides et al., 2015), camalexin (Mucha et al., 2019), and cyanogenic glucoside biosynthesis (Laursen et al., 2016). Hence, designing complexes of pathway biosynthetic enzymes could be an effective engineering strategy to efficiently facilitate substrate channeling and improve titers of products at the protein level. Several designs have been attempted to facilitate substrate channeling, including enzyme

co-localization and complex assembly. Enzyme colocalization can be achieved by altering the enzyme target peptides, whereas enzyme assembly techniques involve protein fusions of enzyme cascades, synthetic storage organelles and synthetic scaffolding proteins (Table 5).

Rerouting biosynthetic pathways by modifying the subcellular localization

In addition to use tissue-specific promoter to optimize natural product production at the transcription level, an alternative strategy is to alter the subcellular location of heterologously expressed protein by addition, removal or modification of target peptides (Reed and Osbourn, 2018). Target peptides are short peptide chains with 3-70 amino acids, which can direct the transport of proteins to specific subcellular sites in the cell, including the nucleus, plastid, mitochondria, endoplasmic reticulum (ER), peroxisome and plasma membrane (Keservani et al., 2015). Rerouting biosynthetic enzymes to the same cellular compartment by modifying the target peptides, the limitation of intracellular organelle separation of upstream and downstream enzymes in natural product synthesis can be alleviated (Figure 4D). This approach has been applied in increasing the yield of various plant metabolites with success.

The MVA pathway in the cytoplasm mainly provides precursors for the biosynthesis of sesquiterpenoids and triterpenoids, whereas the MEP pathway located in the cytosol predominantly offers precursors for the biosynthesis of monoterpenoids, diterpenoids, sesterterpenoids and tetraterpenoids. When directing the natural cytosolic patchoulol synthase (PTS) and FPP synthase (FPPS) to plastids by adding a chloroplast-targeting signal sequence, the accumulation of sesquiterpenes patchoulol and amorpha-4,11-diene was enhanced by more than 1,000-fold in tobacco, accompanied by the increased yield of the monoterpene limonene (Wu et al., 2006). The production of monoterpene geraniol and geraniol-derived products were highest when transiently expressing plastid-targeted GES, followed by mitochondrial- and then cytosolic-targeted GES (Dong et al., 2016). In a very recent study, by redirecting diterpene biosynthesis enzymes GGPPS and diTPSs from the plastidial MEP pathway to an engineered high-flux cytosolic MVA pathway, the production of diterpenes momilactones, forskolin and taxadiene was increased by about 10-fold compared to those in the chloroplast in *N. benthamiana* (De La Peña and Sattely,

2021). In contrast, redirecting the ER-mounted taxadiene-5α-hydroxylase (T5αH) and cytochrome P450 reductase (CPR) to the chloroplast where taxadiene synthases (TS) functions could also improve yields of the oxidative products of taxadiene, taxadiene-5α-ol in *N. benthamiana*, when combined with enhanced engineered precursor supply (Li et al., 2019). As for improving phenylpropanoids production, the accumulation of tropane alkaloids (i.e., hyoscyamine and scopolamine) could be improved when heterologously expressing the bacteria *Vitreoscilla* hemoglobin (VHb), an oxygen carrier and transporter in plastids of *H. niger* hairy roots transgenic lines (Guo et al., 2018).

Substrate channeling by enzyme assembly

Enzyme assembly, by ligand binding (e.g., protein fusions) or physically sequestrating enzymes (e.g., synthetic scaffolds)**,** can bring key pathway enzymes closer spatially to enhance the flux of a metabolic pathway. Fusion proteins can be generated either by directly fusing two or more enzymes or with a linker (Figure 4E). Linker size, protein arrangement orders and directions all have impacts on the activity of the generated enzyme fusion. Transient expression of enzyme fusion of *FARNESYLDIPHOSPHATE SYNTHASE* (*FDS*) and *EPI-ARISTOLOCHENE SYNTHASE* (*EAS*) with a short peptide (Gly-Ser-Gly) linker enabled higher titer of sesquiterpene capsidiol production than when the two were individually expressed in *N. tabacum* (Brodelius et al., 2002). Using the same strategy, expressing fusion of *FDS* and *AMORPHADIENE SYNTHASE* (*ADS*) in *A. annua* also led to maximum accumulation of amorpha-4,11-diene and significant increase of artemisinin titer (Han et al., 2016). Although enzyme fusion can effectively facilitate enzyme aggregation, it has its own limitations, e.g. fusion proteins may not fold properly, resulting in decreased catalytic activity of enzymes.

2A self-cleaving peptides, or 2A peptides, are 18–22 amino-acid-long viral peptides, and share a core sequence motif of GDVEXNPGP at the C-terminus (Ahier and Jarriault 2014; Liu et al., 2017). These 2A peptides facilitate formation of polyproteins by causing the ribosome skipping to fail at making a peptide bond during translation in eukaryotic cells (Figure 4E) (Donnelly et al., 2001). Although 2A peptide technology cannot guarantee physical proximity of enzymes, it allows simultaneous expression of different enzyme proteins. The technique of 2A peptides has been utilized for the production of

natural products in bactera. For example, triterpene friedelin was produced by expressing its synthase with tHMG1 linked by the 2A peptide of ERBV-1 (Souza-Moreira et al., 2018). In plants, the "cleavage" efficiencies of different 2As and the effects of gene position on protein expression level in bi-, tri-, and quad-cistronic 2A constructs have been explored (Liu et al., 2017). In a multiple gene expression vector, four different 2A peptides (F2A, E2A, T2A and P2A) were used to link four genes (*ZmBz1*, *ZmBz2*, *ZmC1* and *ZmR2*) involved in anthocyanin biosynthesis and seven reporter genes (GFP, mCherry, YFP, CFP, GUS, CP53 and AO), with all these parts driven by a maize endosperm specific bidirectional promoter (*PZmBD1*). The transgenic maize lines harbouring this vector showed accumulation of anthocyanin in embryo and endosperm, and the total anthocyanin content reaches 2.91 g/kg DW (Liu et al., 2018).

Scaffolding proteins are natural or artifical proteins, which can be used to bring together two or more key biosynthetic enzymes in a relatively stable conformation (Figure 4G). The naturally present twin-arginine translocation (Tat) complex is responsible for the active translocation of folded proteins across a lipid bilayer in plant thylakoids and prokaryotes (Figure 4H) (Patel et al., 2014). In one study, in order to improve channeling of tyrosine into dhurrin, three dhurrin enzymes were co-localized in the thylakoid membrane of chloroplasts by using subunits of the Tat complex as a protein scaffold (Henriques de Jesus et al., 2017). Specifically, exchanging the natural membrane anchors of the two P450s with TatB and fusing a soluble UDP-glucosyltransferase (UGT) to the transmembrane protein TatC via a flexible linker. By using this approach, the yield of dhurrin increased 5-fold, together with reduced side products in *N. benthamiana* (Henriques de Jesus et al., 2017).

Synthetic storage organelles, such as synthetic lipid (or hydrophobic) droplets, have been developed as an emerging strategy to co-localize enzymes for enhanced production of natural products, especially to sequester lipophilic compounds, such as terpenoids (Figure 4F). Lipid droplets are hydrophobic particles that predominantly occur in oil-rich seeds. With the identification of key genes required for the formation of lipid bodies in seeds, lipid droplet formation can be engineered in tissues besides seeds. By co-expression of *Arabidopsis* diacylglycerol acyltransferase 1 (*DGAT1*), *Arabidopsis* TFs

(*WRINKL1*) and oleosins (*OLE1*) from castor bean, the lipid droplets can be formed in leaf mesophyll tissue in *N. benthamiana* plants (Delatte et al., 2018). Further co-expression of α-bisabolol synthase with these three genes *DGAT1*, *WRINKL1* and *OLE1* promoted storage of α-bisabolol more than 17-fold (~ 140μg/g DW) (Delatte et al., 2018). Similarly, cytosolic lipid droplets were constructed via ectopic expression of *WRINKLED1* together with a microalgal lipid droplet surface protein *No*LDSP (Sadre et al., 2019). By fusing terpenoid enzymes to the lipid droplet surface protein, terpenoid production is successfully re-targeted to lipid droplets, resulting in enhanced accumulation of both target sesquiterpenoids and diterpenoids in leaves of *N. benthamiana*. In another study, in order to prevent plastid synthesized squalene "leak" out of chloroplast and the downstream modification by squalene epoxidase in the cytosol, the terpene biosynthesis enzymes (FPS and SQS) and synthetic lipid droplet proteins were colocalized to chloroplast (Zhao et al., 2018). Based on computational modeling, oleosin proteins were redesigned by replacing the N-terminal domain with a transit peptide for chloroplast localization, and removing the C-terminal domain to form a stabilized hydrophobic protein (HP). As a result, the yield of squalene reached 2.6 mg/g (fresh weight) without compromising plant growth in tobacco (Zhao et al., 2018).

Spatiotemporally actuated organelles (or called condensates) can be formed by applying the recently developed liquid–liquid phase separation (LLPS) technique, which is facilitated by using of self-associating intrinsically disordered protein regions (IDRs) (Bracha et al., 2018). These IDRs work together to promote LLPS by acting as "sticky" biopolymers through short film interacting residues patterned along their unstructured chains (Bracha et al., 2019). LLPS strategy can increase the metabolic flux by substrate channeling, redistributing existing enzymes into co-localized clusters or organelles. LLPS technology has just emerged in recent years, and haven't found applications in plants yet. Zhao et al. (2019) engineered the deoxyviolacein biosynthesis in yeast by coupling two pathway enzymes to form condensates using LLPS. Using this approach, the yield of desired products enhanced by six folds, with product specificity increased up to 18 folds as a result of reduced flux towards competing pathways.

Rational design or directed evolution for improved enzymatic performance

Optimizing the performance of each enzyme in the pathway is crucial in metabolic engineering as it determines the flux in a metabolic pathway. To meet this demand, rational design and directed evolution have been developed, by applying artificial selection pressure, to improve the substrate specificity, stability, quantity and catalytic properties of each enzymatic step (Figure 4I). Rational design is a strategy of creating novel biomolecules with certain functionality, which requires detailed knowledge of the structure and function of the protein or biomolecule, while directed evolution refers to methods to alter enzyme function using mutagenesis and selection with no prior knowledge required (Nannemann et al., 2011).

Site-directed mutagenesis has been frequently employed to rationally improve the performance of an enzyme, especially in microorganisms (Greenhagen et al., 2006). For instance, site-directed mutagenesis followed by biochemical assays identified the functions of many active-site residues of Bialaphos resistance protein (BAR), an enzyme conferring resistance to the broad-spectrum herbicide phosphinothricin. Several BAR variants generated display significantly reduced nonspecific activities (Christ et al., 2017). On the other hand, directed evolution is a powerful tool for modifying key enzymes with improved catalytic properties for the biosynthesis of natural products, which can lead to higher diversity of natural products by generating novel and more potent analogs (Williams et al., 2003). Directed evolution have been successfully applied in the production of secondary metabolites in model microorganisms, including enhanced production of flavonoids (Pandey et al., 2016), lycopene (Wang et al., 2000; Hong et al., 2019), carotenoid (Schmidt-Dannert et al., 2000; Umeno et al., 2005) and taxol (Li et al., 2017). However, the implementation of directed evolution in plants is difficult, due to their large genome size, long life cycles, metabolic complexity and difficulty for genetic transformations. Among these difficulties, the biggest obstacle lies in generating large transgenic populations with high variabilities and subjecting them to high-throughput screening. It is therefore more practical to generate mutant library of enzymes and screen for improved catalytic efficiency in bacteria or yeast, followed by reintroducing the mutant back into the plant via genome editing or other approaches. For example, in order

to improve carbon fixation of photosynthesis, the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), a key CO_2 fixing enzyme, was firstly evolved by directed evolution in *E.coli* (Wilson et al., 2018). Later, the Rubiscos with amino acid changes was codon-optimized and transformed to tobacco. Recently, two microbial platforms, OrthoRep in *S. cerevisiae* and EvolvR in *E. coli*, have been adapted for directed evolution of plant enzymes (Gionfriddo et al., 2019). However, proteins evolved in microorganisms may not exhibit the same behavior in plants, due to the different codon usage, proper folding and the complexity of the metabolic environment. An alternative approach of directly evolving the proteins in plants is now feasible with the developing of CRISPR–Cas systems. CRISPR-based directed evolution methods harness the power to achieve targeted random mutagenesis of a plant gene of interest, by coupling Cas9 with a gene-specific sgRNA library (Rao et al., 2021). Furthermore, whole plants can be regenerated from selected plant tissues or single cells. Several studies using CRISPR-Cas9 for directed evolution was successfully implemented in rice, which acquired enhanced resistance to different herbicides (Garcia-Garcia et al., 2020; Kuang et al., 2020; Li et al., 2020).

CONCLUSIONS AND PERSPECTIVES

With our ever-increasing demand for food, medicines, flavors, cosmetics and renewable fuels, plants as synthetic biology platforms hold great promises to meet the sustainable and cost-effective production of high-valuable products. Although the engineering strategies at different levels were described individually, these strategies are not independent of each other. Instead, in order to achieve the maximum production yield of natural products, several strategies are generally combined and deployed for whole plant metabolic engineering. For example, the modified gene transformation method, combinatorial supertransformation of transplastomic recipient lines (COSTREL), was applied to generate a large tobacco population (Fuentes et al., 2016). Specifically, by introducing the core pathway of artemisinic acid biosynthesis into the chloroplast genome followed by combinatorially supertransforming five additional nuclear transgenes, transplastomic tobacco showed up to 77-fold increase in artemisinic acid production (120 mg/kg DW) (Fuentes et al., 2016).

Compared to using microorganisms, plants as chassis to produce high levels of valuable metabolites is lagged behind. The major constraint is the difficulty in target gene delivery limited by plant genetic transformation techniques. The expansion of plant species can be genetically modified, improvement of transformation efficiency, and development of novel delivery machinery (i.e., carrier molecules and carbon nanotubes), would promote the rapid development and application of plant chassis. Another hurdle for developing plant chassis is the lack of available artificial parts and limited plant natural regulatory elements (*cis*-regulatory elements, promoters and terminators). The upstream open reading frames (uORFs) are reported to have crucial functions in primary metabolism, thereby enhancing the yield of desired metabolites (Robin et al., 2016). However, the function of uORFs in secondary metabolism needs further exploration. Considering the tight endogenous regulation in plants, both plant native and artificially synthesized regulators are in urgent need of being identified or designed. An integrative approach to combine multi-omics (genomic, transcriptomic, metabolomics) data, and applying machine learning approach can aid in uncovering novel genes encoding enzymes, transporters, and regulators involved in plant specialized metabolism (Luo et al., 2022). Metabolome genome-wide association study (mGWAS) targeting large populations, linking global metabolites changes to genetic variants (single-nucleotide polymorphisms (SNPs)) could also enable the discovery of key genes in the biosynthetic pathway. Furthermore, functionally characterized genes can be deposited in the 'tool box' for metabolic engineering.

Another hurdle for plant metabolic engineering is the complex plant pathways distributed across organelles and tissues, and the lack of information about metabolite transport. Determining the precise subcellular locations for all enzymes and metabolites of plant specialized metabolism is still a major challenge. The combination of mass spectrometry imaging (MSI) and live Single-cell MS can be applied to pinpoint the metabolites accumulated in different cell types (Yamamoto et al., 2016). In addition, the traditional methods, such as *in situ* hybridization, immunostaining, and the recently developed single-cell RNA sequencing technique can help dissect the gene expression and protein accumulation of biosynthetic pathway enzymes in different cell types.

With more bioactive compounds and associated genes being characterized using multi-omics techniques (Luo et al., 2022), the scope for plants as a unique platform for engineering and production of high-value compounds will expand gradually. The discovery of bioactive phytochemicals in plants and their functions in promoting plant health can potentially contribute to sustainable agriculture in many different aspects, especially in terms of weathering biotic and abiotic stresses. For instance, the plant hormone strigolactones have been proved to be capable of promoting arbuscular mycorrhizal fungus growth, especially under conditions of phosphate deprivation, indicating that strigolactones can be used to facilitate phosphate acquisition by those plants that can form symbiotic relationship with arbuscular mycorrhizal fungus under phosphate starvation (Kapulnik and Koltai, 2016). In a recent study, a volatile compound CPH was discovered and proved to function in protecting plants against *Empoasca* leafhoppers in *Nicotiana attenuate*. Synthetic biology can be used to reconstruct novel biosynthetic pathways in crops, which would accumulate desired phytochemicals (i.e., strigolactones and CPH), thereby protecting plants against biotic and abiotic stress conditions. Future advances in aspects of plant metabolism, together with the emerging computational approaches, genetic engineering tools, precise control of multiple stacking genes, host development, and cost reduction in DNA synthesis will inform the design of more comprehensive plant metabolic engineering strategies. The advances in plant synthetic biology are painting a picture that diverse bioactive metabolites can be massively and routinely produced, and promote drug discovery, sustainable agriculture and renewable energy.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (grant no. 32061143023), Shenzhen Municipal Startup Fund and the Key Laboratory of Molecular Design for Plant Cell Factory of Guangdong Higher Education Institutes (grantno.2019KSYS006). The figures were created via BioRender.com.

REFERENCES

Aharoni, A., Giri, A. P., Deuerlein, S., Griepink, F., de Kogel, W. J., Verstappen,

F. W., Verhoeven, H. A., Jongsma, M. A., Schwab, W., and Bouwmeester, H. J. (2003) Terpenoid metabolism in wild-type and transgenic *Arabidopsis* plants. Plant Cell **15:** 2866-2884.

Ahier, A., and Jarriault, S. (2014) Simultaneous expression of multiple proteins under a single promoter in Caenorhabditis elegans via a versatile 2A-based toolkit. Genetics **196:** 605-613.

Akashi, T., Aoki, T., and Ayabe, S. (2005) Molecular and biochemical characterization of 2-hydroxyisoflavanone dehydratase. Involvement of carboxylesterase-like proteins in leguminous isoflavone biosynthesis. Plant Physiol. **137:** 882-891.

Andersen-Ranberg, J., Kongstad, K. T., Nafisi, M., Staerk, D., Okkels, F. T., Mortensen, U. H., Lindberg Møller, B., Frandsen, R. J. N., and Kannangara, R. (2017) Synthesis of C-glucosylated octaketide anthraquinones in *Nicotiana benthamiana* by using a multispecies-based biosynthetic pathway. ChemBioChem **18:** 1893-1897.

Andersson, M., Turesson, H., Olsson, N., Fält, A. S., Ohlsson, P., Gonzalez, M. N., Samuelsson, M., and Hofvander, P. (2018) Genome editing in potato via CRISPR-Cas9 ribonucleoprotein delivery. Physiol. Plant **164:** 378-384.

Bai, Y., Yang, C., Halitschke, R., Paetz, C., Kessler, D., Burkard, K., Gaquerel, E., Baldwin, I. T., and Li, D. (2022) Natural history-guided omics reveals plant defensive chemistry against leafhopper pests. Science **375:** eabm2948.

Baltes, N. J., Gil-Humanes, J., and Voytas, D. F. (2017) Genome engineering and agriculture: Opportunities and challenges. Prog. Mol. Biol. Transl. Sci. **149:** 1-26.

Banf, M., Zhao, K., and Rhee, S. Y. (2019) METACLUSTER-an R package for context-specific expression analysis of metabolic gene clusters. Bioinformatics **35:** 3178-3180.

Beekwilder, J., van Houwelingen, A., Cankar, K., van Dijk, A. D., de Jong, R. M., Stoopen, G., Bouwmeester, H., Achkar, J., Sonke, T., and Bosch, D. (2014) Valencene synthase from the heartwood of nootka cypress (*Callitropsis nootkatensis*) for biotechnological production of valencene. Plant Biotechnol. J. **12:** 174-182.

Belcher, M. S., Vuu, K. M., Zhou, A., Mansoori, N., Agosto Ramos, A., Thompson, M. G., Scheller, H. V., Loqué, D., and Shih, P. M. (2020) Design of orthogonal regulatory systems for modulating gene expression in plants. Nat. Chem. Biol. **16:** 857-865.

Bertea, C. M., Voster, A., Verstappen, F. W., Maffei, M., Beekwilder, J., and Bouwmeester, H. J. (2006) Isoprenoid biosynthesis in *Artemisia annua*: Cloning and heterologous expression of a germacrene A synthase from a glandular trichome cDNA library. Arch. Biochem. Biophys. **448:** 3-12.

Beyraghdar Kashkooli, A., van der Krol, A. R., Rabe, P., Dickschat, J. S., and Bouwmeester, H. (2019) Substrate promiscuity of enzymes from the sesquiterpene biosynthetic pathways from *Artemisia annua* and

Tanacetum parthenium allows for novel combinatorial sesquiterpene production. Metab. Eng. **54:** 12-23.

Blazeck, J., Alper, H. S. (2013) Promoter engineering: Recent advances in controlling transcription at the most fundamental level. Biotechnol. J. **8:** 46-58.

Bock, R. (2015) Engineering plastid genomes: Methods, tools, and applications in basic research and biotechnology. Annu. Rev. Plant Biol. **66:** 211-241.

Boehm, C. R., and Bock, R. (2019) Recent advances and current challenges in synthetic biology of the plastid genetic system and metabolism. Plant Physiol. **179:** 794-802.

Bonawitz, N. D., Kim, J. I., Tobimatsu, Y., Ciesielski, P. N., Anderson, N. A., Ximenes, E., Maeda, J., Ralph, J., Donohoe, B. S., Ladisch, M., and Chapple, C. (2014) Disruption of mediator rescues the stunted growth of a lignin-deficient *Arabidopsis* mutant. Nature **509:** 376-380.

Bouwmeester, H. J., Wallaart, T. E., Janssen, M. H., van Loo, B., Jansen, B. J., Posthumus, M. A., Schmidt, C. O., De Kraker, J. W., König, W. A., and Franssen, M. C. (1999) Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. Phytochemistry **52:** 843-854.

Bovy, A., de Vos, R., Kemper, M., Schijlen, E., Almenar Pertejo, M., Muir, S., Collins, G., Robinson, S., Verhoeyen, M., Hughes, S., Santos-Buelga, C., and van Tunen, A. (2002) High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes *LC* and *C1*. Plant Cell **14:** 2509-2526.

Bracha, D., Walls, M. T., and Brangwynne, C. P. (2019) Probing and engineering liquid-phase organelles. Nat. Biotechnol. **37:** 1435-1445.

Bracha, D., Walls, M. T., Wei, M. T., Zhu, L., Kurian, M., Avalos, J. L., Toettcher, J. E., and Brangwynne, C. P. (2018) Mapping local and global liquid phase behavior in living cells using photo-oligomerizable seeds. Cell **175:** 1467-1480.e1413.

Brodelius, M., Lundgren, A., Mercke, P., and Brodelius, P. E. (2002) Fusion of farnesyldiphosphate synthase and epi-aristolochene synthase, a sesquiterpene cyclase involved in capsidiol biosynthesis in *Nicotiana tabacum*. Eur. J. Biochem. **269:** 3570-3577.

Brown, P. D., Tokuhisa, J. G., Reichelt, M., and Gershenzon, J. (2003) Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. Phytochemistry **62:** 471-481.

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

Butelli, E., Titta, L., Giorgio, M., Mock, H. P., Matros, A., Peterek, S., Schijlen, E. G., Hall, R. D., Bovy, A. G., Luo, J., and Martin, C. (2008) Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat. Biotechnol. **26:** 1301-1308.

Cai, Y., Jia, J. W., Crock, J., Lin, Z. X., Chen, X. Y., Croteau, R. (2002) A cDNA

clone for beta-caryophyllene synthase from *Artemisia annua*. Phytochemistry **61:** 523-529.

Calgaro-Kozina, A., Vuu, K. M., Keasling, J. D., Loqué, D., Sattely, E. S., and Shih, P. M. (2020) Engineering plant synthetic pathways for the biosynthesis of novel antifungals. ACS Cent. Sci. **6:** 1394-1400.

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

Cardi, T., Lenzi, P., and Maliga, P. (2010) Chloroplasts as expression platforms for plant-produced vaccines. Expert Rev. Vaccines **9:** 893-911.

Chae, L., Kim, T., Nilo-Poyanco, R., and Rhee, S. Y. (2014) Genomic signatures of specialized metabolism in plants. Science **344:** 510-513.

Chang, K., Qiu, F., Chen, M., Zeng, L., Liu, X., Yang, C., Lan, X., Wang, Q., and Liao, Z. (2014) Engineering the MEP pathway enhanced ajmalicine biosynthesis. Biotechnol. Appl. Biochem. **61:** 249-255.

Chari, R., Yeo, N. C., Chavez, A., and Church, G. M. (2017) sgRNA Scorer 2.0: A species-independent model to predict CRISPR/Cas9 activity. ACS Synth. Biol. **6:** 902-904.

Chen, F., Tholl, D., Bohlmann, J., and Pichersky, E. (2011) The family of terpene synthases in plants: A mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. Plant J. **66:** 212-229.

Chen, M., Yan, T., Shen, Q., Lu, X., Pan, Q., Huang, Y., Tang, Y., Fu, X., Liu, M., Jiang, W., Lv, Z., Shi, P., Ma, Y. N., Hao, X., Zhang, L., Li, L., and Tang, K. (2017) GLANDULAR TRICHOME-SPECIFIC WRKY 1 promotes artemisinin biosynthesis in *Artemisia annua*. New Phytol. **214:** 304-316.

Chen, Q. J., Xie, M., Ma, X. X., Dong, L., Chen, J., and Wang, X. C. (2010) MISSA is a highly efficient in vivo DNA assembly method for plant multiple-gene transformation. Plant Physiol. **153:** 41-51.

Chiasson, D., Giménez-Oya, V., Bircheneder, M., Bachmaier, S., Studtrucker, T., Ryan, J., Sollweck, K., Leonhardt, H., Boshart, M., Dietrich, P., and Parniske, M. (2019) A unified multi-kingdom Golden Gate cloning platform. Sci. Rep. **9:** 10131.

Choi, K. R., Jang, W. D., Yang, D., Cho, J. S., Park, D., and Lee, S. Y. (2019) Systems metabolic engineering strategies: Integrating systems and synthetic biology with metabolic engineering. Trends Biotechnol. **37:** 817-837.

Christ, B., Hochstrasser, R., Guyer, L., Francisco, R., Aubry, S., Hortensteiner, S., and Weng, J. K. (2017) Non-specific activities of the major herbicide-resistance gene *BAR*. Nat. Plants **3:** 937-945.

Christianson, D. W. (2017) Structural and chemical biology of terpenoid cyclases. Chem. Rev. **117:** 11570-11648.

Chu, Y., Kwon, T., and Nam, J. (2014) Enzymatic and metabolic engineering for

efficient production of syringin, sinapyl alcohol 4-O-glucoside, in *Arabidopsis thaliana*. Phytochemistry **102:** 55-63.

Chuai, G., Ma, H., Yan, J., Chen, M., Hong, N., Xue, D., Zhou, C., Zhu, C., Chen, K., Duan, B., Gu, F., Qu, S., Huang, D., Wei, J., and Liu, Q. (2018) DeepCRISPR: optimized CRISPR guide RNA design by deep learning. Genome Biol. **19:** 80.

Clarke, J. L., and Daniell, H. (2011) Plastid biotechnology for crop production: Present status and future perspectives. Plant Mol. Biol. **76:** 211-220.

Coll, A., Wilson, M. L., Gruden, K., and Peccoud, J. (2015) Rule-based design of plant expression vectors using genoCAD. PLoS ONE **10:** e0132502.

Crocoll, C., Mirza, N., Reichelt, M., Gershenzon, J., and Halkier, B. A. (2016) Optimization of engineered production of the glucoraphanin precursor dihomomethionine in *Nicotiana benthamiana*. Front. Bioeng. Biotechnol. **4:** 14.

Dastmalchi, M., Chang, L., Chen, R., Yu, L., Chen, X., Hagel, J. M., and Facchini, P. J. (2019) Purine permease-type benzylisoquinoline alkaloid transporters in opium poppy. Plant Physiol. **181:** 916-933.

De La Peña, R., and Sattely, E. S. (2021) Rerouting plant terpene biosynthesis enables momilactone pathway elucidation. Nat. Chem. Biol. **17:** 205-212.

De Marchis, F., Wang, Y., Stevanato, P., Arcioni, S., and Bellucci, M. (2009) Genetic transformation of the sugar beet plastome. Transgenic Res. **18:** 17-30.

De Meester, B., de Vries, L., Özparpucu, M., Gierlinger, N., Corneillie, S., Pallidis, A., Goeminne, G., Morreel, K., De Bruyne, M., De Rycke, R., Vanholme, R., and Boerjan, W. (2018) Vessel-specific reintroduction of CINNAMOYL-COA REDUCTASE1 (CCR1) in dwarfed *ccr1* mutants restores vessel and xylary fiber integrity and increases biomass. Plant Physiol. **176:** 611-633.

de Oliveira, M. V. V., Jin, X., Chen, X., Griffith, D., Batchu, S., and Maeda, H. A. (2019) Imbalance of tyrosine by modulating TyrA arogenate dehydrogenases impacts growth and development of *Arabidopsis thaliana*. Plant J. **97:** 901-922.

Deavours, B. E., and Dixon, R. A. (2005) Metabolic engineering of isoflavonoid biosynthesis in alfalfa. Plant Physiol. **138:** 2245-2259.

Decaestecker, W., Buono, R. A., Pfeiffer, M. L., Vangheluwe, N., Jourquin, J., Karimi, M., Van Isterdael, G., Beeckman, T., Nowack, M. K., and Jacobs, T. B. (2019) CRISPR-TSKO: A technique for efficient mutagenesis in specific cell types, tissues, or organs in *Arabidopsis*. Plant Cell **31:** 2868-2887.

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

Demurtas, O. C., de Brito Francisco, R., Diretto, G., Ferrante, P., Frusciante, S., Pietrella, M., Aprea, G., Borghi, L., Feeney, M., Frigerio, L., Coricello, A., Costa, G., Alcaro, S., Martinoia, E., and Giuliano, G. (2019) ABCC transporters mediate the vacuolar accumulation of crocins in saffron stigmas. Plant Cell **31:** 2789-2804.

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

Dong, W., Stockwell, V. O., Goyer, A. (2015) Enhancement of thiamin content in *Arabidopsis thaliana* by metabolic engineering. Plant Cell Physiol. **56:** 2285-2296.

Donnelly, M. L. L., Luke, G., Mehrotra, A., Li, X., Hughes, L. E., Gani, D., and Ryan, M. D. (2001) Analysis of the aphthovirus 2A/2B polyprotein 'cleavage' mechanism indicates not a proteolytic reaction, but a novel translational effect: A putative ribosomal 'skip'. J. Gen. Virol. **82:** 1013-1025.

Dräger, B. (2002) Analysis of tropane and related alkaloids. J. Chromatogr. A **978:** 1-35.

Dubouzet, J. G., Matsuda, F., Ishihara, A., Miyagawa, H., and Wakasa, K. (2013) Production of indole alkaloids by metabolic engineering of the tryptophan pathway in rice. Plant Biotechnol. J. **11:** 1103-1111.

Dufourmantel, N., Tissot, G., Goutorbe, F., Garçon, F., Muhr, C., Jansens, S., Pelissier, B., Peltier, G., and Dubald, M. (2005) Generation and analysis of soybean plastid transformants expressing *Bacillus thuringiensis* Cry1Ab protoxin. Plant Mol. Biol. **58:** 659-668.

Dupuy, L., Mackenzie, J., Rudge, T., and Haseloff, J. (2008) A system for modelling cell-cell interactions during plant morphogenesis. Ann. Bot. **101:** 1255-1265.

Endo, A., Masafumi, M., Kaya, H., and Toki, S. (2016) Efficient targeted mutagenesis of rice and tobacco genomes using Cpf1 from *Francisella novicida*. Sci. Rep. **6:** 38169.

Engler, C., Kandzia, R., and Marillonnet, S. (2008) A one pot, one step, precision cloning method with high throughput capability. PLoS ONE **3:** e3647.

Ennajdaoui, H., Vachon, G., Giacalone, C., Besse, I., Sallaud, C., Herzog, M., and Tissier, A. (2010) Trichome specific expression of the tobacco (*Nicotiana sylvestris*) cembratrien-ol synthase genes is controlled by both activating and repressing cis-regions. Plant Mol. Biol. **73:** 673-685.

Federici, F., Dupuy, L., Laplaze, L., Heisler, M., and Haseloff, J. (2012) Integrated genetic and computation methods for in planta cytometry. Nat. Methods **9:** 483-485.

Field, B., and Osbourn, A. E. (2008) Metabolic diversification--independent assembly of operon-like gene clusters in different plants. Science **320:** 543-547.

Forestier, E. C. F., Czechowski, T., Cording, A. C., Gilday, A. D., King, A. J., Brown, G. D., and Graham, I. A. (2021) Developing a *Nicotiana benthamiana* transgenic platform for high-value diterpene production and candidate gene evaluation. Plant Biotechnol. J. **19:** 1614-1623.

Franke, J., Kim, J., Hamilton, J. P., Zhao, D., Pham, G. M., Wiegert-Rininger, K., Crisovan, E., Newton, L., Vaillancourt, B., Tatsis, E., Buell, C. R., and O'Connor, S. E. (2019) Gene discovery in gelsemium highlights conserved gene clusters in monoterpene indole alkaloid biosynthesis. ChemBioChem **20:** 83-87.

Fu, X., Shi, P., He, Q., Shen, Q., Tang, Y., Pan, Q., Ma, Y., Yan, T., Chen, M., Hao, X., Liu, P., Li, L., Wang, Y., Sun, X., and Tang, K. (2017) AaPDR3, a PDR transporter 3, is involved in sesquiterpene β-Caryophyllene transport in *Artemisia annua*. Front. Plant Sci. **8:** 723.

Fuentes, P., Zhou, F., Erban, A., Karcher, D., Kopka, J., and Bock, R. (2016) A new synthetic biology approach allows transfer of an entire metabolic pathway from a medicinal plant to a biomass crop. eLife **5:** e13664.

Fujino, N., Tenma, N., Waki, T., Ito, K., Komatsuzaki, Y., Sugiyama, K., Yamazaki, T., Yoshida, S., Hatayama, M., Yamashita, S., Tanaka, Y., Motohashi, R., Denessiouk, K., Takahashi, S., and Nakayama, T. (2018) Physical interactions among flavonoid enzymes in snapdragon and torenia reveal the diversity in the flavonoid metabolon organization of different plant species. Plant J. **94:** 372-392.

Garcia-Garcia, J. D., Joshi, J., Patterson, J. A., Trujillo-Rodriguez, L., Reisch, C. R., Javanpour, A. A., Liu, C. C., and Hanson, A. D. (2020) Potential for applying continuous directed evolution to plant enzymes: An exploratory study. Life (Basel) **10:** 179.

Gatica-Arias, A., Farag, M. A., Stanke, M., Matoušek, J., Wessjohann, L., and Weber, G. (2012) Flavonoid production in transgenic hop (*Humulus lupulus* L.) altered by PAP1/MYB75 from *Arabidopsis thaliana* L. Plant Cell Rep. **31:** 111-119.

Geck, M. K., and Kirsch, J. F. (1999) A novel, definitive test for substrate channeling illustrated with the aspartate aminotransferase/malate dehydrogenase system. Biochemistry **38:** 8032-8037.

Geu-Flores, F., Nielsen, M. T., Nafisi, M., Møldrup, M. E., Olsen, C. E., Motawia, M. S., and Halkier, B. A. (2009) Glucosinolate engineering identifies a gamma-glutamyl peptidase. Nat. Chem. Biol. **5:** 575-577.

Gibson, D. G., Glass, J. I., Lartigue, C., Noskov, V. N., Chuang, R. Y., Algire, M. A., Benders, G. A., Montague, M. G., Ma, L., Moodie, M. M., Merryman, C., Vashee, S., Krishnakumar, R., Assad-Garcia, N., Andrews-Pfannkoch, C., Denisova, E. A., Young, L., Qi, Z. Q., Segall-Shapiro, T. H., Calvey, C. H., Parmar, P. P., Hutchison, C. A., 3rd, Smith, H. O., and Venter, J. C. (2010) Creation of a bacterial cell controlled by a chemically synthesized genome. Science **329:** 52-56.

Gionfriddo, M., De Gara, L., and Loreto, F. (2019) Directed evolution of plant

processes: Towards a green (r)evolution? Trends Plant Sci. **24:** 999-1007.

Giovinazzo, G., D'Amico, L., Paradiso, A., Bollini, R., Sparvoli, F., and DeGara, L. (2005) Antioxidant metabolite profiles in tomato fruit constitutively expressing the grapevine stilbene synthase gene. Plant Biotechnol. J. **3:** 57-69.

Gou, M., Ran, X., Martin, D. W., and Liu, C. J. (2018) The scaffold proteins of lignin biosynthetic cytochrome P450 enzymes. Nat. Plants **4:** 299-310.

Greenhagen, B. T., O'Maille, P. E., Noel, J. P., and Chappell, J. (2006) Identifying and manipulating structural determinates linking catalytic specificities in terpene synthases. Proc. Natl. Acad. Sci. U.S.A. **103:** 9826-9831.

Grotewold, E. (2008) Transcription factors for predictive plant metabolic engineering: Are we there yet? Curr. Opin. Biotechnol. **19:** 138-144.

Guillon, S., Trémouillaux-Guiller, J., Pati, P. K., Rideau, M., and Gantet, P. (2006) Hairy root research: Recent scenario and exciting prospects. Curr. Opin. Plant Biol. **9:** 341-346.

Guo, Z., Tan, H., Lv, Z., Ji, Q., Huang, Y., Liu, J., Chen, D., Diao, Y., Si, J., and Zhang, L. (2018) Targeted expression of Vitreoscilla hemoglobin improves the production of tropane alkaloids in *Hyoscyamus niger* hairy roots. Sci. Rep. **8:** 17969.

Hamberger, B., and Bak, S. (2013) Plant P450s as versatile drivers for evolution of species-specific chemical diversity. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **368:** 20120426.

Hamberger, B., Ellis, M., Friedmann, M., Souza, C. A. S. d. A., Barbazuk, B., and Douglas, C. J. D. J. (2007) Genome-wide analyses of phenylpropanoid-related genes in *Populus trichocarpa*, *Arabidopsis thaliana*, and *Oryza sativa*: The *Populus* lignin toolbox and conservation and diversification of angiosperm gene families. Can. J. Bot. **85:** 1182-1201.

Han, J., Wang, H., Kanagarajan, S., Hao, M., Lundgren, A., and Brodelius, P. E. (2016) Promoting artemisinin biosynthesis in *Artemisia annua* plants by substrate channeling. Mol. Plant **9:** 946-948.

Han, J., Wang, H., Lundgren, A., and Brodelius, P. E. (2014) Effects of overexpression of AaWRKY1 on artemisinin biosynthesis in transgenic *Artemisia annua* plants. Phytochemistry **102:** 89-96.

Hartley, J. L., Temple, G. F., and Brasch, M. A. (2000) DNA cloning using *in vitro* site-specific recombination. Genome Res. **10:** 1788-1795.

Hemmerlin, A., Harwood, J. L., and Bach, T. J. (2012) A raison d'être for two distinct pathways in the early steps of plant isoprenoid biosynthesis? Prog. Lipid. Res. **51:** 95-148.

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

Herrmann, K. M., and Weaver, L. M. (1999) The shikimate pathway. Annu. Rev. Plant Physiol. Plant Mol. Biol. **50:** 473-503.

Holstein, S. A., and Hohl, R. J. (2004) Isoprenoids: Remarkable diversity of form and function. Lipids **39:** 293-309.

Hong, J., Park, S. H., Kim, S., Kim, S. W., and Hahn, J. S. (2019) Efficient production of lycopene in *Saccharomyces cerevisiae* by enzyme engineering and increasing membrane flexibility and NAPDH production. Appl. Microbiol. Biotechnol. **103:** 211-223.

Hong, Y., Zhou, Q., Hao, Y., and Huang, A. C. (2021) Crafting the plant root metabolome for improved microbe-assisted stress resilience. New Phytol. **234:** 1945-1950

Hou, B. K., Zhou, Y. H., Wan, L. H., Zhang, Z. L., Shen, G. F., Chen, Z. H., and Hu, Z. M. (2003) Chloroplast transformation in oilseed rape. Transgenic Res. **12:** 111-114.

Huchelmann, A., Boutry, M., and Hachez, C. (2017) Plant glandular trichomes: Natural cell factories of high biotechnological interest. Plant Physiol. **175:** 6-22.

Hwang, H. J., Lee, S. Y., and Lee, P. C. (2018) Engineering and application of synthetic nar promoter for fine-tuning the expression of metabolic pathway genes in *Escherichia coli*. Biotechnol. Biofuels **11:** 103.

Jelesko, J. G. (2012) An expanding role for purine uptake permease-like transporters in plant secondary metabolism. Front. Plant Sci. **3:** 78.

Jeong, Y. J., An, C. H., Woo, S. G., Park, J. H., Lee, K. W., Lee, S. H., Rim, Y., Jeong, H. J., Ryu, Y. B., and Kim, C. Y. (2016) Enhanced production of resveratrol derivatives in tobacco plants by improving the metabolic flux of intermediates in the phenylpropanoid pathway. Plant Mol. Biol. **92:** 117-129.

Ji, Y., Xiao, J., Shen, Y., Ma, D., Li, Z., Pu, G., Li, X., Huang, L., Liu, B., Ye, H., and Wang, H. (2014) Cloning and characterization of AabHLH1, a bHLH transcription factor that positively regulates artemisinin biosynthesis in *Artemisia annua*. Plant Cell Physiol. **55:** 1592-1604.

Jiang, W., Fu, X., Pan, Q., Tang, Y., Shen, Q., Lv, Z., Yan, T., Shi, P., Li, L., Zhang, L., Wang, G., Sun, X., and Tang, K. (2016) Overexpression of AaWRKY1 leads to an enhanced content of artemisinin in *Artemisia annua*. Biomed. Res. Int. **2016:** 7314971.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., and Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science **337:** 816-821.

Jones, J. A., Toparlak Ö, D., and Koffas, M. A. (2015) Metabolic pathway balancing and its role in the production of biofuels and chemicals. Curr. Opin. Biotechnol. **33:** 52-59.

Han, J.L., Liu, B.Y., Ye, H.C., Wang, H., Li, Z.Q., and Li, G.F. (2006). Effects of overexpression of the endogenous farnesyl diphosphate synthase on the artemisinin content in *Artemisia annua* L. J. Integr. Plant Biol. **48:** 482-487.

Kapulnik, Y., and Koltai, H. (2016) Fine-tuning by strigolactones of root response to low phosphate. J. Integr. Plant Biol. **58:** 203-212.

Kautsar, S. A., Suarez Duran, H. G., Blin, K., Osbourn, A., and Medema, M. H. (2017) plantiSMASH: Automated identification, annotation and expression analysis of plant biosynthetic gene clusters. Nucleic Acids Res. **45:** W55-W63.

Keservani, R. K., Sharma, A. K., and Jarouliya, U. (2015) Protein and peptide in drug targeting and its therapeutic approach. Ars Pharmaceutica (Internet) **56:** 165-177.

Kim, H. K., Min, S., Song, M., Jung, S., Choi, J. W., Kim, Y., Lee, S., Yoon, S., and Kim, H. H. (2018) Deep learning improves prediction of CRISPR-Cpf1 guide RNA activity. Nat. Biotechnol. **36:** 239-241.

Kim, Y. J., Lee, O. R., Oh, J. Y., Jang, M. G., and Yang, D. C. (2014) Functional analysis of 3-hydroxy-3-methylglutaryl coenzyme a reductase encoding genes in triterpene saponin-producing ginseng. Plant Physiol. **165:** 373-387.

King, A. J., Brown, G. D., Gilday, A. D., Larson, T. R., and Graham, I. A. (2014) Production of bioactive diterpenoids in the euphorbiaceae depends on evolutionarily conserved gene clusters. Plant Cell **26:** 3286-3298.

Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A., and Liu, D. R. (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature **533:** 420-424.

Krieg, T., Sydow, A., Faust, S., Huth, I., and Holtmann, D. (2018) CO_2 to terpenes: Autotrophic and electroautotrophic α-humulene production with *Cupriavidus necator*. Angew. Chem. Int. Ed. Engl. **57:** 1879-1882.

Kuang, Y., Li, S., Ren, B., Yan, F., Spetz, C., Li, X., Zhou, X., and Zhou, H. (2020) Base-editing-mediated artificial evolution of osals1 in planta to develop novel herbicide-tolerant rice germplasms. Mol. Plant **13:** 565-572.

Kubo, M., Udagawa, M., Nishikubo, N., Horiguchi, G., Yamaguchi, M., Ito, J., Mimura, T., Fukuda, H., and Demura, T. (2005) Transcription switches for protoxylem and metaxylem vessel formation. Genes Dev. **19:** 1855-1860.

Lakshmanan, M., Kodama, Y., Yoshizumi, T., Sudesh, K., and Numata, K. (2013) Rapid and efficient gene delivery into plant cells using designed peptide carriers. Biomacromolecules **14:** 10-16.

Lam, E., and Michael, T. P. (2022) Wolffia, a minimalist plant and synthetic biology chassis. Trends Plant Sci. **27:** 430-439.

Lange, B. M., Rujan, T., Martin, W., and Croteau, R. (2000) Isoprenoid biosynthesis: The evolution of two ancient and distinct pathways across genomes. Proc. Natl. Acad. Sci. U.S.A. **97:** 13172-13177.

Lange, I., Poirier, B. C., Herron, B. K., and Lange, B. M. (2015) Comprehensive Assessment of Transcriptional Regulation Facilitates Metabolic Engineering of Isoprenoid Accumulation in Arabidopsis. Plant Physiol. **169:** 1595-1606.

Lau, W., Sattely, E. S. (2015) Six enzymes from mayapple that complete the

biosynthetic pathway to the etoposide aglycone. Science **349:** 1224-1228.

Laursen, T., Borch, J., Knudsen, C., Bavishi, K., Torta, F., Martens, H. J., Silvestro, D., Hatzakis, N. S., Wenk, M. R., Dafforn, T. R., Olsen, C. E., Motawia, M. S., Hamberger, B., Møller, B. L., and Bassard, J. E. (2016) Characterization of a dynamic metabolon producing the defense compound dhurrin in sorghum. Science **354:** 890-893.

Lee, A. R., Kwon, M., Kang, M. K., Kim, J., Kim, S. U., and Ro, D. K. (2019) Increased sesqui- and triterpene production by co-expression of HMG-CoA reductase and biotin carboxyl carrier protein in tobacco (*Nicotiana benthamiana*). Metab. Eng. **52:** 20-28.

Li, B. J., Wang, H., Gong, T., Chen, J. J., Chen, T. J., Yang, J. L., and Zhu, P. (2017) Improving 10-deacetylbaccatin III-10-β-O-acetyltransferase catalytic fitness for taxol production. Nat. Commun. **8:** 15544.

Li, C., Zhang, R., Meng, X., Chen, S., Zong, Y., Lu, C., Qiu, J. L., Chen, Y. H., Li, J., and Gao, C. (2020) Targeted, random mutagenesis of plant genes with dual cytosine and adenine base editors. Nat. Biotechnol. **38:** 875-882.

Li, J., Mutanda, I., Wang, K., Yang, L., Wang, J., and Wang, Y. (2019) Chloroplastic metabolic engineering coupled with isoprenoid pool enhancement for committed taxanes biosynthesis in *Nicotiana benthamiana*. Nat. Commun. **10:** 4850.

Li, Z., Xing, A., Moon, B. P., Burgoyne, S. A., Guida, A. D., Liang, H., Lee, C., Caster, C. S., Barton, J. E., Klein, T. M., and Falco, S. C. (2007) A Cre/loxP-mediated self-activating gene excision system to produce marker gene free transgenic soybean plants. Plant Mol. Biol. **65:** 329-341.

Liu, D.H., Ren, W.W., Cui, L.J., Zhang, L.D., Sun, X.F., and Tang, K.X. (2011) Enhanced accumulation of catharanthine and vindoline in *Catharanthus roseus* hairy roots by overexpression of transcriptional factor ORCA2. African J. Biotechnol. **10:** 3260-3268.

Liu, H., Sun, Y., Ramos, K. R., Nisola, G. M., Valdehuesa, K. N., Lee, W. K., Park, S. J., and Chung, W. J. (2013) Combination of Entner-Doudoroff pathway with MEP increases isoprene production in engineered *Escherichia coli*. PLoS ONE **8:** e83290.

Liu, J., Osbourn, A., and Ma, P. (2015) MYB transcription factors as regulators of phenylpropanoid metabolism in plants. Mol. Plant **8:** 689-708.

Liu, W., and Stewart, C. N., Jr. (2015) Plant synthetic biology. Trends Plant Sci. **20:** 309-317.

Liu, X., Li, S., Yang, W., Mu, B., Jiao, Y., Zhou, X., Zhang, C., Fan, Y., and Chen, R. (2018) Synthesis of seed-specific bidirectional promoters for metabolic engineering of anthocyanin-rich maize. Plant Cell Physiol. **59:** 1942-1955.

Liu, X., Ma, X., Wang, H., Li, S., Yang, W., Nugroho, R. D., Luo, L., Zhou, X., Tang, C., Fan, Y., Zhao, Q., Zhang, J., and Chen, R. (2021) Metabolic engineering of astaxanthin-rich maize and its use in the production of biofortified eggs. Plant Biotechnol. J. **19:** 1812-1823.

Liu, Z., Chen, O., Wall, J. B. J., Zheng, M., Zhou, Y., Wang, L., Vaseghi, H. R., Qian, L., and Liu, J. (2017) Systematic comparison of 2A peptides for cloning multi-genes in a polycistronic vector. Sci. Rep. **7:** 2193.

Lloyd, A. M., Walbot, V., and Davis, R. W. (1992) *Arabidopsis* and *Nicotiana anthocyanin* production activated by maize regulators R and C1. Science **258:** 1773-1775.

Lu, X., Zhang, L., Zhang, F., Jiang, W., Shen, Q., Zhang, L., Lv, Z., Wang, G., and Tang, K. (2013) AaORA, a trichome-specific AP2/ERF transcription factor of *Artemisia annua*, is a positive regulator in the artemisinin biosynthetic pathway and in disease resistance to *Botrytis cinerea*. New Phytol. **198:** 1191-1202.

Luo, F., Yu, Z., Zhou, Q., and Huang, A. (2022) Multi-omics-based discovery of plant signaling molecules. Metabolites **12:** 76.

Luo, J., Butelli, E., Hill, L., Parr, A., Niggeweg, R., Bailey, P., Weisshaar, B., and Martin, C. (2008) AtMYB12 regulates caffeoyl quinic acid and flavonol synthesis in tomato: Expression in fruit results in very high levels of both types of polyphenol. Plant J. **56:** 316-326.

Luo, Y., Li, B. Z., Liu, D., Zhang, L., Chen, Y., Jia, B., Zeng, B. X., Zhao, H., Yuan, Y. J. (2015) Engineered biosynthesis of natural products in heterologous hosts. Chem. Soc. Rev. **44:** 5265-5290.

Lv, Z., Guo, Z., Zhang, L., Zhang, F., Jiang, W., Shen, Q., Fu, X., Yan, T., Shi, P., Hao, X., Ma, Y., Chen, M., Li, L., Zhang, L., Chen, W., and Tang, K. (2019a) Interaction of bZIP transcription factor TGA6 with salicylic acid signaling modulates artemisinin biosynthesis in *Artemisia annua*. J. Exp. Bot. **70:** 3969-3979.

Lv, Z., Jiang, R., Chen, J., and Chen, W. (2020) Nanoparticle-mediated gene transformation strategies for plant genetic engineering. Plant J. **104:** 880-891.

Lv, Z., Wang, Y., Liu, Y., Peng, B., Zhang, L., Tang, K., and Chen, W. (2019b) The SPB-Box transcription factor AaSPL2 positively regulates artemisinin biosynthesis in *Artemisia annua* L. Front. Plant Sci. **10:** 409.

Ma, Y. N., Xu, D. B., Li, L., Zhang, F., Fu, X. Q., Shen, Q., Lyu, X. Y., Wu, Z. K., Pan, Q. F., Shi, P., Hao, X. L., Yan, T. X., Chen, M. H., Liu, P., He, Q., Xie, L. H., Zhong, Y. J., Tang, Y. L., Zhao, J. Y., Zhang, L. D., Sun, X. F., and Tang, K. X. (2018) Jasmonate promotes artemisinin biosynthesis by activating the TCP14-ORA complex in *Artemisia annua*. Sci. Adv. **4:** eaas9357.

Maier, A., Schrader, A., Kokkelink, L., Falke, C., Welter, B., Iniesto, E., Rubio, V., Uhrig, J. F., Hülskamp, M., and Hoecker, U. (2013) Light and the E3 ubiquitin ligase COP1/SPA control the protein stability of the MYB transcription factors PAP1 and PAP2 involved in anthocyanin accumulation in *Arabidopsis*. Plant J. **74:** 638-651.

Malhotra, K., Subramaniyan, M., Rawat, K., Kalamuddin, M., Qureshi, M. I., Malhotra, P., Mohmmed, A., Cornish, K., Daniell, H., and Kumar, S.

(2016) Compartmentalized metabolic engineering for artemisinin biosynthesis and effective malaria treatment by oral delivery of plant cells. Mol. Plant **9:** 1464-1477.

Malik, M. R., Tang, J., Sharma, N., Burkitt, C., Ji, Y., Mykytyshyn, M., Bohmert-Tatarev, K., Peoples, O., and Snell, K. D. (2018) Camelina sativa, an oilseed at the nexus between model system and commercial crop. Plant Cell Rep. **37:** 1367-1381.

Malnoy, M., Viola, R., Jung, M. H., Koo, O. J., Kim, S., Kim, J. S., Velasco, R., and Nagamangala Kanchiswamy, C. (2016) DNA-free genetically edited grapevine and apple protoplast using CRISPR/Cas9 ribonucleoproteins. Front. Plant Sci. **7:** 1904.

Matsuura, H. N., and Fett-Neto, A. G. (2015) Plant alkaloids: Main features, toxicity, and mechanisms of action. *Plant Toxins*. Springer, Netherland. pp. 1-15

McKnight, T. D., Roessner, C. A., Devagupta, R., Scott, A. I., and Nessler, C. L. (1990) Nucleotide sequence of a cDNA encoding the vacuolar protein strictosidine synthase from Catharanthus roseus. Nucleic Acids Res. **18:** 4939.

Meadows, A. L., Hawkins, K. M., Tsegaye, Y., Antipov, E., Kim, Y., Raetz, L., Dahl, R. H., Tai, A., Mahatdejkul-Meadows, T., Xu, L., Zhao, L., Dasika, M. S., Murarka, A., Lenihan, J., Eng, D., Leng, J. S., Liu, C. L., Wenger, J. W., Jiang, H., Chao, L., Westfall, P., Lai, J., Ganesan, S., Jackson, P., Mans, R., Platt, D., Reeves, C. D., Saija, P. R., Wichmann, G., Holmes, V. F., Benjamin, K., Hill, P. W., Gardner, T. S., and Tsong, A. E. (2016) Rewriting yeast central carbon metabolism for industrial isoprenoid production. Nature **537:** 694-697.

Mehrotra, S., and Goyal, V. (2012) *Agrobacterium*-mediated gene transfer in plants and biosafety considerations. Appl. Biochem. Biotechnol. **168:** 1953-1975.

Mercke, P., Crock, J., Croteau, R., and Brodelius, P. E. (1999) Cloning, expression, and characterization of epi-cedrol synthase, a sesquiterpene cyclase from *Artemisia annua* L. Arch. Biochem. Biophys. **369:** 213-222.

Miettinen, K., Dong, L., Navrot, N., Schneider, T., Burlat, V., Pollier, J., Woittiez, L., van der Krol, S., Lugan, R., Ilc, T., Verpoorte, R., Oksman-Caldentey, K. M., Martinoia, E., Bouwmeester, H., Goossens, A., Memelink, J., and Werck-Reichhart, D. (2014) The seco-iridoid pathway from *Catharanthus roseus*. Nat. Commun. **5:** 3606.

Mitsuda, N., Iwase, A., Yamamoto, H., Yoshida, M., Seki, M., Shinozaki, K., and Ohme-Takagi, M. (2007) NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of *Arabidopsis*. Plant Cell **19:** 270-280.

Mohanta, T. K., Bashir, T., Hashem, A., Abd Allah, E. F., and Bae, H. (2017) Genome editing tools in plants. Genes (Basel) **8:** 399.

Moore, B. M., Wang, P., Fan, P., Leong, B., Schenck, C. A., Lloyd, J. P.,

Lehti-Shiu, M. D., Last, R. L., Pichersky, E., and Shiu, S. H. (2019) Robust predictions of specialized metabolism genes through machine learning. Proc. Natl. Acad. Sci. U.S.A. **116:** 2344-2353.

Morris, W. L., Ducreux, L. J., Shepherd, T., Lewinsohn, E., Davidovich-Rikanati, R., Sitrit, Y., and Taylor, M. A. (2011) Utilisation of the MVA pathway to produce elevated levels of the sesquiterpene α-copaene in potato tubers. Phytochemistry **72:** 2288-2293.

Mortimer, J. C. (2019) Plant synthetic biology could drive a revolution in biofuels and medicine. Exp. Biol. Med. (Maywood) **244:** 323-331.

Moser, S., and Pichler, H. (2019) Identifying and engineering the ideal microbial terpenoid production host. Appl. Microbiol. Biotechnol. **103:** 5501-5516.

Mucha, S., Heinzlmeir, S., Kriechbaumer, V., Strickland, B., Kirchhelle, C., Choudhary, M., Kowalski, N., Eichmann, R., Hückelhoven, R., Grill, E., Kuster, B., and Glawischnig, E. (2019) The formation of a camalexin biosynthetic metabolon. Plant Cell **31:** 2697-2710.

Muir, S. R., Collins, G. J., Robinson, S., Hughes, S., Bovy, A., Ric De Vos, C. H., van Tunen, A. J., and Verhoeyen, M. E. (2001) Overexpression of petunia chalcone isomerase in tomato results in fruit containing increased levels of flavonols. Nat. Biotechnol. **19:** 470-474.

Muñoz-Bertomeu, J., Arrillaga, I., Ros, R., and Segura, J. (2006) Up-regulation of 1-deoxy-D-xylulose-5-phosphate synthase enhances production of essential oils in transgenic spike lavender. Plant Physiol. **142:** 890-900.

Nakatsuka, T., Abe, Y., Kakizaki, Y., Yamamura, S., and Nishihara, M. (2007) Production of red-flowered plants by genetic engineering of multiple flavonoid biosynthetic genes. Plant Cell Rep. **26:** 1951-1959.

Nannemann, D. P., Birmingham, W. R., Scism, R. A., and Bachmann, B. O. (2011) Assessing directed evolution methods for the generation of biosynthetic enzymes with potential in drug biosynthesis. Future Med. Chem. **3:** 809-819.

Napier, J. A., Haslam, R. P., Beaudoin, F., and Cahoon, E. B. (2014) Understanding and manipulating plant lipid composition: Metabolic engineering leads the way. Curr. Opin. Plant Biol. **19:** 68-75.

Nes, W. D. (2011) Biosynthesis of cholesterol and other sterols. Chem. Rev. **111:** 6423-6451.

Nishida, K., Arazoe, T., Yachie, N., Banno, S., Kakimoto, M., Tabata, M., Mochizuki, M., Miyabe, A., Araki, M., Hara, K. Y., Shimatani, Z., and Kondo, A. (2016) Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. Science **353:** aaf8729.

Nogueira, M., Enfissi, E. M. A., Welsch, R., Beyer, P., Zurbriggen, M. D., and Fraser, P. D. (2019) Construction of a fusion enzyme for astaxanthin formation and its characterisation in microbial and plant hosts: A new tool for engineering ketocarotenoids. Metab. Eng. **52:** 243-252.

Nour-Eldin, H. H., Andersen, T. G., Burow, M., Madsen, S. R., Jørgensen, M. E., Olsen, C. E., Dreyer, I., Hedrich, R., Geiger, D., and Halkier, B. A.

(2012) NRT/PTR transporters are essential for translocation of glucosinolate defence compounds to seeds. Nature **488:** 531-534.

Nour-Eldin, H. H., Madsen, S. R., Engelen, S., Jørgensen, M. E., Olsen, C. E., Andersen, J. S., Seynnaeve, D., Verhoye, T., Fulawka, R., Denolf, P., and Halkier, B. A. (2017) Reduction of antinutritional glucosinolates in Brassica oilseeds by mutation of genes encoding transporters. Nat. Biotechnol. **35:** 377-382.

Nützmann, H. W., Huang, A., and Osbourn, A. (2016) Plant metabolic clusters - from genetics to genomics. New Phytol. **211:** 771-789.

Ochoa-Villarreal, M., Howat, S., Hong, S., Jang, M. O., Jin, Y. W., Lee, E. K., and Loake, G. J. (2016) Plant cell culture strategies for the production of natural products. BMB Rep. **49:** 149-158.

Oksman-Caldentey, K. M. (2007) Tropane and nicotine alkaloid biosynthesis-novel approaches towards biotechnological production of plant-derived pharmaceuticals. Curr. Pharm. Biotechnol. **8:** 203-210.

Onoyovwe, A., Hagel, J. M., Chen, X., Khan, M. F., Schriemer, D. C., and Facchini, P. J. (2013) Morphine biosynthesis in opium poppy involves two cell types: Sieve elements and laticifers. Plant Cell **25:** 4110-4122.

Owen, C., Patron, N. J., Huang, A., and Osbourn, A. (2017) Harnessing plant metabolic diversity. Curr. Opin. Chem. Biol. **40:** 24-30.

Oyarce, P., De Meester, B., Fonseca, F., de Vries, L., Goeminne, G., Pallidis, A., De Rycke, R., Tsuji, Y., Li, Y., Van den Bosch, S., Sels, B., Ralph, J., Vanholme, R., and Boerjan, W. (2019) Introducing curcumin biosynthesis in Arabidopsis enhances lignocellulosic biomass processing. Nat. Plants **5:** 225-237.

Paddon, C. J., Westfall, P. J., Pitera, D. J., Benjamin, K., Fisher, K., McPhee, D., Leavell, M. D., Tai, A., Main, A., Eng, D., Polichuk, D. R., Teoh, K. H., Reed, D. W., Treynor, T., Lenihan, J., Jiang, H., Fleck, M., Bajad, S., Dang, G., Dengrove, D., Diola, D., Dorin, G., Ellens, K. W., Fickes, S., Galazzo, J., Gaucher, S. P., Geistlinger, T., Henry, R., Hepp, M., Horning, T., Iqbal, T., Kizer, L., Lieu, B., Melis, D., Moss, N., Regentin, R., Secrest, S., Tsuruta, H., Vazquez, R., Westblade, L. F., Xu, L., Yu, M., Zhang, Y., Zhao, L., Lievense, J., Covello, P. S., Keasling, J. D., Reiling, K. K., Renninger, N. S., and Newman, J. D. (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. Nature **496:** 528-532.

Paine, J. A., Shipton, C. A., Chaggar, S., Howells, R. M., Kennedy, M. J., Vernon, G., Wright, S. Y., Hinchliffe, E., Adams, J. L., Silverstone, A. L., and Drake, R. (2005) Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nat. Biotechnol. **23:** 482-487.

Pandey, R. P., Parajuli, P., Koffas, M. A. G., and Sohng, J. K. (2016) Microbial production of natural and non-natural flavonoids: Pathway engineering, directed evolution and systems/synthetic biology. Biotechnol. Adv. **34:** 634-662.

Panicot, M., Minguet, E. G., Ferrando, A., Alcázar, R., Blázquez, M. A., Carbonell, J., Altabella, T., Koncz, C., and Tiburcio, A. F. (2002) A polyamine metabolon involving aminopropyl transferase complexes in *Arabidopsis*. Plant Cell **14:** 2539-2551.

Park, S. U., Yu, M., and Facchini, P. J. (2003) Modulation of berberine bridge enzyme levels in transgenic root cultures of California poppy alters the accumulation of benzophenanthridine alkaloids. Plant Mol. Biol. **51:** 153-164.

Patel, R., Smith, S. M., and Robinson, C. (2014) Protein transport by the bacterial Tat pathway. Biochim. Biophys. Acta **1843:** 1620-1628.

Paul, P., Singh, S. K., Patra, B., Sui, X., Pattanaik, S., and Yuan, L. (2017) A differentially regulated AP2/ERF transcription factor gene cluster acts downstream of a MAP kinase cascade to modulate terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. New Phytol. **213:** 1107-1123.

Pauw, B., Hilliou, F. A., Martin, V. S., Chatel, G., de Wolf, C. J., Champion, A., Pré, M., van Duijn, B., Kijne, J. W., van der Fits, L., and Memelink, J. (2004) Zinc finger proteins act as transcriptional repressors of alkaloid biosynthesis genes in *Catharanthus roseus*. J. Biol. Chem. **279:** 52940-52948.

Payne, R. M., Xu, D., Foureau, E., Teto Carqueijeiro, M. I., Oudin, A., Bernonville, T. D., Novak, V., Burow, M., Olsen, C. E., Jones, D. M., Tatsis, E. C., Pendle, A., Ann Halkier, B., Geu-Flores, F., Courdavault, V., Nour-Eldin, H. H., and O'Connor, S. E. (2017) An NPF transporter exports a central monoterpene indole alkaloid intermediate from the vacuole. Nat. Plants **3:** 16208.

Peebles, C. A., Hughes, E. H., Shanks, J. V., and San, K. Y. (2009) Transcriptional response of the terpenoid indole alkaloid pathway to the overexpression of ORCA3 along with jasmonic acid elicitation of *Catharanthus roseus* hairy roots over time. Metab. Eng. **11:** 76-86.

Pham, J. V., Yilma, M. A., Feliz, A., Majid, M. T., Maffetone, N., Walker, J. R., Kim, E., Cho, H. J., Reynolds, J. M., Song, M. C., Park, S. R., and Yoon, Y. J. (2019) A review of the microbial production of bioactive natural products and biologics. Front. Microbiol. **10:** 1404.

Phillips, I., and Silver, P. (2006) *A New Biobrick Assembly Strategy Designed For Facile Protein Engineering***.** Dspace, http://hdl.handle.net/1721.1/32535.

Piatek, A., Ali, Z., Baazim, H., Li, L., Abulfaraj, A., Al-Shareef, S., Aouida, M., and Mahfouz, M. M. (2015) RNA-guided transcriptional regulation in planta via synthetic dCas9-based transcription factors. Plant Biotechnol. J. **13:** 578-589.

Picaud, S., Brodelius, M., and Brodelius, P. E. (2005) Expression, purification and characterization of recombinant (E)-beta-farnesene synthase from *Artemisia annua*. Phytochemistry **66:** 961-967.

Polturak, G., Grossman, N., Vela-Corcia, D., Dong, Y., Nudel, A., Pliner, M., Levy, M., Rogachev, I., and Aharoni, A. (2017) Engineered gray mold resistance, antioxidant capacity, and pigmentation in betalain-producing crops and ornamentals. Proc. Natl. Acad. Sci. U.S.A. **114:** 9062-9067.

Portela, R. M., Vogl, T., Kniely, C., Fischer, J. E., Oliveira, R., and Glieder, A. (2017) Synthetic core promoters as universal parts for fine-tuning expression in different yeast species. ACS Synth. Biol. **6:** 471-484.

Püllmann, P., Ulpinnis, C., Marillonnet, S., Gruetzner, R., Neumann, S., and Weissenborn, M. J. (2019) Golden mutagenesis: An efficient multi-site-saturation mutagenesis approach by Golden Gate cloning with automated primer design. Sci. Rep. **9:** 10932.

Rai, K. M., Ghose, K., Rai, A., Singh, H., Srivastava, R., and Mendu, V. (2019) Genome engineering tools in plant synthetic biology. In: Singh, S.P., Pandey, A., Du, G., Kumar, S. eds. *Current Developments in Biotechnology and Bioengineering.* Elsevier, pp. 47-73.

Rajniak, J., Barco, B., Clay, N. K., and Sattely, E. S. (2015) A new cyanogenic metabolite in *Arabidopsis* required for inducible pathogen defence. Nature **525:** 376-379.

Rao, G. S., Jiang, W., Mahfouz, M. (2021) Synthetic directed evolution in plants: Unlocking trait engineering and improvement. Synth. Biol. **6:** ysab025.

Redding-Johanson, A. M., Batth, T. S., Chan, R., Krupa, R., Szmidt, H. L., Adams, P. D., Keasling, J. D., Lee, T. S., Mukhopadhyay, A., and Petzold, C. J. (2011) Targeted proteomics for metabolic pathway optimization: Application to terpene production. Metab. Eng. **13:** 194-203.

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

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

Reski, R., Bae, H., and Simonsen, H. T. (2018) Physcomitrella patens, a versatile synthetic biology chassis. Plant Cell Rep. **37:** 1409-1417.

Richardson, S. M., Wheelan, S. J., Yarrington, R. M., and Boeke, J. D. (2006) GeneDesign: Rapid, automated design of multikilobase synthetic genes. Genome Res. **16:** 550-556.

Robin, A. Y., Giustini, C., Graindorge, M., Matringe, M., and Dumas, R. (2016) Crystal structure of norcoclaurine-6-O-methyltransferase, a key rate-limiting step in the synthesis of benzylisoquinoline alkaloids. Plant J. **87:** 641-653.

Rontein, D., Onillon, S., Herbette, G., Lesot, A., Werck-Reichhart, D., Sallaud, C., and Tissier, A. (2008) CYP725A4 from yew catalyzes complex structural rearrangement of taxa-4(5),11(12)-diene into the cyclic ether 5(12)-oxa-3(11)-cyclotaxane. J. Biol. Chem **283:** 6067-6075.

Sabate, R., de Groot, N. S., and Ventura, S. (2010) Protein folding and aggregation in bacteria. Cell. Mol. Life Sci. **67:** 2695-2715.

Sadre, R., Kuo, P., Chen, J., Yang, Y., Banerjee, A., Benning, C., and Hamberger, B. (2019) Cytosolic lipid droplets as engineered organelles for production and accumulation of terpenoid biomaterials in leaves. Nat. Commun. **10:** 853.

Sallaud, C., Giacalone, C., Töpfer, R., Goepfert, S., Bakaher, N., Rösti, S., and Tissier, A. (2012) Characterization of two genes for the biosynthesis of the labdane diterpene Z-abienol in tobacco (*Nicotiana tabacum*) glandular trichomes. Plant J. **72:** 1-17.

Samuni-Blank, M., Izhaki, I., Dearing, M. D., Gerchman, Y., Trabelcy, B., Lotan, A., Karasov, W. H., and Arad, Z. (2012) Intraspecific directed deterrence by the mustard oil bomb in a desert plant. Curr. Biol. **22:** 1218-1220.

Sarrion-Perdigones, A., Vazquez-Vilar, M., Palací, J., Castelijns, B., Forment, J., Ziarsolo, P., Blanca, J., Granell, A., and Orzaez, D. (2013) GoldenBraid 2.0: A comprehensive DNA assembly framework for plant synthetic biology. Plant Physiol. **162:** 1618-1631.

Schläpfer, P., Zhang, P., Wang, C., Kim, T., Banf, M., Chae, L., Dreher, K., Chavali, A. K., Nilo-Poyanco, R., Bernard, T., Kahn, D., and Rhee, S. Y. (2017) Genome-wide prediction of metabolic enzymes, pathways, and gene clusters in plants. Plant Physiol. **173:** 2041-2059.

Schmidt-Dannert, C., Umeno, D., and Arnold, F. H. (2000) Molecular breeding of carotenoid biosynthetic pathways. Nat. Biotechnol. **18:** 750-753.

Schultz, B. J., Kim, S. Y., Lau, W., and Sattely, E. S. (2019) Total biosynthesis for milligram-scale production of etoposide intermediates in a plant chassis. J. Am. Chem. Soc. **141:** 19231-19235.

Scotti, N., Rigano, M. M., and Cardi, T. (2012) Production of foreign proteins using plastid transformation. Biotechnol. Adv. **30:** 387-397.

Shen, Q., Lu, X., Yan, T., Fu, X., Lv, Z., Zhang, F., Pan, Q., Wang, G., Sun, X., and Tang, K. (2016) The jasmonate-responsive AaMYC2 transcription factor positively regulates artemisinin biosynthesis in *Artemisia annua*. New Phytol. **210:** 1269-1281.

Shi, P., Fu, X., Liu, M., Shen, Q., Jiang, W., Li, L., Sun, X., and Tang, K. (2017) Promotion of artemisinin content in *Artemisia annua* by overexpression of multiple artemisinin biosynthetic pathway genes. Plant Cell Tiss. Org. **129:** 251-259.

Shitan, N., Bazin, I., Dan, K., Obata, K., Kigawa, K., Ueda, K., Sato, F., Forestier, C., and Yazaki, K. (2003) Involvement of CjMDR1, a plant multidrug-resistance-type ATP-binding cassette protein, in alkaloid transport in *Coptis japonica*. Proc. Natl. Acad. Sci. U.S.A. **100:** 751-756.

Shitan, N., Hayashida, M., and Yazaki, K. (2015) Translocation and accumulation of nicotine via distinct spatio-temporal regulation of nicotine transporters in *Nicotiana tabacum*. Plant Signal Behav. **10:** e1035852.

Shoji, T. (2014) ATP-binding cassette and multidrug and toxic compound

extrusion transporters in plants: A common theme among diverse detoxification mechanisms. Int. Rev. Cell Mol. Biol. **309:** 303-346.

Shoji, T., Inai, K., Yazaki, Y., Sato, Y., Takase, H., Shitan, N., Yazaki, K., Goto, Y., Toyooka, K., Matsuoka, K., and Hashimoto, T. (2009) Multidrug and toxic compound extrusion-type transporters implicated in vacuolar sequestration of nicotine in tobacco roots. Plant Physiol. **149:** 708-718.

Shukal, S., Chen, X., and Zhang, C. (2019) Systematic engineering for high-yield production of viridiflorol and amorphadiene in auxotrophic *Escherichia coli*. Metab. Eng. **55:** 170-178.

Sidorov, V. A., Kasten, D., Pang, S. Z., Hajdukiewicz, P. T., Staub, J. M., and Nehra, N. S. (1999) Technical advance: Stable chloroplast transformation in potato: Use of green fluorescent protein as a plastid marker. Plant J. **19:** 209-216.

Singh, A. K., Verma, S. S., and Bansal, K. C. (2010) Plastid transformation in eggplant (*Solanum melongena* L.). Transgenic Res. **19:** 113-119.

Skirycz, A., Jozefczuk, S., Stobiecki, M., Muth, D., Zanor, M. I., Witt, I., and Mueller-Roeber, B. (2007) Transcription factor AtDOF4;2 affects phenylpropanoid metabolism in *Arabidopsis thaliana*. New Phytol. **175:** 425-438.

Sleight, S. C., Bartley, B. A., Lieviant, J. A., and Sauro, H. M. (2010) In-Fusion BioBrick assembly and re-engineering. Nucleic Acids Res. **38:** 2624-2636.

Souza-Moreira, T. M., Navarrete, C., Chen, X., Zanelli, C. F., Valentini, S. R., Furlan, M., Nielsen, J., and Krivoruchko, A. (2018) Screening of 2A peptides for polycistronic gene expression in yeast. FEMS Yeast Res. **18:** foy036.

Stamp, N. (2003) Out of the quagmire of plant defense hypotheses. Q Rev. Biol. **78:** 23-55.

Staniek, A., Bouwmeester, H., Fraser, P. D., Kayser, O., Martens, S., Tissier, A., van der Krol, S., Wessjohann, L., and Warzecha, H. (2013) Natural products - modifying metabolite pathways in plants. Biotechnol. J. **8:** 1159-1171.

Stavrinides, A., Tatsis, E. C., Foureau, E., Caputi, L., Kellner, F., Courdavault, V., and O'Connor, S. E. (2015) Unlocking the diversity of alkaloids in Catharanthus roseus: Nuclear localization suggests metabolic channeling in secondary metabolism. Chem. Biol. **22:** 336-341.

Stracke, R., Favory, J. J., Gruber, H., Bartelniewoehner, L., Bartels, S., Binkert, M., Funk, M., Weisshaar, B., and Ulm, R. (2010) The *Arabidopsis* bZIP transcription factor HY5 regulates expression of the *PFG1/MYB12* gene in response to light and ultraviolet-B radiation. Plant Cell Environ. **33:** 88-103.

Stracke, R., Ishihara, H., Huep, G., Barsch, A., Mehrtens, F., Niehaus, K., and Weisshaar, B. (2007) Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the *Arabidopsis thaliana* seedling. Plant J. **50:** 660-677.

Sugiyama, A., Linley, P. J., Sasaki, K., Kumano, T., Yamamoto, H., Shitan, N., Ohara, K., Takanashi, K., Harada, E., Hasegawa, H., Terakawa, T., Kuzuyama, T., Yazaki, K. (2011) Metabolic engineering for the production of prenylated polyphenols in transgenic legume plants using bacterial and plant prenyltransferases. Metab. Eng. **13:** 629-637.

Sumit, G. J. (2016) Biosynthesis of structurally diverse triterpenes in plants: The role of oxidosqualene cyclases. Proc. Ind. Nat. Sci. Acad. **82:** 1189-1210.

Sun, Q. Y., Ding, L. W., Lomonossoff, G. P., Sun, Y. B., Luo, M., Li, C. Q., Jiang, L., and Xu, Z. F. (2011) Improved expression and purification of recombinant human serum albumin from transgenic tobacco suspension culture. J. Biotechnol. **155:** 164-172.

Suttipanta, N., Pattanaik, S., Kulshrestha, M., Patra, B., Singh, S. K., and Yuan, L. (2011) The transcription factor CrWRKY1 positively regulates the terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. Plant Physiol. **157:** 2081-2093.

Svitashev, S., Schwartz, C., Lenderts, B., Young, J. K., and Mark Cigan, A. (2016) Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. Nat. Commun. **7:** 13274.

Takanashi, K., Yamada, Y., Sasaki, T., Yamamoto, Y., Sato, F., Yazaki, K. (2017) A multidrug and toxic compound extrusion transporter mediates berberine accumulation into vacuoles in *Coptis japonica*. Phytochemistry **138:** 76-82.

Tan, H., Xiao, L., Gao, S., Li, Q., Chen, J., Xiao, Y., Ji, Q., Chen, R., Chen, W., and Zhang, L. (2015) TRICHOME AND ARTEMISININ REGULATOR 1 is required for trichome development and artemisinin biosynthesis in *Artemisia annua*. Mol. Plant **8:** 1396-1411.

Tetali, S. D. (2019) Terpenes and isoprenoids: A wealth of compounds for global use. Planta **249:** 1-8.

Tholl, D. (2015) Biosynthesis and biological functions of terpenoids in plants. Adv. Biochem. Eng. Biotechnol. **148:** 63-106.

Timoneda, A., Sheehan, H., Feng, T., Lopez-Nieves, S., Maeda, H. A., and Brockington, S. (2018) Redirecting primary metabolism to boost production of tyrosine-derived specialised metabolites in planta. Sci. Rep. **8:** 17256.

Tissier, A., Sallaud, C., Rontein, D. (2013) Tobacco trichomes as a platform for terpenoid biosynthesis engineering. In: Bach, T.J., Rohmer, M. eds. *Isoprenoid Synthesis in Plants and Microorganisms: New Concepts and Experimental Approaches.* Springer New York, New York, NY. pp. 271-283.

Tiwari, R., and Rana, C.S. (2015). Plant secondary metabolites: A review. Int. J. Eng. Res. Gen. Sci. **3:** 661-670.

Tohge, T., Watanabe, M., Hoefgen, R., and Fernie, A. R. (2013) Shikimate and phenylalanine biosynthesis in the green lineage. Front. Plant Sci. **4:** 62.

Töpfer, N., Fuchs, L. M., and Aharoni, A. (2017) The PhytoClust tool for

metabolic gene clusters discovery in plant genomes. Nucleic Acids Res. **45:** 7049-7063.

Torrens-Spence, M. P., Pluskal, T., Li, F. S., Carballo, V., and Weng, J. K. (2018) Complete pathway elucidation and heterologous reconstitution of rhodiola salidroside biosynthesis. Mol. Plant **11:** 205-217.

Tzin, V., Galili, G. (2010) The biosynthetic pathways for shikimate and aromatic amino acids in *Arabidopsis thaliana*. Arabidopsis Book **8:** e0132.

Umeno, D., Tobias, A. V., and Arnold, F. H. (2005) Diversifying carotenoid biosynthetic pathways by directed evolution. Microbiol. Mol Biol. Rev. **69:** 51-78.

Valletta, A., Iozia, L. M., and Leonelli, F. (2021) Impact of environmental factors on stilbene biosynthesis. Plants (Basel) **10:** 90.

Van Wyk, N., Kroukamp, H., and Pretorius, I. (2018) The smell of synthetic biology: engineering strategies for aroma compound production in yeast. Fermentation **4:** 54.

Vanholme, R., Cesarino, I., Rataj, K., Xiao, Y., Sundin, L., Goeminne, G., Kim, H., Cross, J., Morreel, K., Araujo, P., Welsh, L., Haustraete, J., McClellan, C., Vanholme, B., Ralph, J., Simpson, G. G., Halpin, C., and Boerjan, W. (2013) Caffeoyl shikimate esterase (CSE) is an enzyme in the lignin biosynthetic pathway in *Arabidopsis*. Science **341:** 1103-1106.

Vanholme, R., Morreel, K., Darrah, C., Oyarce, P., Grabber, J. H., Ralph, J., and Boerjan, W. (2012) Metabolic engineering of novel lignin in biomass crops. New Phytol. **196:** 978-1000.

Vasilev, N., Schmitz, C., Dong, L., Ritala, A., Imseng, N., Häkkinen, S. T., van der Krol, S., Eibl, R., Oksman-Caldentey, K.-M., Bouwmeester, H., Fischer, R., and Schillberg, S. (2014) Comparison of plant-based expression platforms for the heterologous production of geraniol. Plant Cell Tiss. Org. **117:** 373-380.

Vavitsas, K., Fabris, M., and Vickers, C. E. (2018) Terpenoid metabolic engineering in photosynthetic microorganisms. Genes (Basel) **9:** 520.

Vazquez-Vilar, M., Quijano-Rubio, A., Fernandez-Del-Carmen, A., Sarrion-Perdigones, A., Ochoa-Fernandez, R., Ziarsolo, P., Blanca, J., Granell, A., and Orzaez, D. (2017) GB3.0: A platform for plant bio-design that connects functional DNA elements with associated biological data. Nucleic Acids Res. **45:** 2196-2209.

Villalobos, A., Ness, J. E., Gustafsson, C., Minshull, J., and Govindarajan, S. (2006) Gene Designer: A synthetic biology tool for constructing artificial DNA segments. BMC Bioinformatics **7:** 285.

Vogt, T. (2010) Phenylpropanoid biosynthesis. Mol. Plant **3:** 2-20.

Vranová, E., Coman, D., and Gruissem, W. (2013) Network analysis of the MVA and MEP pathways for isoprenoid synthesis. Annu. Rev. Plant Biol. **64:** 665-700.

Wang, B., Kashkooli, A. B., Sallets, A., Ting, H. M., de Ruijter, N. C. A., Olofsson, L., Brodelius, P., Pottier, M., Boutry, M., Bouwmeester, H.,

and van der Krol, A. R. (2016) Transient production of artemisinin in *Nicotiana benthamiana* is boosted by a specific lipid transfer protein from *A. annua*. Metab. Eng. **38:** 159-169.

Wang, C., Oh, M. K., and Liao, J. C. (2000) Directed evolution of metabolically engineered *Escherichia coli* for carotenoid production. Biotechnol. Prog. **16:** 922-926.

Wang, J., Shen, X., Rey, J., Yuan, Q., and Yan, Y. (2018) Recent advances in microbial production of aromatic natural products and their derivatives. Appl. Microbiol. Biotechnol. **102:** 47-61.

Wang, M., Mao, Y., Lu, Y., Tao, X., and Zhu, J. K. (2017) Multiplex gene editing in rice using the CRISPR-Cpf1 system. Mol. Plant **10:** 1011-1013.

Wang, X., Ye, L., Lyu, M., Ursache, R., Löytynoja, A., and Mähönen, A. P. (2020) An inducible genome editing system for plants. Nat. Plants **6:** 766-772.

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

Williams, G. J., Domann, S., Nelson, A., and Berry, A. (2003) Modifying the stereochemistry of an enzyme-catalyzed reaction by directed evolution. Proc. Natl. Acad. Sci. U.S.A. **100:** 3143-3148.

Wilson, R. H., Martin-Avila, E., Conlan, C., and Whitney, S. M. (2018) An improved Escherichia coli screen for Rubisco identifies a protein-protein interface that can enhance CO(2)-fixation kinetics. J. Biol. Chem. **293:** 18-27.

Wink, M., Alfermann, A. W., Franke, R., Wetterauer, B., Distl, M., Windhövel, J., Krohn, O., Fuss, E., Garden, H., Mohagheghzadeh, A., Wildi, E., and Ripplinger, P. (2005) Sustainable bioproduction of phytochemicals by plant in vitro cultures: anticancer agents. Plant Genetic Resources **3:** 90-100.

Wu, S., Schalk, M., Clark, A., Miles, R. B., Coates, R., and Chappell, J. (2006) Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. Nat. Biotechnol. **24:** 1441-1447.

Xiang, L., Jian, D., Zhang, F., Yang, C., Bai, G., Lan, X., Chen, M., Tang, K., and Liao, Z. (2019) The cold-induced transcription factor bHLH112 promotes artemisinin biosynthesis indirectly via ERF1 in *Artemisia annua*. J. Exp. Bot. **70:** 4835-4848.

Xie, K., Minkenberg, B., and Yang, Y. (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc. Natl. Acad. Sci. U.S.A. **112:** 3570-3575.

Yamamoto, K., Takahashi, K., Mizuno, H., Anegawa, A., Ishizaki, K., Fukaki, H., Ohnishi, M., Yamazaki, M., Masujima, T., and Mimura, T. (2016) Cell-specific localization of alkaloids in *Catharanthus roseus* stem tissue measured with Imaging MS and Single-cell MS. Proc. Natl. Acad. Sci. U.S.A. **113:** 3891-3896.

Yang, K., Stracquadanio, G., Luo, J., Boeke, J. D., and Bader, J. S. (2016) BioPartsBuilder: A synthetic biology tool for combinatorial assembly of biological parts. Bioinformatics **32:** 937-939.

Yu, Z. X., Li, J. X., Yang, C. Q., Hu, W. L., Wang, L. J., and Chen, X. Y. (2012) The jasmonate-responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in *Artemisia annua* L. Mol. Plant **5:** 353-365.

Zhang, F., Fu, X., Lv, Z., Lu, X., Shen, Q., Zhang, L., Zhu, M., Wang, G., Sun, X., Liao, Z., and Tang, K. (2015a) A basic leucine zipper transcription factor, AabZIP1, connects abscisic acid signaling with artemisinin biosynthesis in *Artemisia annua*. Mol. Plant **8:** 163-175.

Zhang, H. Y., Wang, X. H., Dong, L., Wang, Z. P., Liu, B., Lv, J., Xing, H. L., Han, C. Y., Wang, X. C., and Chen, Q. J. (2017) MISSA 2.0: An updated synthetic biology toolbox for assembly of orthogonal CRISPR/Cas systems. Sci. Rep. **7:** 41993.

Zhang, L., Jing, F., Li, F., Li, M., Wang, Y., Wang, G., Sun, X., and Tang, K. (2009) Development of transgenic *Artemisia annua* (Chinese wormwood) plants with an enhanced content of artemisinin, an effective anti-malarial drug, by hairpin-RNA-mediated gene silencing. Biotechnol. Appl. Biochem. **52:** 199-207.

Zhang, Q., Liang, M., Zeng, J., Yang, C., Qin, J., Qiang, W., Lan, X., Chen, M., Lin, M., and Liao, Z. (2022) Engineering tropane alkaloid production and glyphosate resistance by overexpressing AbCaM1 and G2-EPSPS in *Atropa belladonna*. Metab. Eng. **72:** 237-246.

Zhang, Q., Wu, N., Jian, D., Jiang, R., Yang, C., Lan, X., Chen, M., Zhang, F., and Liao, Z. (2019) Overexpression of AaPIF3 promotes artemisinin production in *Artemisia annua*. Ind. Crops Prod. **138:** 111476.

Zhang, Y., Butelli, E., Alseekh, S., Tohge, T., Rallapalli, G., Luo, J., Kawar, P. G., Hill, L., Santino, A., Fernie, A. R., and Martin, C. (2015b) Multi-level engineering facilitates the production of phenylpropanoid compounds in tomato. Nat. Commun. **6:** 8635-8635.

Zhang, Y., Zhou, L., Tang, K., Xu, M., and Miao, Z. (2020) Matching is the key factor to improve the production of patchoulol in the plant chassis of *Marchantia paleacea*. ACS Omega **5:** 33028-33038.

Zhang, Y. H. (2011) Substrate channeling and enzyme complexes for biotechnological applications. Biotechnol. Adv. **29:** 715-725.

Zhao, C., Kim, Y., Zeng, Y., Li, M., Wang, X., Hu, C., Gorman, C., Dai, S. Y., Ding, S. Y., and Yuan, J. S. (2018) Co-compartmentation of terpene biosynthesis and storage via synthetic droplet. ACS Synth. Biol. **7:** 774-781.

Zhao, E. M., Suek, N., Wilson, M. Z., Dine, E., Pannucci, N. L., Gitai, Z., Avalos, J. L., and Toettcher, J. E. (2019) Light-based control of metabolic flux through assembly of synthetic organelles. Nat. Chem. Biol. **15:** 589-597.

Zhao, M., and Qu, H. (2009) Human liver rate-limiting enzymes influence

metabolic flux via branch points and inhibitors. BMC Genomics **10** Suppl 3**:** S31.

Zhao, S., Tuan, P. A., Li, X., Kim, Y. B., Kim, H., Park, C. G., Yang, J., Li, C. H., and Park, S. U. (2013) Identification of phenylpropanoid biosynthetic genes and phenylpropanoid accumulation by transcriptome analysis of *Lycium chinense*. BMC Genomics **14:** 802.

Zhong, Y., Li, L., Hao, X., Fu, X., Ma, Y., Xie, L., Shen, Q., Kayani, S., Pan, Q., Sun, X., and Tang, K. (2018) AaABF3, an abscisic acid-responsive transcription factor, positively regulates artemisinin biosynthesis in *Artemisia annua*. Front. Plant Sci. **9:** 1777.

Zhu, Q., Yu, S., Zeng, D., Liu, H., Wang, H., Yang, Z., Xie, X., Shen, R., Tan, J., Li, H., Zhao, X., Zhang, Q., Chen, Y., Guo, J., Chen, L., and Liu, Y. G. (2017) Development of "purple endosperm rice" by engineering anthocyanin biosynthesis in the endosperm with a high-efficiency transgene stacking system. Mol. Plant **10:** 918-929.

Zhu, Q., Zeng, D., Yu, S., Cui, C., Li, J., Li, H., Chen, J., Zhang, R., Zhao, X., Chen, L., and Liu, Y. G. (2018) From golden rice to aSTARice: Bioengineering astaxanthin biosynthesis in rice endosperm. Mol. Plant **11:** 1440-1448.

Ziegler, J., and Facchini, P. J. (2008) Alkaloid biosynthesis: Metabolism and trafficking. Annu. Rev. Plant Biol. **59:** 735-769.

Zvi, M. M. B., Shklarman, E., Masci, T., Kalev, H., Debener, T., Shafir, S., Ovadis, M., and Vainstein, A. (2012) PAP1 transcription factor enhances production of phenylpropanoid and terpenoid scent compounds in rose flowers. New Phytol. **195:** 335-345.

FIGURES

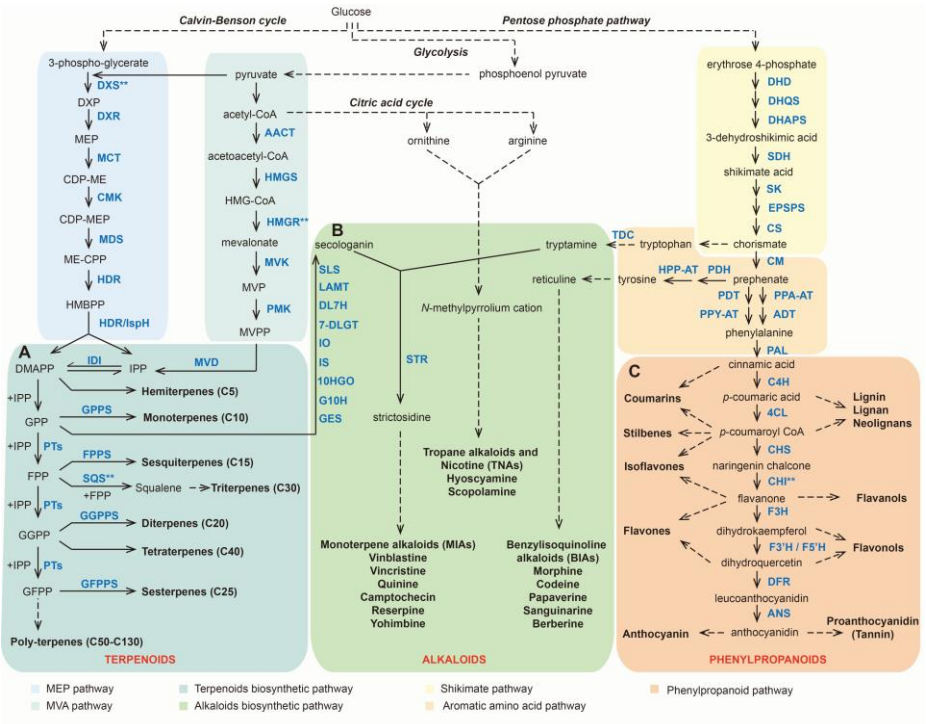

Figure 1. The biosynthetic pathway of three major classes of secondary metabolites (terpenoids (**A**), alkaloids (**B**) and phenylpropanoids (**C**)) **in plants**

The catalytic enzymes are shown in blue, intermediates in black, and the desired products in bold black. Solid arrow represents one step reaction, and dashed arrow represents multiple steps. Rate-limiting enzymes are marked with two asterisks. Abbreviations: DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1deoxy-D-xylulose 5-phosphate reductoisomerase; MCT, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; CMK, 4-(cytidine 5'diphospho)-2-Cmethyl-D-erythritol kinase; MDS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS, 4-hydroxy-3-methylbut-2-enyl-diphosphate synthase; HDR/IspH, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; AACT, acetyl-CoA acetyltransferase; HMGS, hydroxymethylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MVK, mevalonate kinase; PMK, phosphomevalonate kinase; MVD, diphosphomevalonate decarboxylase; IDI, isopentenyl diphosphate isomerase; GPP, geranyl diphosphate; GPPS, GPP synthase; FPP, farnesyl diphosphate; FPPS, FPP synthase; SQS, squalene synthase; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; GFPP, geranylfarnesyl diphosphate; GFPPS, GFPP synthase; GES, geraniol synthase; G10H, geraniol 10-hydroxylase; 10HGO, 10-hydroxygeraniol dehydrogenase; IS, iridoid synthase; IO, iridoid oxidase; 7-DLGT, 7-deoxyloganetic acid glucosyltransferase; DL7H, 7-deoxyloganic acid hydroxylase; LAMT, loganic acid methyltransferase; SLS, secologanin synthase; TDC, tryptophan decarboxylase; STR, strictosidine synthase; DHD, dehydroquinate dehydratase; DHQS, 3-dehydroquinate synthase; DAHPS, 3-deoxy-D-arabino-heptulosonate7-phosphatesynthase; SDH, shikimate dehydrogenase; SK, shikimate kinase; EPSPS, 5-ENOLYPYRUVYLSHIKIMATE 3-PHOSPHATE SYNTHASE; CS, chorismate synthase; CM, chorismate mutase; PDH, prephenate dehydrogenase; HPP-AT, 4-hydroxyphenylpyruvate aminotransferase; PPA-AT, prephenate aminotransferase; PDT, prephenate dehydratase; PPY-AT, phenylpyruvate aminotransferase; ADT, arogenate dehydratase; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; CHS, chalcone synthese; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid-3′-hydroxylase; F5′H, flavonoid-5′-hydroxylase; DFR, dihydroflavonol reductase; ANS, Anthocyanidin synthese.

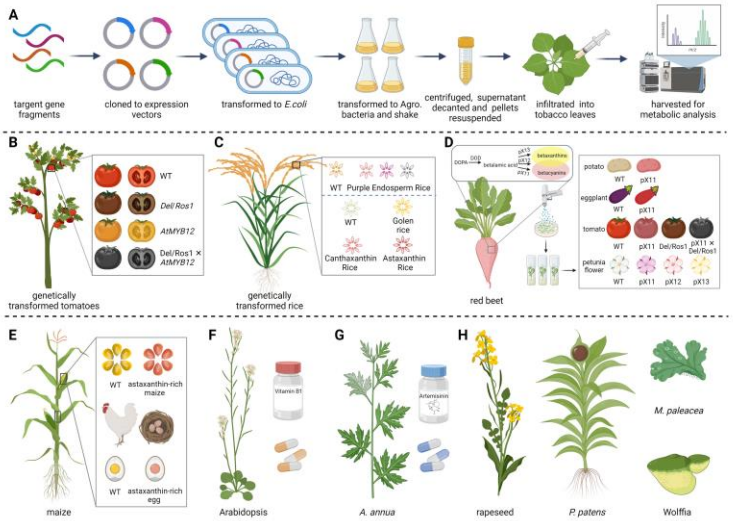

Figure 2. Plant chassis used for the production of natural products

(A) The workflow of transient expression of genes in tobacco using the Agrobacterium-mediated infiltration approach. **(B)** Enhanced phenylpropanoid production in tomato fruits by co-expression of *AtMYB12* with other transcription factors. **(C)** Pathway engineering to produce more anthocyanins (Purple Endosperm Rice), β-Carotene (Golden rice), canthaxanthin, and astaxanthin in rice endosperm. **(D)** Heterologous production of betalain biosynthetic pathway (from red beet) in tomato, potato, eggplant and tobacco flowers. **(E)** Engineered maize accumulate astaxanthin in seeds. Hens feed with astaxanthin-rich seeds laid astaxanthin-rich egg yolks. **(F)** *Arabidopsis* has been engineered to produce vitamin B1. **(G)** *A. annua* has been engineered to enhance the production of artemisinin. **(H)** Other promising plant chassis, including poplars, rapeseeds, *P. paten*, *M. paleacea* and wolffian.

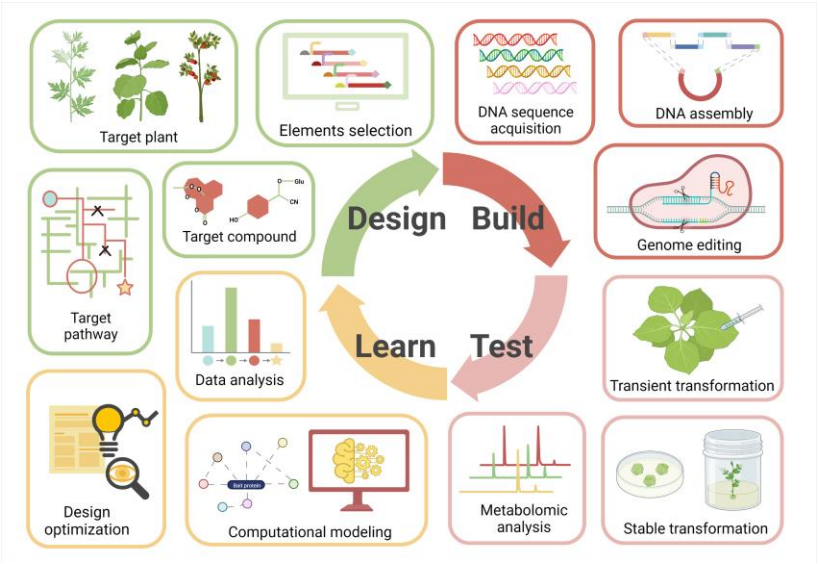

Figure 3. Schematic representation of the Design–Build-Test–Learn (DBTL) cycle in plant synthetic biology

In the "Design" phase, the targeted plant species, targeted compounds or the biosynthetic

pathway to be engineered or modified is selected and each of the necessary building blocks are designed. In the "Build" phase, the genetic parts are synthesized and the constructs are assembled. In the "Test" phase, the constructs are transformed into the selected plant hosts, modified organism analyzed and data collected. In the "learn" phase, the data from the Test phase are analyzed, and used to predict or optimize the design in the next round of the DBTL cycle.

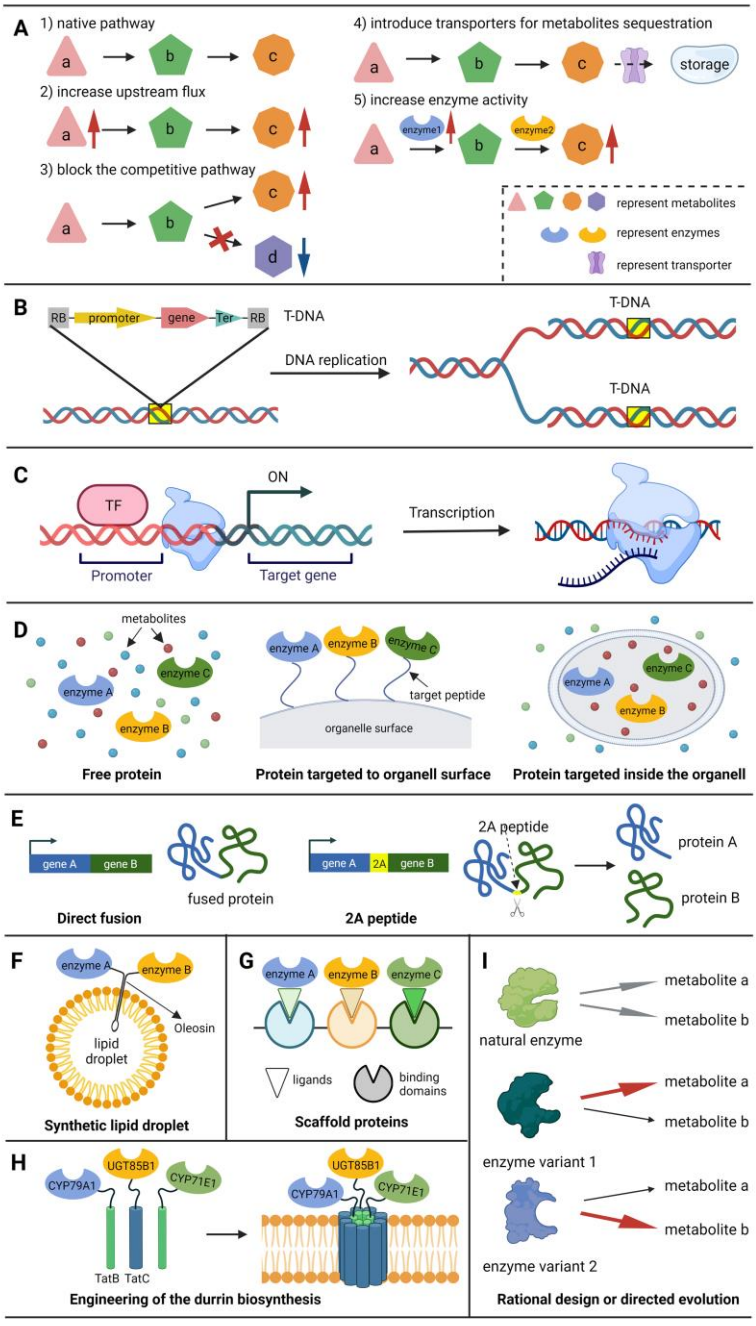

Figure 4. The synthetic biology-based strategies for engineering plants for the production of natural products

(A) The general strategies to enhance metabolite production. **(B)** Increase the total enzyme activity to improve natural product accumulation by genome editing at the DNA

level. **(C)** Engineering transcription factors and promoters to improve natural product production at the RNA level. **(D)** Compartmentalizing or rerouting biosynthetic enzymes to the same cellular compartment by modifying the targeting peptides. **(E)** Enzyme assembly by fusion protein and 2A peptide. **(F)** Co-localization of enzymes to artificial organelles, such as lipid droplets. **(G)** Enzyme assembly by using scaffold proteins. **(H)** Engineering the dhurrin biosynthesis by co-localizing pathway enzymes onto the thylakoid membrane of chloroplasts using subunits of the Tat complex (TatB and TatC) as protein scaffolds. **(I)** Rational design or directed evolution to improve the substrate specificity, stability, and catalytic activity of certain enzymes.

TABLES

Table 1. Comparison of using plants and micro-organisms as hosts for production of natural products

	Plants as hosts	Microorganisms as hosts
Pros	Rely on renewable atmospheric carbon, water, essential nutrients, and sunlight for efficient growth;	Simplicity of genetic modification;
	Readily available common enzyme cofactors and metabolic precursors;	Fast growth and short life cycles;
	Have differentiated cells and tissues, can be used for storage of assorted metabolites;	Controlled production condition;
	Partially shared pathways in different plant species facilitate pathway transfer;	
	Plant cell culture and hairy root culture provide a fast platform for production of natural products.	
Cons	Long life cycles;	Rely on exogenous carbohydrates (sugars) and energy;
	Require demanding growth conditions;	Lack of essential membrane compartments, protein misfolding;
	Difficulty in domestication;	Difficulty in correct expression of plant enzymes (e.g., P450s);
	Limitation in genetic modification;	Low tolerance of toxic products.
	Tight metabolic regulations and intricate feedback regulation mechanisms.	

Table 2. Commonly used rate-limiting enzymes for enhancement of compounds production in plant metabolic engineering

Classes	Secondary metabolites	Enzymes	Approach	Outcomes	Species/ tissue	References

			Overexpression of endogenous *PgHMGR1*	Enhancing the production of ginsenosides to 2-fold	*P. ginseng* adventitious root cultures	Kim et al., 2014
	Sesquiterpenes and triterpenes	*HMGR*, 3-hydroxy-3-methylglutaryl-CoA reductase	Transient co-expression of a truncated *tHMGR* with β-amyrin synthase *SAD1*	Increasing β-amyrin production by 4-fold	Tobacco leaves	Reed et al., 2017
Terpenoids	Triterpenes	*SQS*, qualene synthase	Transient expression of an oat *SQS*	Elevating yields of β-amyrin by 2-3-fold	Tobacco leaves	Reed et al., 2017
	Monoterpenoids, diterpenoids, sesterterpenoids and tetraterpenoids	*DXS*, 1-deoxy-d-xylulose 5-phosphate synthase	Overexpression of an *Arabidopsis DXS*	Increasing accumulation of essential oils (mainly monoterpenes)	*Spike lavender*	Muñoz-Bertomeu et al., 2006
			Co-expression of a tomato *DXS* and a tobacco *GGPPS*	Increasing the production of diterpenoid cembratrienol by 3.5-fold	*N. tabacum*	Brückner and Tissier, 2013
	Flavonol	*CHI*, chalcone isomerase	Ectopic overexpression of a *Petunia CHI* gene in tomato	Increasing total flavonols in fruit peels by 78-fold	Tomato	Muir et al., 2001
Phenylpropanoids	Isoflavonoid	*IFS*, isoflavonone synthase	Overexpression of a *M. truncatula IFS*	Increasing the production of genistein glucoside up to 50 nmol/ g FW	Alfalfa (*Medicago sativa*)	Deavours and Dixon, 2005

	Anthocyanin	*DFR*, dihydroflavonol 4-reductase	Overexpression of a gerbera *DFR*, suppression of flavonol synthase *FLS* and flavonoid 3′-hydroxylase *F3'H*	Enhancing accumulation of pelargonidin-based anthocyanin	Tobacco	Nakatsuka et al., 2007
	trans-resveratrol	*StSy*, stilbene synthase	Overexpression of *StSy*	Trans-resveratrol was enhanced	Tomato	Giovinazzo et al., 2005
	MIAs	*TDC*, tryptophan decarboxylase; *SA1D*, anthranilate synthase	Overexpression of *OsTDC* and *OsSA1D*	Accumulation of serotonin and serotonin-derived indole compounds	Rice	Dubouzet et al., 2013
Alkaloids						
	BIAs	*BBE*, berberine bridge enzyme	overexpression of *BBE*	BIAs, such as macarpine, dihydromacarpine and dihydrochelilutine are increased	*Eschscholzia californica* root cultures	Park et al., 2003

Table 3. Examples of transcription factors applied for plant engineering

Type of transcription factors	Genes	Secondary metabolites	References
Myeloblastosis (MYB)	MYB-type R and MYB-type C1	Anthocyanin, kaempferol and flavanone naringenin	Lloyd et al., 1992; Bovy et al., 2002
	IbMYB1a	Resveratrol compounds	Jeong et al., 2016
	PAP1 and *PAP2*	Flavonoids and volatile terpenoids	Gatica-Arias et al., 2012; Maier et al., 2013
	MYB11, *MYB12* and *MYB111*	Flavonoids and volatile terpenoids	Stracke et al., 2007

Basic helix–loop–helix (bHLH)	*AabHLH1*	Artemisinin	Ji et al., 2014
	bHLH112	Artemisinin	Xiang et al., 2019
	AaMYC2	Artemisinin	Shen et al., 2016
	AaPIF3	Artemisinin	Zhang et al., 2019
	CrMYC2	Serpentine	Suttipanta et al., 2011
APETALA2/Ethylene-Responsive-Factor (AP2/ERF)	*AaORA*	Artemisinin	Lu et al., 2013; Ma et al., 2018
	ORCA2,	Catharanthine and vindoline	Liu et al., 2011
	ORCA3	Ajmalicine and serpentine	Peebles et al., 2009
	ORCA4	MIAs, especially tabersonine	Paul et al., 2017
	AaERF1, AaERF2	Artemisinin	Yu et al., 2012
	TAR1	Artemisinin	Tan et al., 2015
WRKY	*AaWRKY1*	Artemisinin	Han et al., 2014; Jiang et al., 2016
	AaGSW1	Artemisinin	Chen et al., 2017
	CrWRKY1	Serpentine	Suttipanta et al., 2011
Basic Leucine Zipper (bZIP)	*AabZIP1*	Artemisinin	Zhang et al., 2015a
	AaABF3	Artemisinin	Zhong et al., 2018
	AaTGA6	Artemisinin	Lv et al., 2019a
SQUAMOSA promoter-binding protein-like (SPL)	*AaSPL2*	Artemisinin	Lv et al., 2019b
Teosinte branched 1/Cycloidea/Proliferating cell factor (TCP)	*AaTCP14*	Artemisinin	Ma et al., 2018
Zinc finger transcription factor	*DOF4;2*	Phenylpropanoids	Skirycz et al., 2007
	ZCT1, *ZCT2*, and	Negative regulators of terpene indole alkaloids	Pauw et al., 2004

	ZCT3		
NAC-domain transcription factors	*NST1*, *NST3*, *VND6* and *VND7*	Phenylpropanoids	Kubo et al., 2005; Mitsuda et al., 2007
Mediator complex	MED5a and MED5b	Negative regulators of lignin biosynthesis	Bonawitz et al., 2014

Table 4. Examples of promoters used for plant engineering

Type of transcription factors	*cis*-regulatory elements	Origins	Applications	References
Trichome-specific promoters	Cembratrienol synthase (*pCBTS*) promoter	*Nicotiana sylvestris*	Expression of *TS* and *CS* specifically in *N. tabacum* trichomes increased the production of taxa-4(5),11(12)-diene and casbene, respectively.	Rontein et al., 2008; Tissier et al., 2013
			Coexpression of *NtCPS2* and the *NtABS* resulted in a yield of about 35 ug/g Z-abienol.	Sallaud et al., 2012
Tuber-specific promoter	Patatin	*Solanum tuberosum*	Overexpressing a potato α-copaene synthase gene enhanced sesquiterpene α-copaene in tubers up to 15-fold.	Morris et al., 2011
Secondary wall-specific promoter	Secondary wall NAC binding element (SNBE)	Artificial promoter	Drive the expression of *CCR1* gene in the *Arabidopsis ccr1* mutant, rescued its vascular collapse and increased biomass yield.	De Meester et al., 2018
	Cell wall cellulose synthase promoter (*pCesA4*)	*A. thaliana*	Coexpression of *DCS* and *CURS2* leads to production of curcumin in Arabidopsis.	Oyarce et al., 2019

Fruit-specific promoter	E8	*Lycopersicon esculentum*	Expression of *AtMYB12* enhanced accumulation of polyphenol in tomato fruits.	Zhang et al., 2015b

Table 5. Examples of metabolic engineering at the protein level

Classes	Strategy	Approach	Outcomes	References
Sesquiterpene	Signal peptides	Redirect cytosolic *PTS* and *FPPS* to plastids	Patchoulol and amorpha-4,11-diene enhanced more than 1000-fold in tobacco	Wu et al., 2006
Monoterpene	Signal peptides	*PaGDPS1* was targeted expressed to plastids, cytosol, or mitochondria respectively	Geraniol and geraniol-derived products were highest when transiently expressing plastid-targeted GES, followed by mitochondrial- and then cytosolic-targeted GES in tobacco	Dong et al., 2016
Diterpene	Signal peptides	Redirect diterpene biosynthesis from the plastidial MEP pathway to an engineered high-flux cytosolic MVA pathway	Momilactones, forskolin and taxadiene had increased about 10-fold in tobacco	De La Peña and Sattely 2021
Diterpene	Signal peptides	Redirect *T5αH*, and *CPR* to the chloroplast	The yields of taxadiene and taxadiene-5α-ol are 56.6μg/g and 1.3 μg/g FW, respectively, in tobacco	Li et al., 2019
Phenylpropanoid	Signal peptides	Bacterial prenyltransferases were introduced to cytosol, plastids and mitochondria of legumes	Prenylated polyphenols was obtained particularly by plastidic targeting	Sugiyama et al., 2011

Alkaloid	Signal peptides	Bacteria *Vitreoscilla hemoglobin* (*VHb*) was expressed in different subcellular locations of *H. niger* hairy roots	The content of hyoscyamine and scopolamine was highest with targeted plastidial expression	Guo et al., 2018
Sesquiterpene	Fusion proteins	Transient expression of *FDS* and *EAS* with a short peptide (Gly-Ser-Gly) linker	The production of capsidiol was increased in *N. tabacum*	Brodelius et al., 2002
Sesquiterpene	Fusion proteins	Transient expression of *FDS* and *ADS* with a short peptide (Gly-Ser-Gly) linker	The accumulation of amorpha-4,11-diene and artemisinin titer was increased in *A. annua*	Han et al., 2016
Triterpene	2A peptides	Coexpression of friedelin synthase and *tHMG1*, which linked by the 2A peptide of ERBV-1	Friedelin titers was comparable to monocistronic expression in yeast	Souza-Moreira et al., 2018
Cyanogenic glycoside	Scaffolding proteins, fusion proteins	Fuse two P450s with TatB and a soluble UGT to the transmembrane protein TatC via a flexible linker	The production of dhurrin was increased 5-fold in *N. benthamiana*	Henriques de Jesus et al., 2017
Sesquiterpene	Synthetic lipid droplets	Co-expression of α-bisabolol synthase with three genes (*DGAT1*, *WRINKL1* and *OLE1*) required to form lipid droplets	The accumulation of α-bisabolol was more than 17-fold in *N. benthamiana*	Delatte et al., 2018
Sesquiterpene and diterpenes	Synthetic lipid droplets, signal peptides	Fuse terpenoid enzymes to the lipid droplet surface protein	The yield of targeted diterpenoids was increased up to 2.5-fold in *N. benthamiana*	Sadre et al., 2019
Sesquiterpene	synthetic Lipid droplets, signal	Terpene enzymes and synthetic lipid droplet proteins were colocalized to	The yield of squalene is 2.6 mg/g FW in *N. tabacum*	Zhao et al., 2018

peptides chloroplast

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