

Medicinal Plants: Guests and Hosts in the Heterologous Expression of High-Value Products

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ABSTRACT

Medicinal plants play an important dual role in the context of the heterologous expression of high-value pharmaceutical products. On the one hand, the classical biochemical and modern omics approaches allowed for the discovery of various genes encoding biosynthetic pathways in medicinal plants. Recombinant DNA technology enabled introducing these genes and regulatory elements into host organisms and enhancing the heterologous production of the corresponding secondary metabolites. On the other hand, the transient expression of foreign DNA in plants facilitated the production of numerous proteins of pharmaceutical importance. This review summarizes several success stories of the engineering of plant metabolic pathways in heterologous hosts. Likewise, a few examples of recombinant protein expression in plants for therapeutic purposes are also highlighted. Therefore, the importance of medicinal plants has grown immensely as sources for valuable products of low and high molecular weight. The next step ahead for bioengineering is to achieve more success stories of industrial-scale production of secondary plant metabolites in microbial systems and to fully exploit plant cell factories' commercial potential for recombinant proteins.

Introduction

Plants have been utilized as remedies since antiquity. The earliest documented record of using plants as medicines dates back to the culture of the Neanderthal people (~ 60 000 BC). Some of the ancient knowledge of medicinal plants reached us owing to the written documents or books like *Ebers Papyrus* (1500 BC), *History of Plants* (Theophrastus; ~ 300 BC), *De Materia Medica* (Dioscorides; ~ 100 AD), and *Canon medicinae* (Avicenna; 1025 AD) [1].

Many of the current drugs originate from natural products [2]. Today, about 80% of the population in developing countries rely on herbal medicinal products as a primary source of healthcare and traditional medical practice [3]. The survey by Newman and Cragg [4] revealed that out of the 1602 new chemical entities approved as drugs between 1981 and 2019, 751 were with natural origin (i. e., “unaltered natural products, botanical drugs [defined

mixtures], natural product derivatives [or mimics] or made by total synthesis but the pharmacophore is from a natural product”).

Native plants are among the most common sources of bioactive natural products (► **Table 1**). For instance, 12 000 *Taxus brevifolia* trees were chopped down to provide the 2 kg substance necessary for the studies at the beginning of the Taxol study [5]. Therefore, a sustainable balance needs to be established between exploiting the plants as resources for natural products and environmental protection. Galantamine content reaches 0.1–0.2% based on the dry weight of daffodil bulbs. Although the total chemical synthesis is possible, galantamine is also produced currently from plants because its synthetic route is difficult and expensive. Furthermore, the last stages of the galantamine synthesis have to be executed carefully to avoid any health risk due to the sensitization potential of narwedine [6]. In another recent study, the price of ginsenosides obtained from *Panax* plants was rela-

► **Table 1** Plant-derived natural products of importance for the pharmaceutical industry (adapted from [2]).

Compound	Plant species	Need (to/y)	Price USD\$/kg	Reference
Artemisinin	<i>A. annua</i>	50–60	100	[109, 110]
Paclitaxel	<i>T. brevifolia</i>	0.5	26 000–38 000	[2]
Docetaxol	<i>T. brevifolia</i>	0.3	8200–43 200	[2]
Resveratrol	<i>V. vinifera</i>	10 000	600	[111]
Ajmalicine	<i>R. serpentina</i>	0.3	1500	[112]
Anthocyanins	<i>V. vinifera</i>	2.0	2000	[2, 113]
Vincristine	<i>V. minor</i>	0.8	350 000	[2, 113]
Colchicine	<i>C. autumnale</i>	5.0	6000	[2, 113]
Galantamine	<i>G. nivalis</i>	34*	50 000	[6, 114–116]
	<i>N. pseudonarcissus</i>			
	<i>L. aestivum</i>			
	<i>P. maritimum</i>			
Ginsenosides	<i>P. ginseng</i>	1500**	41 000	[7, 117]

* The annual demand is estimated, considering the forecast for Alzheimer's disease prevalence for 2020, prescription share, and the well-tolerated daily dose (See the corresponding literature references in the table above for more details); ** The overall demand per year is calculated based on the worldwide ginseng production and average ginsenoside content in roots (See the literature references in the table above for additional information)

tively more expensive (25–57 USD/mg) in comparison to the cost of yeast-produced ginsenosides (0.5–25 USD/mg) [7]. Numerous natural products are also derived from plant foods and have additional health benefits beyond their basic nutritional value, thus preventing a wide range of chronic diseases [8]. The increased pharmaceutical and nutraceutical demand for valuable plant natural products, in general, has propelled the advancement of alternative biomanufacturing solutions based on metabolic engineering and synthetic biology [9].

Plants have been used today to produce both low and high molecular weight compounds of medicinal importance through DNA recombinant technology. As a result, the experimental workflow takes different steps depending on whether low or high molecular substances need to be produced. However, the overall cellular engineering process is based on design, build, test, and learn iterations, named the DBTL cycle [10].

The literature search strategy employed in this review comprises the recent developments of heterologous expression related to medicinal plants. However, specific details concerning plant biosynthesis of secondary metabolites or the production of recombinant proteins in plants are not included in this survey due to space limitations. Therefore, the reader is also referred to several interesting reviews on the plant secondary metabolites [11–19] and the emerging field of molecular farming [20–22], respectively.

The first part of this review is focused on the production of small molecule compounds, and the second part centers on therapeutic proteins. The exemplary cases, for which most steps of the biosynthesis are already known, have been selected for the first part. This knowledge about the genes of interest encoding a partial or entire metabolic pathway in plants may also favor transferring the target metabolite production into a host organism and

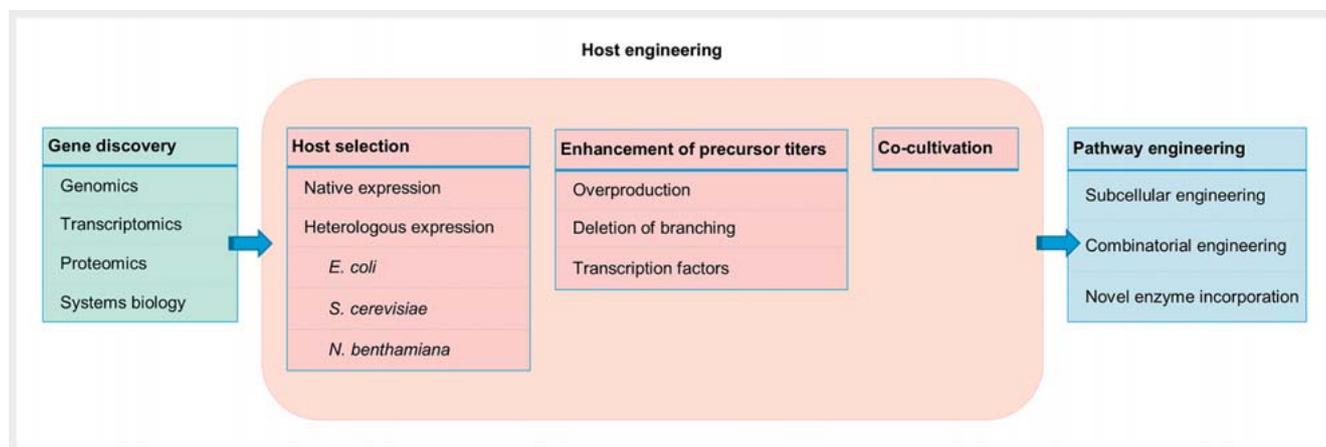
triggering potential industrial application. Several selected stories are included in the second part, showing the successful expression of recombinant proteins in plants. This review highlights the growing potential of medicinal plants for the biomanufacturing of high-value pharmaceutical products.

Approaches for Gene Candidate Discovery in Plant Secondary Metabolism

The metabolic reconstruction process goes through successive steps: gene discovery, host engineering, and pathway engineering (► **Fig. 1**). Therefore, the discovery of gene candidates is the first committed step in the manipulation of biosynthetic pathways.

Classical biochemical and recent integrative approaches based on *omics* technologies (genomics, transcriptomics, proteomics, and metabolomics) have contributed to identifying promising candidate genes that belong to plant metabolic pathways of pharmaceutical significance.

The classical (reductionist) approach involves isolation and purification of an unknown enzyme, followed by a protein-mass spectrometric analysis, screening of cDNA libraries to identify the corresponding gene, and functional assay with the metabolite of interest at the end. For example, such a traditional approach was applied for cloning and characterization of norcoclaurine synthase, an enzyme catalyzing the initial step in benzyloisoquinoline alkaloid (BIA) biosynthesis [23]. Degenerate primers were designed based on peptide sequences from the purified native norcoclaurine synthase (NCS). Next, the target nucleotide sequence was amplified by polymerase chain reaction using the aforementioned primers and a full-length cDNA isolated from a *Thalictrum flavum* cell culture as a template. The NCS enzyme is responsible



► **Fig. 1** Common steps and features of metabolic engineering.

for the condensation of dopamine and 4-hydroxyphenylacetaldehyde to (*S*)-norcoclaurine from which all BIAs are derived.

The reduced sequencing costs and bioinformatics tools advancements led to the increased quantity of whole-genome sequences of medicinal plants in recent years [24].

Genome-wide association studies (GWAS) are a highly effective means of gene discovery in model plants and, together with expression quantitative trait loci (eQTLs), may explain a substantial fraction of phenotypic variation [25]. The most significant agronomic traits (i. e., grain size [weight], grain number, cold/salt tolerance, disease resistance, etc.) are controlled by multiple genes (namely, QTLs) and are strongly dependent on the environment [26]. GWAS were performed for 14 agronomic traits in *Oryza sativa*, which led to the detection of loci that explain ~ 36% of the phenotypic variance. Furthermore, 6 loci were closely related to previously known genes. This study shows the power of the next generation of genome sequencing and GWAS for dissecting complex traits [27]. Metabolic gene clusters are a common hallmark in microbial genomes. Such functional gene clusters are also occasionally found in plant genomes. One example of this gene cluster phenomenon is the discovery of genes that encode the biosynthesis of monoterpenes and diterpenes in *Solanum lycopersicum* [28]. Notably, high-resolution co-expression analyses based on genome sequencing revealed a coordinated biosynthesis of 3 distinct clusters within the major components of the monoterpene-derived indole alkaloid (MIA) pathway in *Catharanthus roseus* [29]. Hence, genomic sequences mining is a valuable tool for identifying metabolic gene clusters and, thus, for a partial or entire biosynthetic pathway [24].

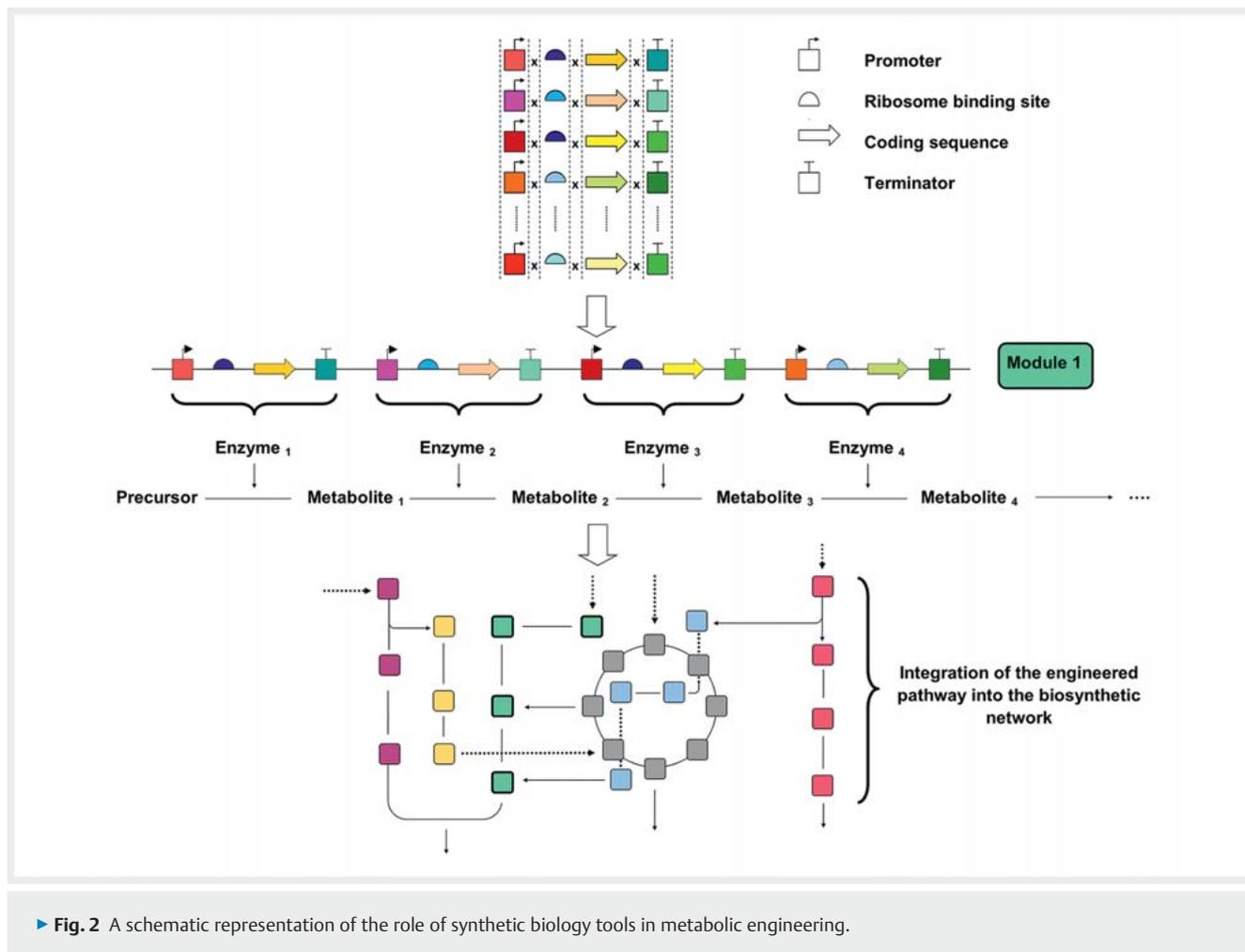
Transcriptomics or global transcriptome profiling has modernized the field of phytochemistry [30]. RNA-seq-based transcriptome analysis provides valuable information about the active metabolic processes. Unlike microarray analysis, it does not require preliminary genomic information, which is also of key importance for the functional characterization of non-model plants. The appropriate statistical method may also lead to the identification of candidate genes from the target biosynthetic pathway. RNA-seq-based transcriptome analysis, especially in combination with metabolite profiling data, is a powerful tool for gene discov-

ery. As an example, this approach enabled the detection of a locus with 10 clustered genes from the noscapine biosynthesis and virus-induced gene silencing validated the gene functions in the HN1 locus [31].

Proteomics can also facilitate gene discovery in biosynthetic pathways. Furthermore, it can contribute to the correct annotations of plant genomes. One example is the identification of the 4 enzymes in the secologanin pathway of *C. roseus* by using an integrated transcriptomics and proteomics approach. The whole strictosidine pathway was successfully reconstructed in a single *N. benthamiana* organ, despite its localization in various cell types like internal phloem-associated parenchyma or epidermis [32]. The screening of the pathogenesis-related 10 protein family, whose representatives are abundant in opium poppy latex, led to the discovery of neopinone isomerase, an important step in the biosynthesis of opiate alkaloids that was hypothesized in the past to be spontaneous [33].

Metabolomics is the principal tool for the unraveling of secondary metabolism in many plant families. Plant metabolomics is a complex methodology that investigates the global spectrum of natural compounds of plant origin with a molecular weight of less than 1000 Da, which are collectively named “metabolome” [34]. None of the genomics, transcriptomics, or proteomics can render any structural information about metabolic analytes. Several instrumentation or technological advancements are responsible for the key role of metabolomics in the gene discovery process of plant biosynthetic pathways; these include increased sensitivity, improved mass resolution, high-throughput automation, development of bioinformatics tools, and specialized online metabolite databases. Despite this progress, with metabolomics, it is not possible to measure the entire metabolome of a plant organism with a single analytical technique, unlike genomics and transcriptomics. However, this obstacle can be overcome by combining different separation techniques (gas chromatography, high-pressure liquid chromatography, and capillary electrophoresis) and detection systems (mass spectrometers, NMR, UV/VIS absorbance, fluorescence, IR absorbance).

The data from genomics, transcriptomics, proteomics, and metabolomics studies can also be integrated to obtain a global



► **Fig. 2** A schematic representation of the role of synthetic biology tools in metabolic engineering.

view of the response in a biological system. However, this systems biology approach encounters great technical challenges like scaling, noise removal, sensitivity, resolution, experimental design suitability, etc. [24]. Therefore, transcriptome-metabolome or metabolome-proteome integrations have been utilized more frequently for gene discovery in plant secondary metabolism.

Strategies for Reconstruction of Plant Biosynthetic Pathways

Synthetic biology is a future technology that may help preserve the environment through the sustainable production of natural plant products. While synthetic biology provides elements like promoters, coding sequences, terminators, transcriptional factors, binding sequences, etc., metabolic engineering uses all this information toward the optimized biosynthesis of the target metabolite [35]. Therefore, synthetic biology can significantly support metabolic engineering with its tools [36]. The synthetic biology approach frequently relies on the combination of components from diverse sources or species (► **Fig. 2**). This method redirects the biochemical resources of the organism to allow the efficient heterologous expression of the target metabolite(s).

The reconstitution of any plant biosynthetic pathway includes several key aspects for consideration. Once the target genes are discovered using classical or modern omics tools, the next step in any engineering strategy is identifying a suitable host organism. Several factors are important for selecting a host system: availability of techniques for cloning and culturing, suitability of a precursor pool, ease of cloning and culturing, suitability for an industrial scale-up application, etc. A first host option for producing valuable natural products with a plant origin potentially comprises plant cells or organisms, in which plant-specific subcellular compartments, substrate pools, protein processing, cofactors supply, and transcription regulation are probably conserved. Genetic modifications of plants are extremely difficult compared to microorganisms, which are often to prefer [37].

The heterologous hosts for the reconstitution of plant secondary metabolic pathways are recognized as a convenient and inexpensive alternative to the native producer. There are 3 major heterologous systems for accommodating plant metabolism: *Escherichia coli*, *Saccharomyces cerevisiae*, and *Nicotiana benthamiana*. *E. coli* is a well-characterized expression system with a shorter doubling period (3–4 times) than *S. cerevisiae*. However, some plant enzymes, such as cytochrome P450s, are transmembrane proteins, which can be problematic for prokaryotic hosts like *E. coli*. *S. cerevisiae* has several advantages: efficient homologous

recombination rates and cellular organelles as a eukaryotic microorganism and can express cytochrome P450s. *N. benthamiana* is amenable to transient and stable transformation and can be applied to express a lengthy biosynthetic pathway due to its gene-stacking feature [38].

The subsequent step after selecting a host organism is to increase the pool of biosynthetic precursors to better produce the molecules of interest. The enhanced substrate titers can be achieved by the overproduction of precursor flux, downregulation of undesired side pathways, or manipulation of transcription factors expression. Modifications in the central yeast metabolism increased the supply of the BIA precursor tyrosine, resulting in a 60-fold enhancement in the production of the early benzyloquinoline precursors. The next engineering steps led to the accumulation of the key intermediate reticuline. These reconstructed reticuline strains may serve as a production platform for the biosynthesis of various natural and novel BIAs [39]. In another study dedicated to strictosidine, a common precursor in the monoterpene indole alkaloids metabolism, 3 yeast genes were deleted to diminish the flux feeding of competing biosynthetic branches [40]. Transcription factors regulate the gene expression of entire metabolic pathways and deliver effective tools for engineering high metabolites levels. Two snapdragon transcription factors were overexpressed in tomatoes, and as a result, the fruit of the plants accumulated anthocyanins close to the concentrations in blackberries and blueberries [41].

Co-cultivation is an interesting host engineering strategy for the biosynthesis of a wide range of plant metabolites. For instance, reticuline, an important constituent in BIAs biosynthesis, was produced first in *E. coli* cells. Next, several BIAs were synthesized from reticuline in *S. cerevisiae* cells as some plant enzymes are not expressed properly in bacteria. Such combined systems can decrease the metabolic burden in the host from the heterologous pathway and are beneficial for the expression of plant enzymes localized in cytosol and the endoplasmic reticulum (ER) [42].

Once all enzymes are identified, combined, and introduced into the heterologous host, the multistep pathway should be validated. The transition to a functional biosynthetic route in a heterologous host is a challenging process. The reduced activity of a heterologous enzyme may be due to one of the following reasons: improper folding of the enzyme, misprocessing of posttranslational modifications, suboptimal pH, product feedback inhibition, etc. Subcellular engineering is one possible solution that can contribute to the proper intracellular localization of the enzyme, favorable pH and substrate conditions, etc. [37]. A new biosynthetic branch to neopine and neomorphine was found in an engineered yeast strain, carrying genes for opiates biosynthesis. This alternative route redirected pathway flux from morphine and other target metabolites. The main reason for the branching from morphine to nontarget neomorphine was the intermediate enzymatic step between thebaine 6-O-demethylase (T6ODM) and codeinone reductase (COR). Therefore, COR was targeted in the ER to allow a longer time for the spontaneous conversion of neopinone to codeinone and to enhance specificity for morphine compared to neomorphine biosynthesis [43]. The pathway manipulation by applying a combinatorial biosynthesis approach afforded novel compounds that are not found in the host, *P. somniferum*. Specifi-

cally, CYP82Y1 yielded 1-hydroxycanadine instead of the common product 1-hydroxy-N-methylcanadine when the preceding N-methyltransferase is not present.

Similarly, swapping of CYP82Y1 with CYP82X2 in the native cascade led to the biosynthesis of N-methylphiocarpine, an isomer of the native 1-hydroxy-N-methylcanadine [44]. Another study shows how unnatural compounds can be synthesized through novel enzyme integration into the native plant biosynthesis. A chlorination biosynthetic pathway from soil bacteria was inserted into *C. roseus*, yielding chlorinated tryptophan, which was then transferred into monoterpene indole alkaloid metabolism to produce chlorinated alkaloids [45].

Engineering of Plant Secondary Metabolic Pathways

The success in metabolic reconstruction depends largely on the thorough knowledge of the plant biosynthetic pathways. Therefore, genetic manipulations are successful mainly for secondary metabolic pathways with elucidated steps [24]. ► **Table 2** summarizes several exemplary natural products with their heterologous expression titers in yeast. This section presents examples of engineered plant metabolic pathways that are also heterologously expressed in microbial systems.

Benzoisoquinoline Alkaloids

The BIAs are a large group of biologically active compounds that attracted much attention to their pharmaceutical relevance and the development of microbial-based production systems. Moreover, most of the biosynthetic steps in the BIAs metabolism are unraveled so far. In one of the earliest studies on the reconstruction of a BIA pathway, transgenic *E. coli* cells, expressing a combination of 5 microbial and plant enzymes, produced (*S*)-reticuline from dopamine with a final yield of 55 mg/L within 1 h. Several types of BIAs, including magnoflorine (7.2 mg/L) and scoulerine (8.3 mg/L), were synthesized from reticuline in the second step by using *S. cerevisiae* cells because several plant enzymes were not properly expressed in bacteria [42]. Yeast cells were engineered to produce reticuline and downstream BIA metabolites from the common substrate norlaudanoline. The reticuline yields varied from \approx 10 to 150 mg/L depending on the enzyme combination. The yeast strains were also engineered to synthesize BIA metabolites along 2 of the major branches from reticuline: the sanguinarine and berberine branch and the morphinan branch [46]. Moreover, the *E. coli* fermentation system was constructed to yield (*S*)-reticuline at levels of 46 mg/L from simple carbon sources like glucose and glycerol without additional substrates [47, 48].

S. cerevisiae strains were engineered with genes from *P. somniferum* and *Pseudomonas putida* M10 to yield naturally occurring and semisynthetic opioids from the final steps of opiate biosynthesis. The fermentation production resulted in 131 mg/L total opioid levels. The development of this production platform is an important step toward sustainable opioid biomanufacturing in yeast [43]. In another study, gene discovery from opium poppy root and stem transcriptomes led to the reconstitution of a 10-

► **Table 2** Heterologous titers of selected plant natural products (adapted from [2]).

Compound	Host organism	Titer	Reference
Resveratrol	<i>E. coli</i>	1.4 g/L	[111]
	<i>S. cerevisiae</i>	5 g/L	[2]
Vanillin	<i>S. cerevisiae</i>	45 mg/L	[118, 119]
Naringenin	<i>S. cerevisiae</i>	474 mg/L	[120]
Dihydroartemisinic acid	<i>S. cerevisiae</i>	100 mg/L	[121]
Artemisinic acid	<i>S. cerevisiae</i> (+ semi-synthesis)	25 g/L	[122]
Morphine	<i>S. cerevisiae</i>	131 mg/mL	[43]
THCA	<i>S. cerevisiae</i>	2.3 mg/L	[62]
CBDA	<i>S. cerevisiae</i>	4.2 µg/L	[62]
Ginsenoside Rh2	<i>S. cerevisiae</i>	2.25 g/L	[66]

gene BIA cascade in yeast to yield dihydrosanguinarine and sanguinarine from the commercial substrate (*R,S*)-norlaudanosoline [49].

An enzyme-coupled biosensor for L-3,4-dihydroxyphenylalanine (L-DOPA) was developed to facilitate the finding of an active tyrosine hydroxylase in yeast (L-DOPA). Using this sensor and subsequent PCR mutagenesis, a highly active tyrosine hydroxylase was created with increased titers for L-DOPA (2.8-fold) and dopamine (7.4-fold). This innovative research fully recovered the 7-step pathway from L-tyrosine to (*S*)-reticuline [50]. The isomerization of neopinone to codeinone in opium poppy was formerly assumed spontaneous. However, it was found recently that this critical step in morphine biosynthesis is catalyzed by neopinone isomerase (NISO) [33]. Together with another recent discovery of thebaine synthase [51], NISO considerably increased codeine synthesis in yeast on account of neopine.

One comprehensive study showed that it is possible to biomanufacture thebaine and hydrocodone in yeast starting from sugar. As a result of this manipulation in opioid biosynthesis, yeast strains expressed 21 (thebaine) and 23 (hydrocodone) enzymes from plants, mammals, bacteria, and yeast itself. This synthetic biology approach emphasizes the potential of yeast as a production chassis for BIAs. However, rigorous improvements are still required to reach economically feasible levels of production [52].

Another study demonstrates that *E. coli* may also serve as a platform for opiates synthesis. Thebaine production based on 4 engineered strains and starting from glycerol achieved yields of 2.1 mg/L, which is a 300-fold increase from other yeast systems [53].

Although the cultivation of opium poppy is still the only source of morphinans, the utilization of genetically modified microbes as cell factories represents an increasingly valuable strategy in the biomanufacturing of *P. somniferum* alkaloids. All the studies above are viewed as proof-of-principle, and yet many efforts are needed to accelerate the development of heterologous systems that can

produce high-value BIAs at a commercial scale. Thus, biotechnological production may help to circumvent any uncertainties in the opiates supply due to seasonal, environmental or political factors and avoid any illicit use of this clinically important class of plant-derived narcotic analgesics.

Monoterpenoid indole alkaloids

The MIAs represent a class of plant secondary metabolites with more than 2000 compounds with pharmacological activity [54]. The alkaloids with antineoplastic properties from this large class of compounds, vinblastine and vincristine, are found in trace amounts in the leaves of *C. roseus* (Madagascar periwinkle, family Apocynaceae).

These valuable alkaloids are formed through condensation from 2 precursors: catharanthine and vindoline. The MIA biosynthesis is quite complex because it includes 31 enzymatic steps from geranyl pyrophosphate (GPP) [9]. Furthermore, the MIA metabolism is localized in different plant tissues (phloem-associated parenchyma, epidermis, mesophyll, laticifer) and subcellular organelles (i.e., plastids, nucleus, ER, and vacuoles) [54–56]. The production of the MIAs in yeast consists of 3 parts: the early stage (from the plastid methylerythritol 4-phosphate (MEP) pathway to the central metabolite strictosidine), the central stage (from strictosidine to tabersonine or catharanthine), and the final stage (the conversion of tabersonine to vindoline) [57].

It has been comprehensively shown that strictosidine can be synthesized *de novo* in an *S. cerevisiae* from 14 known MIA pathway genes. Seven gene additions and 3 gene deletions boost the MIA secondary metabolism [40]. This yeast system accumulated strictosidine at levels of ≈ 0.5 mg/L. Despite the low titers of strictosidine in this yeast strain, the reconstruction of the early stage from MIA metabolism is a critical first step toward the overall heterologous production of vinblastine and vincristine. Furthermore, this heterologous system can be used to examine the differential intracellular compartmentalization of MIA biosynthetic enzymes and its impact on production levels. For example, strictosidine synthase is located in the vacuole, and geraniol synthase is expressed in the chloroplast of plant cells. Therefore, the truncated versions of both genes without a localization signal sequence were used to circumvent potential bottlenecks regarding intracellular membrane transport.

After completing the first stage of the MIA biosynthesis, several bridging enzymes, which form the middle part of the MIA multistep biosynthesis, have been identified. Six genes were described and functionally characterized in yeast, resulting in the conversion of 19*E*-geissoschizine to O-acetylstemmadenine [58]. Both missing steps to tabersonine were elucidated independently by 2 research groups [59, 60].

The identification of tabersonine 3-oxygenase (T3O) and tabersonine 3-reductase (T3R) completes the molecular and biochemical characterization of the only remaining unknown reactions in the tabersonine-to-vindoline pathway (i.e., in the terminal MIA pathway stage). T3O and T3R were expressed in yeast together with 5 formerly identified genes from *C. roseus* to yield 1.1 mg/L of vindoline after feeding with tabersonine [61].

The identification of these missing components secured the framework for the future reconstruction of the whole MIA path-

way. Hopefully, a fully *de novo* pathway to vinblastine in a proto-type will be available soon in microbial systems, followed by a further improvement of end-product titers [57].

Cannabinoids

Cannabinoids have emerged as an attractive target for microbial biosynthesis due to their growing pharmaceutical significance. The entire biosynthesis of the major cannabinoids was realized heterologously for the first time in *S. cerevisiae* from the simple sugar galactose [62]. This reconstitution utilized the yeast hexanoyl-CoA biosynthetic machinery, to which was introduced a series of *C. sativa* genes, encoding a tetraketide synthase, an olivetolic acid cyclase, and an acyl-activating enzyme to produce olivetolic acid (OA). The crucial step in the reconstruction was the formation of the intermediate from the central hub in the cannabinoid biosynthesis: cannabigerolic acid (CBGA). CBGA is synthesized through the coupling of OA and the mevalonate acid (MVA) pathway intermediate GPP. Additionally, a GPP-overexpressing strain and a mutant version of the endogenous farnesyl pyrophosphate synthase *ERG20* were constructed to produce GPP.

However, the patented enzyme from *C. sativa* (CsPT1), responsible for this step, failed to show any activity. Therefore, other genes with a similar function from other organisms were screened. Among those potential candidates was also a soluble prenyltransferase NphB from *Streptomyces* sp. strain CL190, previously used to replace the native CBGA synthase from *C. sativa* [63]. The transcriptome mining ended up with a gene candidate whose truncated version yielded 1.4 mg/L CBGA from galactose. The *Cannabis* synthases, responsible for the terminal steps from CBGA to tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), were expressed with a vacuolar localization tag to enable accumulation levels of 2.3 mg/L THCA and 4.2 µg/L CBDA.

Despite the low cannabinoid levels in *S. cerevisiae*, this work represents a starting point for further optimization studies leading to commercial production of THCA and CBDA, which can be converted easily into tetrahydrocannabinol and cannabidiol after heat exposure. Additionally, the same research group members explored the opportunity to produce unnatural cannabinoids with modified side groups via the established cannabinoid pathway.

The glycosylation of cannabinoids represents another promising opportunity for the biosynthesis of unnatural cannabinoids with improved water solubility, bioavailability, and site-specific drug targeting. For instance, it was shown for the first time that the UDP-glycosyltransferases from *Stevia rebaudiana* and *Oryza sativa* were able to produce cannabinoid mono-, di-, tri-, and tetraglycosides *in vitro* [64].

Ginsenosides

Ginsenosides are glycosylated triterpenes isolated from the *Panax* species that exert various beneficial biological effects. However, it takes about 6 years for the roots to reach a harvesting stage and produce the commercial ginseng material. Therefore, microbial systems producing ginsenosides may become an interesting manufacturing alternative to the wild ginseng roots and plant cell cultures.

Ginsenosides are divided into 2 groups of natural compounds: dammarane (tetracyclic)-type and oleanane (pentacyclic)-type.

Dammarane-type ginsenosides are the major constituents that are obtained from 2 aglycones: protopanaxadiol (Rb1, Rb2, Rc, Rd, Rh2, and Rg3) and protopanaxatriol (Re, Rf, and Rg1) [65].

The highest reported ginsenoside aglycone titer in a heterologous system was obtained recently in yeast for protopanaxadiol: 529.0 mg/L of PPD in shake flasks and 11.02 g/L in 10 L fed-batch fermentation. This remarkable yield was achieved by overexpression of the MVA genes and optimization of the activities of cytochrome P450 enzymes in yeast. Moreover, the C3-OH glycosylation efficiency was improved to produce ginsenoside Rh2 by increasing the copy number of UDP-glycosyltransferase (UGT) Pg45, engineering its promoter, *in vivo*-directed evolution and searching for more active UGTs from other plant species. Following this optimization strategy, the yeast cell factory reached an outstanding Rh2 level of 179.3 mg/L in shake flasks and 2.25 g/L in 10 L fed-batch fermentation. This is the highest yield of ginsenoside Rh2 in engineered microbes as well as a glycosylated natural product. However, it is necessary to conduct additional pilot plant tests in larger fermenters to validate the commercial feasibility of this approach [66]. Furthermore, similar production levels still need to be achieved for the other ginsenoside types.

Paclitaxel

The diterpenoid paclitaxel (Taxol), an efficient therapeutic agent for treating several types of cancer, was isolated initially from the bark of Pacific yew (*T. brevifolia*) more than 5 decades ago. However, the accumulation levels of paclitaxel in yew plants are very low: about 0.01% of the dry weight of bark [67]. As a result, *T. brevifolia* has become a plant species in a near-threatened state due to its overharvesting [17]. Industrial production using total chemical synthesis is also hampered because of its complexity. Therefore, different strategies are employed to produce this valuable compound, such as plant cells cultivation, semi-synthesis, or metabolic engineering of microbial cells [68].

The manufacturing of paclitaxel on an industrial scale is directed toward the semi-synthesis from 2 precursors, baccatin III and 10-deacetylbaaccatin III, obtained from renewable sources like the needles of the Himalayan yew or *Taxus* plant cell cultures [69]. *Taxus* cell culture can also produce paclitaxel entirely, following methyl jasmonate elicitation, to reach accumulation levels of 110 mg/L [70].

Most steps from paclitaxel biosynthesis are known, and the corresponding enzymes and encoding genes have already been characterized in different heterologous systems. However, the metabolic pathway of paclitaxel is rather complicated; therefore, its alternative recombinant production remains still in its infancy [71]. There is a partial success only in the initial part of the paclitaxel biosynthesis to taxadiene. The highest taxadiene titer reported so far is about 1 g/L in fed-batch bioreactor fermentation with *E. coli* after engineering the native upstream MEP-pathway (forming isopentenyl pyrophosphate) and the heterologous downstream pathway (forming terpenoid). However, when taxadiene-5 α -hydroxylase (T5 α H), responsible for the oxidation of taxadiene to taxadien-5 α -ol, was introduced in the next step, the titers reduced significantly to 50 mg/L of taxadiene-5 α -ol [72].

Therefore, the research efforts in recent years are focused on resolving the T5 α H bottleneck. Different approaches were applied

to overcome this obstacle (i. e., optimization of T5 α H expression, improvement of the interaction with cytochrome P450 reductase, intracellular compartmentalization, the use of riboregulated switchable feedback promoters, etc.). The latest progress in transcriptomic data mining and the expected assembly of a high-resolution genome of *Taxus* species soon may also contribute to overcoming the T5 α H bottleneck and reconstituting the entire paclitaxel biosynthesis [73].

Heterologous Expression of Therapeutic Proteins in Plants

Plants have been used traditionally either as a commercial source of valuable end-up natural products or as a genetic pool for elucidation of secondary metabolic steps with a subsequent heterologous expression in microbial systems. The advancements in DNA recombinant technology in the past 3 decades also allowed biomanufacturing of therapeutic proteins in plants. Thus, plants became a beneficial production platform for antibodies, vaccines, human blood products, and growth regulators. As a result, a new applied field emerged named “plant molecular farming”. Some authors prefer using the term “plant molecular pharming” (PMP) or even “biopharming” as the majority of these recombinant proteins are plant-derived pharmaceutical proteins or biopharmaceuticals [22, 74, 75].

The most frequently utilized platforms for protein production nowadays are Chinese hamster ovary cells (CHO) and *E. coli*, followed by *S. cerevisiae* and murine myeloma cells [76]. However, plant-based systems have several major advantages over the traditional prokaryotic and eukaryotic protein production systems concerning manufacturing rapidity, cost, and safety. Prokaryote cells are generally used for the recombinant expression of small proteins (< 30 kDa); the fully formed large proteins are easier produced in eukaryote systems such as plants [77]. Noneukaryotic cells display difficulties in producing the correct folding of human proteins, and insoluble protein formation can be observed when the target protein is overexpressed. Plants can correctly fold and assemble full-size immunoglobulins and even secretory antibodies [78].

The posttranslational modifications (PTMs), such as the formation of covalent bonds, disulphide bridges, glycosylation, etc., are essential for protein functionality. Plants can introduce such PTMs that may affect several therapeutic proteins like serum half-life, immunogenicity, effector function, and solubility [79]. The glycosylation capability of plants is an advantage compared to prokaryotic expression systems. The glycosylation capacity might be limited, even in insect and yeast cells [80]. However, the recombinant proteins expressed in plants lack the human type of glycosylation with terminal sialic acid residues and display a typical β 1,2 xylose and α 1,3 fucose pattern of glycosylation, which may provoke an immune response when administered intravenously [81]. The CRISPR/Cas9 system has been recently applied to introduce mutations into 2 copies of *N. benthamiana* *XylT* [82]. Transcription activator-like effector nucleases (TALENs) have also been used to knockout *XylT* and *FucT* genes in an attempt to reduce such an undesired immunogenic response [83]. The development of modern

genome editing tools may lead to the introduction of whole glycosylation pathways to produce therapeutic proteins in plants with defined human *N*- and *O*-glycan patterns [84]. The production of therapeutic proteins in plants also reduces the risk of potential contamination with animal pathogens (prions, viruses, and mycoplasmas).

The cost of PMP-products is only 0.1 % of mammalian cells and 2–10% of microbial systems [21]. The following plant systems for expression of foreign proteins are exploited: transgenic plants with nuclear/chloroplast transformed genomes, cell suspension cultures, and transient expression. The recombinant proteins can be targeted to various subcellular organelles or compartments such as ER, apoplast, cytosol, and chloroplasts to find the most appropriate cellular surroundings for their accumulation [85].

In summary, plant-based platforms are a favorable in-between production system that can produce larger therapeutic proteins (compared to microbial systems). They are easily scalable and cost-effective, with a lower risk of pathogens (compared to mammalian systems) and toxic contaminants [76]. Plant expression systems are unlikely to replace the golden standards in the industry for protein manufacturing, such as *E. coli* and CHO cells, because plants cannot yet compete with the yields of these well-established industrial systems. Other concerns are also the potential risk of environmental containment, the presence of nonhuman glycosylation, and the lack of regulatory approval. Therefore, PMP technologies for both upstream production and downstream processing have to be developed further to comply with the pharmaceutical industry's Good Manufacturing Practice (GMP) requirements. To achieve this goal, the diverse production systems and techniques used in PMP need to be consolidated to establish standardized procedures [86].

Examples from the PMP Showcase

Human growth hormone was the first-plant-derived recombinant therapeutic protein, expressed in sunflower and tobacco cells [87]. The first report about the efficient production and assembly of functional antibodies in transgenic plants occurred 3 years later. A functional murine full-size IgG1 antibody reached 1.3% of total leaf protein. The specific binding of the antigen recognized by these antibodies was similar to the antibodies derived by hybridoma technology [88]. Since then, many pharmaceutical proteins have been expressed and characterized successfully in plants.

The first breakthrough product in the PMP field is the experimental drug called ZMapp, which showed immunological activity during the 2014 Ebola outbreak in West Africa. The plant production technology for therapeutic proteins attracted attention when 5 from 7 health aid workers survived this epidemic after receiving ZMapp [89]. ZMapp is an optimized combination from 2 previous antibody cocktails, ZMAB (consisting of murine monoclonal antibodies (mAbs) m1H3, m2G4, and m4G7) and MB-003 (consisting of human or human-mouse chimeric mAbs c13C6, h13F6, and c6D8). To extend the antibody half-life in humans and to facilitate clinical acceptance, the ZMAB components were first chimerized and then produced in *N. benthamiana* together with MB-003 candidates to select the final therapeutic combination. The end-cocktail represented a triple combination of anti-

bodies (c13C6, c2G4, and c4G7), which was manufactured using the large-scale, GMP-compatible rapid antibody manufacturing platform and magnICON vectors [90–92]. ZMapp demonstrated 100% rescue of rhesus macaques when treatment is initiated up to 5 days post-challenge. These results secured future development of this cocktail against the Ebola virus for clinical use [93].

Anti-Ebola virus therapy was additionally optimized to the chimeric MIL77E mAb combination of only 2 antibodies, based on c13C6 and c2G4 mAbs from ZMapp, containing a few amino acids changes [94]. This cocktail conferred 100% protection in nonhuman primates infected with the Ebola virus and was produced in CHO cells. Although the MIL77E mAb combination is designed for future manufacturing in mammalian cells, the ZMapp story has contributed to the growing popularity and commercialization of PMP.

The transient expression system in *N. benthamiana*, based on viral vectors and the agroinfiltration technique with *Agrobacterium tumefaciens*, is currently the most preferred platform for producing recombinant proteins in plants. The key advantage of this recombinant DNA technology is the high levels of protein expression in plants, achieved within a short duration of time. ▶ **Table 3** summarizes selected PMP examples in different therapeutic areas, following transient expression in *N. benthamiana* plants.

The transient plant expression system can be employed for the rapid production of recombinant proteins at high yields to meet the sudden demand for the production of emergency vaccines during viral outbreaks [95]. Therefore, *N. benthamiana* can also become a promising host for vaccine manufacturing to fight the COVID-19 pandemic [96]. For instance, 2 companies with extensive PMP expertise registered clinical trials for COVID-19 vaccines presently. The clinical studies of Kentucky BioProcessing (ClinicalTrials.gov identifier: NCT04473690) and Medicago (ClinicalTrials.gov Identifier: NCT04636697) have estimated enrollment of 180 and 30,918 participants, respectively. The outcomes of these clinical trials may presumably determine whether other PMP players will also join the combat against this global pandemic.

Commercialization Examples

Different aspects (i. e., the levels of the biologically active natural product in the native producer, the daily intake dose by the patient, the economic feasibility of the manufacturing process, etc.) have been taken into consideration when the acceptable yield for the heterologous production of a certain low molecular compound is determined. In general, titers over 1 g/L are considered outstanding for any heterologous system, producing small molecules [38].

The assessment of yield optimization in a given heterologous production platform must be considered on a case-by-case basis for each natural compound of interest. For example, the reported hydrocodone titers in yeast are < 1 µg/L, while a single dose of hydrocodone, as used in Vicodin (5 mg), would require thousands of liters of fermentation broth. Furthermore, the conversion rate of thebaine to morphine is 1.5%. Therefore, the overall yield improvement of morphine production using such a yeast strain should be of a factor of $\approx 7 \times 10^6$ [52].

One landmark success in metabolic engineering is the complete biosynthesis of artemisinic acid in *S. cerevisiae*, yielding impressive fermentation titers of 25 g/L. Next, a semisynthetic approach was developed to convert artemisinic acid to artemisinin using a chemical source of singlet oxygen instead of specialized photochemical equipment. Based on yeast strain engineering, fermentation and artemisinin synthetic chemistry, this combined production technology paved the way for an industrial application of this valuable antimalarial drug and its independent supply from a botanical source [97]. As a result, the Sanofi company opened a manufacturing site in Italy for artemisinin production, which secured approximately one-third of the global annual demand in 2014 with its 55–60 tons [2].

The only PMP-product that has entered the market is ELEYISO (taliglucerase alfa) from Protalix BioTherapeutics (Israel). In 2012, the US Food and Drug Administration approved this plant-derived enzyme for replacement therapy of adult patients with Gaucher disease, a rare genetic disorder in which the patients fail to produce the enzyme glucocerebrosidase [98]. ELEYISO is produced in genetically engineered carrot cells and naturally contains terminal mannose residues on its complex glycans. Therefore, the plant-produced glucocerebrosidase does not need exposure of mannose residues *in vitro* as required for the market competitor Cerezyme produced in CHO cells [99]. The long-term safety and efficacy of taliglucerase alfa was proved by 6 clinical studies in adults and children with Gaucher disease [100].

Conclusion and Future Prospects

This review aims to show the growing potential of medicinal plants beyond their traditional application as a source of pharmaceutically important natural products. This new potential is also related to recombinant DNA technology. However, the past 30 years of application of DNA recombinant technology in plants have shown that the road to commercially viable titers is neither straightforward nor secure and that significant improvement is necessary before success is realized.

On the other hand, the progress made indicates that metabolic engineering and synthetic biology greatly impact the biomanufacturing of high-value products from plants [38]. The heterologous expression in host organisms represents an increasingly valuable and feasible strategy for exploiting the wide chemical diversity in nature without endangering plant biodiversity.

The recent advancements in high-throughput sequencing, genome editing tools like zinc-finger nucleases, transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9 system are very useful for manipulating secondary metabolism in plants [24]. These genome editing techniques may also enable precision engineering of the therapeutic proteins with bespoke glycan decoration and lead to increased accumulation levels of the target biopharmaceutical by knocking out the genes encoding plant proteases [101]. Cell-free synthesis systems can also be employed to manufacture various protein-based products or metabolites [102–105]. The product optimization based on a simultaneous global exploration of various factors may achieve more reproducible yields than assessing one factor at a time [106]. Such a systematic workflow may be used efficiently to improve both metabo-

► **Table 3** Selected examples of recombinant proteins expressed transiently in *N. benthamiana*.

Therapeutic target	Recombinant protein	Expression level	Reference
Cancer	ML-II lectin	60 mg/kg FW	[123]
	Rituximab	385 mg/kg FW	[124]
	BR55-2	30 mg/kg FW	[125]
Chikungunya virus	CHKV mAb	100 µg/g FW	[126]
Cholera	Cholera toxin B subunit	3.1 g/kg FW	[127]
	Cholera toxin B subunit	0.5–1.5 g/kg FW	[128]
Dengue virus	E60	120 µg/g FW	[129]
	cEDIII	5.2 mg/g FW	[130]
	cEDIII-Co1	4.8 mg/g FW	[130]
Ebola virus	6D8	1.21 mg/g FW	[131]
Erythropoietin	rhEPO	85 mg/kg FW	[132]
Fabry disease	α-GAL	71 nmol/h/µg TP	[133]
	α-NAGAL	5 nmol/h/µg TP	[133]
	α-NAGALEL	7 nmol/h/µg TP	[133]
Influenza	Hemagglutinin VLPs	50 mg/kg FW	[134]
	rHA0	0.2 g/kg FW	[135]
Hepatitis B virus (HBV)	HBV surface antigen	295 µg/g FW	[136]
	HBV core antigen	0.2–1 mg/g FW	[137]
Human epidermal growth factor	hEGF	15.695 µg/g FW	[138]
Human immunodeficiency virus	HIV Env gp140	5–6 mg/kg FW	[139]
	VRC01 _{Fab} -Avaren	40 mg/kg FW	[140]
	CAP256-VRC26.08	489 mg/kg FW	[141]
	CAP256-VRC26.09	487 mg/kg FW	[141]
Herpes simplex virus	HSV8	1.42 mg/g FW	[131]
Malaria	CCT	2 mg/g FW	[142]
Rabies	E559/62–71–3 mAbs	490 mg/kg FW	[143]
SARS-CoV-2	mAb B38	4 µg/g FW	[144]
	mAb H4	35 µg/g FW	[144]
	ACE2-Fc	100 µg/g FW	[145]
	ACE2-Fc	≈ 80 µg/g FW	[146]
	CR3022 mAb	130 µg/g FW	[147]
West Nile virus	pE16	0.74 mg/g FW	[148]
	pE16scFv-CH	0.77 mg/g FW	[148]
	DIII	73 µg/g FW	[149]
	HBcAg-wDIII	1.2 mg/g FW	[150]
	E16 mAb	339.9 µg/g FW	[151]
Zika virus	c2A10G6	1.47 mg/g FW	[131]
	IgG-ZE3	1.5 mg/g FW	[152]
	ZIKV E	160 µg/g FW	[153]

FW – fresh leaf weight; TP – total protein

olite or protein product levels in any heterologous expression system [107, 108].

All these approaches may contribute to further optimization. We hope to see in the near future more industrial examples based on the recombinant DNA technology for manufacturing pharmaceutically valuable products with plant origin.

Contributors' Statement

N.A.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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