Metabolic Engineering of Medicinal Plants and Microorganisms for the Production of Natural Products

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19.1
Introduction

Plants are a rich source of bioactive compounds. Compounds of plant origin are used as drugs and precursors of semisynthetic drugs, and may provide valuable leads for novel drug design. Furthermore, plant extracts have been and are still used to prevent, and to treat, a number of diseases although the mechanism of action is frequently unknown. Finally, there is a global demand for "greener" manufacturing processes, which are economically attractive, to be available in a timely manner [1].

Worldwide more than 50 000 plant species are used for medicinal purposes [2]. The World Health Organization (WHO) estimated that more than 80% of the population in the world in less developed countries depend primarily on herbal medicine for basic healthcare needs [3]. The current herbal drug market has reached a level of US$62 billion, which is forecast to grow to US$5 trillion in 2050 [4]. The world market for herbal medicines shows an annual growth of 5–15% [5]. In the United Kingdom, more than 25% of the population use herbal medicines on a regular basis [3].

In the past 30 years, more than 25% of the new drug entities approved were based on a molecule of plant origin and about one third of the approximately 980 new pharmaceuticals originated from, or were inspired by, natural products [6, 7]. About 50% of the top-selling chemicals are derived from knowledge of plant secondary metabolism [7]. About 40% of the pharmaceuticals in the United States and Europe use plants as raw source material [8].

Besides plants and plant extracts, pure compounds derived from plants play an important role in contemporary pharmacy and medicine. Typical plant compound (Table 19.1 and Figure 19.1) commonly used drugs are terpenoids, alkaloids, polyketides, phenylpropanoids, and flavonoids. For examples, morphine and codeine from *Papaver somniferum* L., artemisinin from *Artemisia annua* L., paclitaxel from *Taxus brevifolia* Nutt, genistein from *Glycine max* L. (Merr.), scopolamine from *Dubosia species*, campothecin from *Camptotheca acuminata* Decne, and podophyllotoxin from *Podophyllum* species.

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Table 19.1 Overview of the production of the plant-derived and medicinally relevant compounds with their metabolic engineering strategies.

Compound	Activity/ function	Plants source	Demand (tons/yr)	Current production	Price US$/kg	Reason for combinatorial biosynthesis	Ref.
Dihydroartemisinic acid (Terpenoid)	Anti-malarial	*Artemisia annua* (0.01–0.86% in aerial parts)	120	*E. coli*	1000	Availability in nature is limited Chemical synthesis–economically not feasible	[9]
Paclitaxel (Terpenoid)	Antitumor	Taxus species	0.3	Plant cell culture Idem ESCA genetics (USA); Phyton Catalytic (USA/ Germany); Nippon Oil (Japan); Samyang Genex (Korea)	28000	Low production in nature Slowly growing tree The bark is non renewable–harvesting the bark results in the death of the tree Chemical synthesis–economically not feasible	[10–12]
Vanillin (phenylpropanoid)	Flavor	*Vanilla planifolia*	>10000	Chemical synthesis	12 (synthetic) 30–120 (vanilla pods)	Green manufacturing	[13]
Genistein (flavonoid)	Fiber	*Gossypium hirsutum*	36000	Isolation from plants	Unknown?	Cottonseed free from gossypol for protein source of human diet	

Podophyllotoxin (Lignan)	Antitumor	*Podophyllum* species (4.3% DW)	–	Plant cell culture (Nippon oil – Japan) From which starting compound/	1490	Endangered species Chemical synthesis – economically not feasible	[10, 14]
Scopolamine (alkaloid)	Anticho-linergic	*Duboisia* species (1.2 –2.4% in leaves)	–	Transgenic hairy root culture of *Hyoscyamus niger* (HnH6H and NtPMT) – 411.2 mg/l *Duboisia* sp. Sumitomo Chemical Industries (Japan)	Unknown?	Multiple chiral centers – chemical synthesis – economically not feasible	[10, 15, 16]
Morphine (alkaloid)	Analgesic	*Papaver* (20% DW in latex; 1.23–2.45 % DW whole plant extracts)	27.8	Isolation from plants	Unknown?	Multiple chiral centers – chemical synthesis – economically not feasible	[12, 17]
Vincristin (alkaloid)	Antitumor	*Catharanthus roseus* (0.0003 % DW in whole plant)	0.3	Isolation of plants	1400	Multiple chiral centers – chemical synthesis – economically not feasible Higher yield	[18]

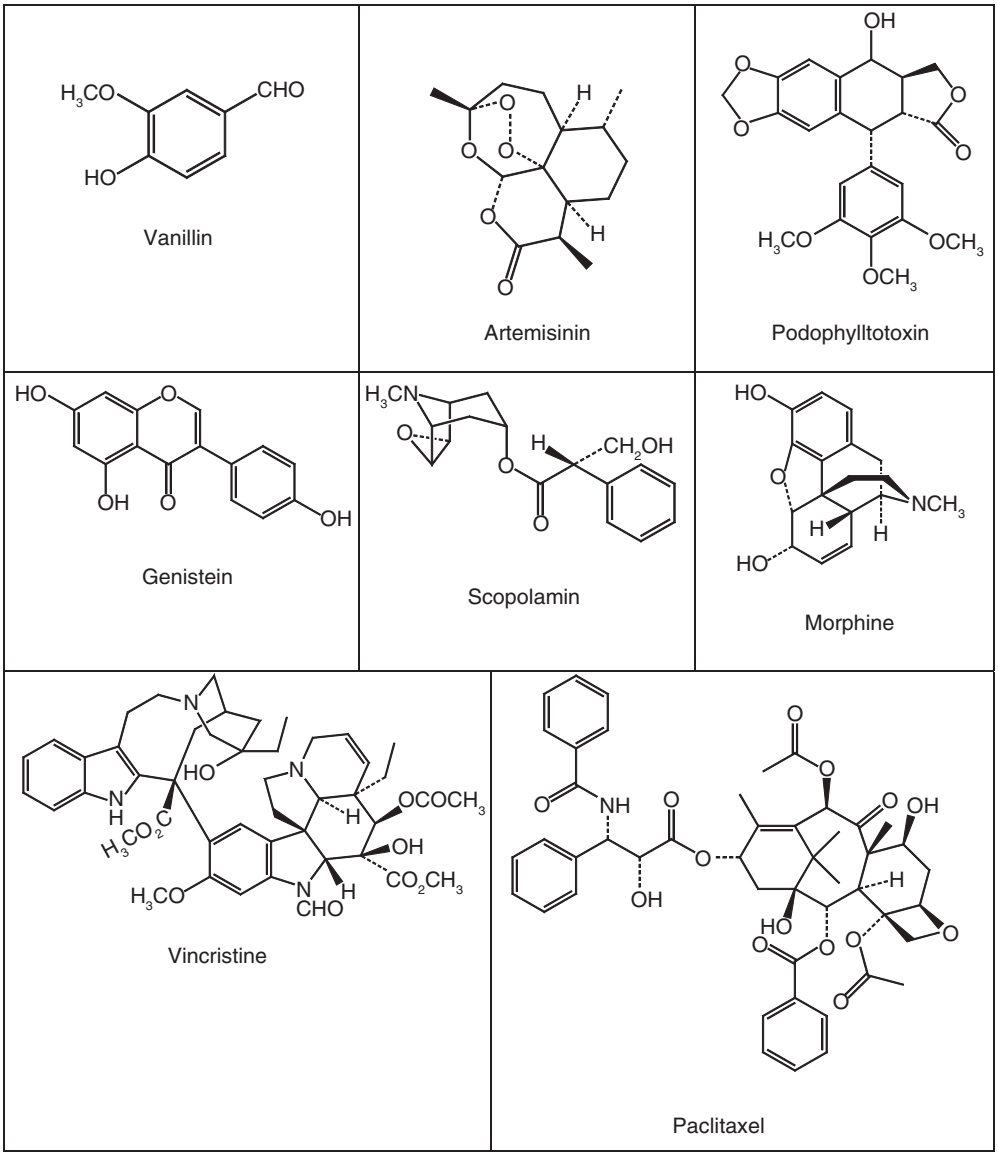

Figure 19.1 Chemical structures of the important plant- derived compounds discussed in this chapter.

Despite the use of and demand for plant-derived compounds, their availability is a major bottleneck in supplying the pharmaceutical needs. Most of these compounds are secondary metabolites, which are present only in low amounts from natural sources. Most medicinal plants are not cultivated, but are collected from the wild and some of them are slow growing. Because of intensive collection from the wild, the current extinction rate of medicinally used plants is estimated

to be 100–1000 times higher than for other plants. As many as 15 000 out of 50 000–70 000 medicinal plant species are now threatened with extinction [19]. Currently between 4000 and 10 000 medicinal plants are on the endangered species list and this number is expected to increase in the future [20].

There are a number of limitations to obtaining plant-derived compounds. They may be restricted to one species or genus, and might be formed only during a particular stage of growth or development or under specific seasonal, stress or nutrient availability conditions [21, 22]. Chemists have also been challenged to synthesize plant-derived compounds via organic chemistry. This is often hampered by the chemical complexity, specific stereochemistry, and the economic feasibility.

Metabolic engineering may offer prospects to overcome the lack of availability of such compounds, through the advancement of molecular biology techniques, including cloning, recombinant DNA, and knowledge of the plant biosynthetic pathways. In this chapter we discuss the major strategies in plant metabolic engineering and their principle approaches and its prospects and limitations for the production of drugs and fine chemicals. Case studies are used as illustrations.

19.2
The Plant as a Source of Natural Products

19.2.1
Plant Cell Cultures

In principle, whole plants, plant organs, and even single cells can be used for the production of natural products. Plant cell culturing was initiated in the 1930s [23] and could offer alternatives to improving the production of the secondary metabolites, as natural harvesting is sometimes bulky and not feasible from an economic point of view. The main advantages of plant cell culturing are easy up-scaling, simple purification schemes due to product secretion, environmental friendliness, and amenability to strict control with regards to meeting FDA manufacturing standards [24]. Plant cell cultures are also not subject to changes in environmental conditions, thus the production of the desired compounds could take place at any location and in any season [25].

In 1959, plant cells were first cultured in a 10 l glass or steel bioreactor [26] and later, in 1977, the first larger scale 20 l stirred tank bioreactor of *N. tabacum* cells was reported [27]. Today, undifferentiated plant cell suspension cultures can easily be scaled up for commercial production purposes, but the productivity is often hampered by the fact that the compounds of interest are not produced in the undifferentiated cells. Currently 14 plant cell cultures have been commercialized for secondary metabolites production for pharmaceutical, food, and cosmetic purposes [10, 28]. Examples are scopolamine from *Dubosia* sp. (Sumitomo Chemical Industries, Japan), ginsenosides from *Panax ginseng* (Nitti Denko, Japan), and paclitaxel from *Taxus* sp. (Phyton Biotech, USA and Samyang Genex, Korea) [10].

One success story of a plant cell culture produced drug is paclitaxel (Taxol® Bristol Myers Squibb). While the plant itself, *Taxus brefolia*, only produces paclitaxel at approximately 0.01% of the dry weight of the bark [29], the plant cell suspension culture has been shown to produce steadily in the region of 140–295 mg/l, reaching 295 mg/l at a maximum under two-stage culture with the elicitation of methyl jasmonate and high density conditions [11].

The main constraints of using plant cell cultures for the production of secondary metabolites include slow growth of plant cells in comparison with microorganisms, no accumulation of the desired metabolites in undifferentiated cultures, compartmentalization of the production of secondary metabolites, low and variable yields, and the decrease of metabolite accumulation as the cell line ages [24, 30–32]. Differentiated cells produce the same product as the plant itself, but in large-scale production, when aiming at an economically attractive route, the yield remains a bottleneck, especially for slow-growing plants. A variety of approaches, such as the growth of differentiated cells (root and shoot culture) and the induction of pathways by elicitors have had limited success so far [22]. The plant production of secondary metabolites is controlled in a tissue-specific manner, thus the dedifferentiation results in loss of production capacity [22] and undifferentiated cell cultures that are genetically unstable, and often lose, partially or totally, their ability to produce secondary products [33, 34].

For example, artimisinin, a potent antimalarial drug, was not found in cell suspension cultures of *Artemisia annua*, while considerable amounts were detected in shoot cultures [35, 36]. Deoxypodophylltoxin, the main lignan in *Anthriscus sylvestris* was also detected in trace amounts in callus and cell suspension cultures [37].

19.2.2 Transgenic Plants

In 1907, Smith and Towsend reported on the cause of crown gall disease of paris daisy (*Chysanthemum frustescens*) by *Bacterium tumefaciens*. Later on the bacterium was classified as *Phytomonas tumefaciens*, and finally as *Agrobacterium tumefaciens*, a gram-negative soil dwelling bacterium [38]. At the end of the 1970s, it was reported that the T-DNA of this microorganism was covalently integrated into the plant nuclear genome in tobacco teratoma cell lines [39, 40]. This has led to many studies to date. Since 1994, transgenic technology has been used and commercialized to produce new crop products with herbicide tolerance, insect resistance, virus resistance, and improved post-harvest quality [41]. Transgenic approaches can be applied to target a rate-limiting step through manipulation of the expression of individual structural genes [5].

There are two transformation approaches commonly used to produce recombinant pharmaceuticals in plants. The first is to subject plants to *Agrobacterium*-mediated transformation, particle bombardment, electroporation, and then secondly to infect plants with recombinant viruses that express transgenes during their replication in the host [42–45]. Genetic transformation of medicinal plants

is usually carried out using *Agrobacterium rhizogenes* to obtain hairy root cultures, or using *Agrobacterium tumefaciens* to produce transformed cells that can be maintained in cell cultures or can be regenerated as whole plants [2].

In principle, the wounded plant tissues, caused by insect or mechanical damage, produce phenolic compounds, which attract *Agrobacterium* by chemotaxis to infect the plant cell on the wounded site and allow the transfer of T-DNA from *Agrobacterium* into the plant nuclear chromosome. The T-DNA contains genes that encode enzymes directing the plant cells to produce peculiar amino acids called opines, and express genes to direct the plant cells to produce plant hormones such as cytokinin and auxin. Opines are used as primary sources of carbon and nitrogen by the cohabiting bacteria, and cytokinins and auxin promote cell division and tumor formation, providing a steadily increasing supply of nutrients for the bacteria [46]. The infection from *A. rhizogenes* in the wounded site will cause a number of small roots to protrude as fine hair growth and proliferate rapidly, causing hairy roots [47]. T-DNA carries the *rol* and *aux* genes. The *rol* genes are responsible for the phenotype of hairy roots and the *aux* genes are involved in root induction by directing auxin synthesis [48].

The major drawbacks of this approach are the unstable gene expression, instability of cell lines that often lose their capacity to produce target molecules over time, and high cost of bioreactors [2]. For example, the alkaloid accumulation in transgenic *Catharanthus roseus* cell cultures quickly returns to the level of the non-transgenic ones [49].

The capacity to regenerate whole plants from single cells without changing the genetic features of the cells and the gene transfer mechanism via *Agrobacterium tumefaciens* facilitates efforts to engineer secondary metabolic pathways [2, 50]. The constraint is the subsequent regeneration of transgenic plants, which remains problematic and time consuming. Unwanted somaclonal variation may be introduced through the tissue culture regeneration system in some cases [51].

Plants have been and are still used as hosts to produce genuine and recombinant proteins and enzymes of industrial and pharmacological value [52]. More than 200 novel antibody-based potential products are in clinical trials worldwide, and the market demand will constrain the capabilities of existing production systems [53]. One would expect the biopharmaceuticals from transgenic plants to be safer and less expensive than those from animal-based sources, which have the potential for contamination with human pathogens [42].

Enhanced productivity of valuable secondary plant metabolites can also be achieved via hairy root cultures [25]. Hairy root cultures can be obtained from transformed root cultures using *Agrobacterium rhizogenes*, a gram-negative soil dwelling bacteria. The term "hairy root" was introduced in 1900 [54] and the first transformation of higher plants using *A. rhizogenes* was achieved in 1973 [55].

Hairy root cultures are genetically stable, capable of unlimited growth without additional hormones, and have an increased capacity for secondary metabolites formation and accumulation [24]. Genetically transformed root cultures have been shown to produce levels of secondary metabolites comparable to those of an intact plant. It has further been shown that hairy root cultures can accumulate

secondary metabolites that normally occur only in the aerial part of the plant. An example is artemisinin in *Artemisia annua* [56]. The transformation of *Artemisia annua* using *A. rhizogenes* carrying the cDNA encoding FDS (farnesyl diphosphate synthase) under a 35S CaMV (cauliflower mosaic virus) promoter yielded fourfold higher artemisinin accumulation compared with untreated control plants [57]. The transformation of *Atropa belladonna* with H6H (hyoscyamine 6β-hydroxylase) from *Hyoscyamus niger*, under the control of the 35S CaMV promoter in a binary plasmid via *A. rhizogenes* mediated transformation, resulted in an accumulation of scopolamine up to fivefold higher compared with untreated control plants [58]. These examples indeed show that hairy root cultures are able to produce the same compounds as in the plant itself. Other examples of hairy root cultures producing secondary metabolites can be found in the work of Srivasta and Srivasta [25].

Another advantage is that transformed roots are able to regenerate whole viable plants, maintain their genetic regeneration, and in addition produce secondary metabolites that are not present in the parent plant [59]. Furthermore, they show fast auxin-independent growth and are suitable for adaptation to bioreactor systems [25]. In addition to production of secondary metabolites, hairy roots are also used to produce human therapeutic proteins, vaccines, and diagnostic monoclonal antibodies. For example, hairy root cultures of potato carrying pBSHER containing the gene for hepatitis B surface antigen (HBsAg) expressed higher levels of HBsAg compared with control cultures [58].

Despite the potentials discussed, challenges during large-scale cultivation, such as unusual rheological properties of hairy root cultures, have to be addressed. Non-optimal fermentation made it necessary to investigate novel approaches to apply hairy root cultures to fermentor [25] and process design [24].

In conclusion, the use of hairy roots as factories for the production of novel plant-based bioactive compounds, vaccines, antibodies, and other therapeutic proteins offers good prospects for feasibility of commercial production.

19.3 Optimizing Biochemical Pathways

19.3.1 Strategies and Goals of Metabolic Engineering

Metabolic engineering is generally defined as the redirection of one or more enzymatic reactions to produce new compounds in an organism, to improve the production of existing compounds, or to mediate the degradation of compounds [60]. Metabolic engineering of plants offers interesting perspectives to improve the productivity of the plant as a cell factory. This approach may create new opportunities in agriculture, environmental applications, production of chemicals, and medicines [22, 51]. The main goal of metabolic engineering in general is to produce the desired natural products in a sustainable and economically attractive

Table 19.2 Important goals of metabolic engineering in general.

Physiological understanding	
Novel compounds	To yield a novel compound in a plant and other precursors by introducing the appropriate heterologous genes [2] To give a new trait (color, taste, smell) to food, flowers or ornamental plants [22]
To increase	To improve production of a desired compound or enzyme in a cell culture and also in the plant itself To achieve production in a related plant species or even in microorganisms To improve agronomic traits, such as resistance of a plant to various stresses, pests, diseases, and to increase the seed yield of a crop plant through the expression of certain metabolites [22, 61]
To decrease	To decrease levels of noxious or antinutritional factors in food and feed crops [2]
Regulatory understanding	
To improve our understanding of pathways regulation and flux when some of the intermediate pathways increase in abundance beyond their usual concentration range [61]	

way [24]. Several more specific goals of metabolic engineering are listed in Table 19.2.

The production level of a compound of interest that is present in trace amounts can be enhanced through the following: by increasing the flux of precursors; by blocking a competitive (parallel) pathway using the same precursor or intermediate compound; by introducing new routes of metabolism; by overcoming rate-limiting steps; by reducing flux through enhancing competing pathways; by over-expressing regulatory genes or transcription factors that induce the pathway; by inhibiting or limiting catabolism of the molecule; or by increasing the number of specialized cells producing the compound [2, 62].

Some of the scientific challenges comprise a better understanding of the partly known secondary metabolite biosynthetic pathways on a genetic level, the generation of heterologous organisms with desirable biosynthetic characteristics, and optimized tools for pathway manipulation such as vectors, synthetic genes, and regulating elements [62]. Moreover, biosynthetic pathways are often species-specific. Features such as cell compartmentalization, tissue differentiation, and multi-enzyme complexes, will make the outcomes unpredictable.

19.3.2 Metabolic Pathways of Interest

A comprehensive understanding of different metabolic pathways and their genetic control is essential for the application of a genomics approach to the improvement of medicinal plants [5]. In this chapter we confine the discussion to the metabolic

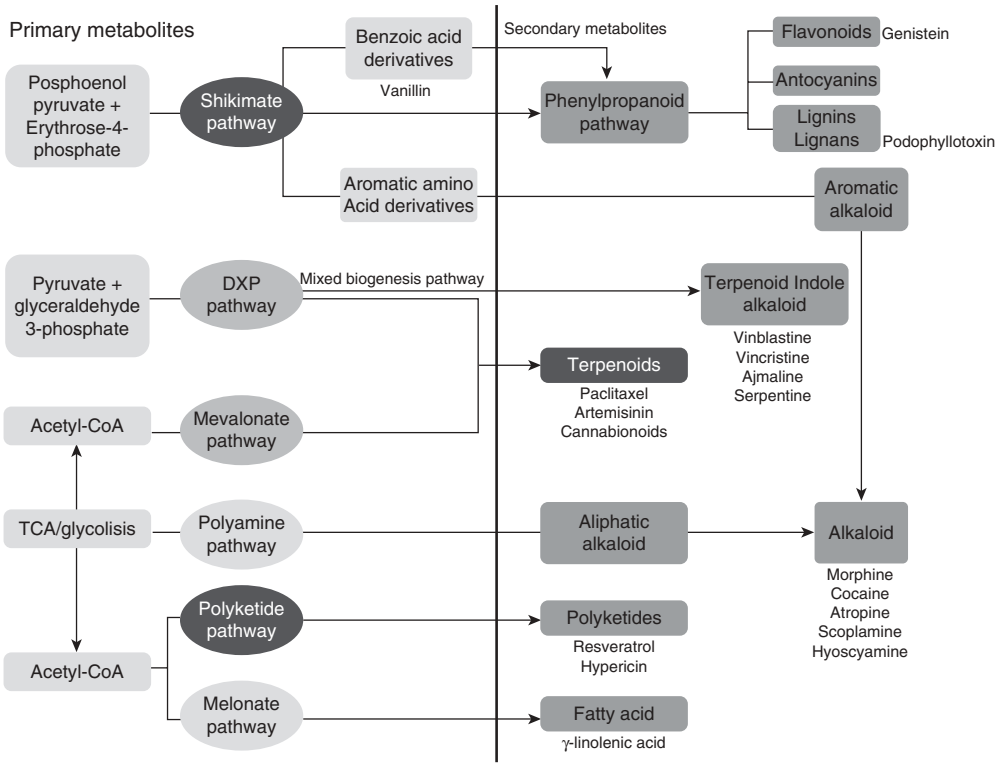

Figure 19.2 Simplified biosynthetic pathways of primary and secondary metabolism in plants (adapted from reference [5]).

pathways of medicinal compounds, among other pathways of interest (Figure 19.2).

Most secondary metabolites are derived from the shikimate, terpenoid, and polyketide pathways. The shikimate pathway is the major source of phenylpropanoids and aromatic compounds [63, 64], such as flavonoids, coumarins, isoquinoline and indole alkaloids, lignans, lignins, and anthocyanins.

The terpenoid pathway leads to more than one-third of all known secondary metabolites, including mono-, seseui-, di-, tri-, and tetraterpenes. It is also the source of the C5-building block (isoprene) in many skeletons from other biosynthetic origins, such as anthraquinones, naphthoquinones, cannabinoids, furanocoumarines, and terpenoid indole alkaloids [65].

The polyketide pathway is a rich source of bioactive molecules such as anthranoids. It is attractive as a model for metabolic engineering studies because the complex structure results from simple C2 units combined in different ways and the modular construction of enzymatic catalysts allows control of enzyme structure [62].

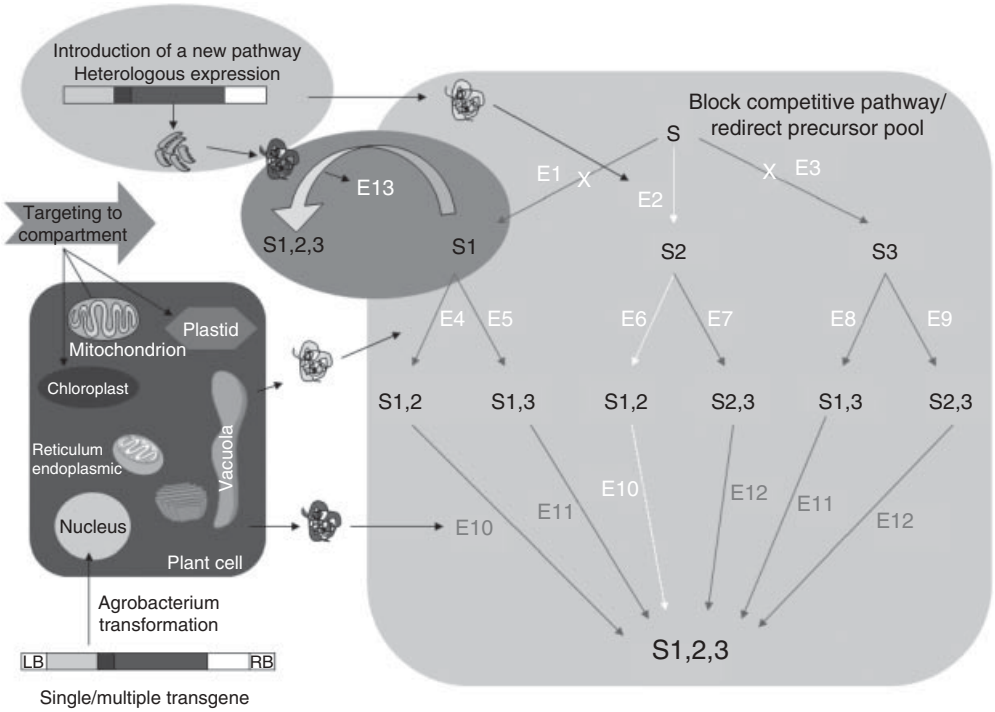

Figure 19.3 Schematic biosynthetic network: S, basic skeleton to which functional group 1, 2, and 3 are added; E1–E13, enzymes that catalyze biosynthetic steps (adapted from reference [66]).

The complexity of a metabolic pathway and some strategies to engineer the pathway are illustrated in Figure 19.3. It is supposed that a basic skeleton S (substrate) is present in which three functional groups can be introduced. If highly specific enzymes would catalyze all steps, 12 enzymes (E) (E1 to E12) could be involved in the formation of three different products with one functional group, three different products with two functional groups, and one final product with all three functional groups. If the specificity of the substrate is broad, it is likely that three different enzymes will be adequate. Heterologous genes (E13) can also be introduced into the plant metabolic pathway, which could catalyze all three functions of the substrate (S1) into product "S1,2,3."

19.3.3
Synthetic Biology

Synthetic biology is a rapidly growing multidisciplinary field among biologists, chemists, physicists, engineers, and mathematicians [67]. It is defined as the design and construction or engineering driven building of new or artificial

biological components or increasingly complex biological entities, such as enzymes, genetic circuits, and cells, or the redesign of existing biological systems for novel applications [68, 69]. The goals are to build complex systems into specific hosts [69], to engineer synthetic organisms [69, 70], to improve understanding of biological systems, and to produce bio-orthogonal systems with new functions [67].

The distinguishing element that differentiates synthetic biology from traditional metabolic engineering is the focus on the design and technological construction of core components (the enzyme, genetic circuit, metabolic pathway, etc., parts), which can be modeled, understood, and tuned to meet specific criteria. The assembly of these components into integrated systems, which enables a systematic forward-engineering of (or parts of) biological systems for improved and novel applications, is a second key issue of synthetic biology [68, 69].

Synthetic biology is categorized into two broad classes. One uses non-natural molecules to reproduce emergent behaviors from natural biology, with the goal of creating artificial life. The other seeks interchangeable parts from natural biology to assemble into systems that function unnaturally [71].

The knowledge of these tools and methods may enable synthetic biologists to design, fabricate, integrate, test, and construct artificial biological systems that originate from the insights discovered by experimental biologists and their holistic perspectives [70].

Although synthetic biology offers promising applications for novel compounds and novel approaches, the success so far is rather limited as it is quite a young science. It is further hindered by the fact that the production processes of the most effective biological components (promoters, gene, plasmids, etc.) have been patented. Royalty payments increase the costs, which make it economically no longer attractive [68]. Another drawback is that living systems are highly complex. Currently, biologists lack information about how the integration of living systems works [70]. The success of synthetic biology depends on its capacity to surpass traditional engineering. It should blend the best features of natural systems with artificial designs that are extensible, comprehensible, user-friendly, and implement stated specifications to fulfill user goals [70].

19.4 Metabolic Engineering Strategies and Techniques in Medicinal Plant Biotechnology

The major metabolic engineering strategies and techniques applied in medicinal plant biotechnology are discussed in detail in this section (see also Figure 19.1). They include up- or down-regulating of pathways, redirecting common precursors, targeting metabolites to specific cellular compartments, and creating storage of overproducing secondary metabolites. Examples of techniques used and their application are given in Table 19.3.

Table 19.3 Techniques used in metabolic engineering and their applications.

Technique	Genes/enzymes	Plant species	Target compounds/ goals	Ref.
Single transgene (biotransformation)	Codeinone reductase (COR)	*Papaver somniferum*	Increase of morphine	[72]
	CYP80B3	*Papaver somniferum*	Increase of morphine alkaloid	[73]
Multiple transgene insertions	10 genes	*Oryza sativa*	Resistant to multiple pathogens, insects, and herbicides, and antibiotic marker free	[74]
Polycistronic vectors (artificial chromosomes)	Cholera toxin β-subunit (*CTB gene*)	*Nicotiana tabacum*	Production of cholera toxin β-subunit	[75, 76]
Transcription factor	ORCA	*Catharanthus roseus*	Increase of terpenoid indole alkaloid	[77]
Sense/antisense suppression	Antisense *CYP80B3*	*Papaver somniferum*	Decrease of benzylisoquinoline alkaloids up to 84% of total alkaloid	[73]
Virus inducing gene silencing (VIGS)	Phytoene desaturase (PapsPDS)	*Papaver somniferum*	Reduction of transcript level of endogenous PapsPDS and photobleach phenotype; and assessing gene function	[78]
	Putrescine *N*-methyltransferase (PMT)	*Nicotiana tabacum*	Reduction of nicotine	[79]
Repressor silencing	Hydrolases, polygalacturonase and pectinesterase	*Solanum lycopersicum*	Altering fruit ripening	[80]
RNAi	Berberine bridge enzyme (BBE)	*Eschscholzia californica*	Accumulation of (s)-reticuline	[81]
	Salutaridinol 7-*O*-acetyltransferase (SalAT)	*Papaver somniferum*	Accumulation of salutaridinol	[82]
	δ-Cadiene synthase	*Gossypium hirsutum*	Reduction of gossypol	[83]

19.4.1 Upregulating of Pathways (Overexpression)

Transcription factors (in multi-enzyme pathways) are regulatory proteins that can be used to regulate multiple steps or even to modulate an entire pathway in order to produce a significant yield of a desired product through sequence-specific DNA binding and protein–protein interactions [61, 84]. They can act as activators or repressors of gene expression, which mediate, respectively, an increase or a decrease in the accumulation of messenger RNA [85]. They are also able to regulate steps for which the enzymes are unknown [84]. Using this approach it is often necessary to increase precursor availability and to understand the coordination of multiple branches or sections of the metabolic pathway. The use of transcription factors requires integrated information from genomics, transcriptomics, proteomics, and metabolomics [24].

Several transcription factors have now been identified. A relevant example is transcription factor MYB12, a flavonol-specific regulator of the phenylpropanoid biosynthesis in developing seedlings. Total flavanol content of the seed was increased when MYB12 was expressed in developing *Arabidopsis thaliana* seedlings. The expression of the genes encoding the four flavonoid biosynthetic enzymes was upregulated, increasing the flux through the flavanone pathway [86]. Three transcription factors – ORCA1, ORCA2, and ORCA3 (octadecanoid-responsive *Catharanthus* AP2-domain) – have been identified in the medicinal plant *Catharanthus roseus* and are involved in biosynthesis of terpenoid indole alkaloids. They belong to the AP2/ERF transcription factor family. The overexpression of ORCA3 in *C. roseus* cultured cells increased the expression of the terpenoid indole alkaloid biosynthesis genes TDC (tryptophan decarboxylase), STR, CPR (cytochrome P450 reductase), D4H (desacetoxyvindoline 4-hydroxylase) [87] and SLS (secologanin synthase) [88]. Moreover, ORCA3 also regulated two genes encoding enzymes (ASα, α-subunit of anthranilate synthase; and DXS, D-1-deoxyxylulose 5-phosphate synthase) in the primary metabolism leading to terpenoid indole alkaloid precursor formation [77].

Next to frequently studied pathways, such as the phenylpropanoid biosynthesis, and the well-characterized MYB transcription factor family, finding a transcription factor that acts on specific pathway genes is very challenging [61]. Transcription factors are difficult to identify in non-model species [84]. An alternative is to design synthetic transcription factors, which target one or more genes of choice [89]. As an example, we mention the design of a synthetic zinc finger protein transcription factor (ZFP-TF) targeted to a methylphytylbenzoquinol methyltransferase (VTE3). The expression of the ZFP-TF increased the activity of native γ-methyltransferase (VTE4) and the α-tocopherol content in *Arabidopsis thaliana* seeds [90]. Transcription factors, natural or synthetic, are used only if the pathway is endogenous to the plant [61].

19.4.2
Redirecting Common Precursors

Many branching points are found in a biosynthetic pathway where enzymes compete for a common precursor. Increasing and redirecting the precursor pool towards the biosynthesis of the target compounds can theoretically increase their production. This can be achieved by blocking the competitive pathway or by inducing overexpression of genes in the precursor pathway [65].

For example, overexpressing the gene that encodes taxadiene synthase in the tomato, a precursor in the carotenoid pathway, increased the production of taxadiene in a tomato mutant. The production was 660–20 000 times higher than in *Arabidopsis thaliana* [91]. By overexpressing genes in precursor pathways in both peppermint [92] and lavender [93] an increase in the monoterpene fraction of the essential oils was found.

However, owing to tight regulation of metabolite accumulation, this approach may also have a limited impact on target products. The increase of intermediate precursor resulted in a limited accumulation of alkaloid target product in *C. roseus* [94]. In this case, the direct overexpression of related genes in the alkaloid pathway was shown to be more effective at increasing the alkaloid accumulation in *C. roseus* [95]. The effect seems to be temporary. It might be due to a result of the same factors, which induce variability in non-transgenic plants [24].

19.4.3
Targeting Metabolites to Specific Plant Cell Compartments

Targeting gene expression to a specific cellular compartment or organelle that contains the precursors could increase the level of the target compounds. Plants are able to express the transgene with organelle targeting signals from the nuclear DNA and the resulting recombinant proteins will be targeted to the appropriate organelles. Specific amino acid sequences required for targeting of proteins to particular organelles and for retention of proteins in organelles have been identified [51]. Thus targeting the enzymes to the compartment of the substrate seems feasible. However, the products formed may cause toxicity problems in a compartment other than the usual one [65].

Using this approach, overexpression of a target gene, either in plastids or the cytosol, allows transport of a sufficient pool of common precursors in the right direction. This leads to a more than 1000-fold increase in concentration of the sesquiterpenes patchouli alcohol and amorpha-4,10-diene, and a 10–30-fold increase of the monoterpene limonene in transgenic tobacco plants compared with untreated control plants [96].

19.4.4
Creation of Storage of Overproduced Secondary Metabolites

A plant may have the capacity to produce secondary metabolites but sometimes it lacks a proper subcellular compartment to store them [97]. Modifications to metabolic storage of products or secondary metabolic pathways have been generally more successful than manipulations of primary and intermediary metabolism [98, 99]. The genes controlling the formation of subcellular compartments have been isolated and characterized in plants [100].

For example, expressing of the *Or* gene encoding a DnaJ cysteine-rich domain-containing protein led to the formation of large membranous chromoplasts in cauliflower curd cells [100]. The expression of the same gene in transgenic potato under the control of a potato granule-bound starch synthase promoter increased the total carotenoid up to sixfold compared with the original, non-transgenic plants [100].

19.4.5
Downregulating of Pathways (Silencing)

The production of a certain compound can be reduced by decreasing the flux towards that product by reducing the level of enzyme in the pathway, increasing catabolism, and increasing flux into competitive pathways [2, 66].

A particular step in the pathway that leads to undesirable compounds can be blocked by suppressing genes that upregulate the pathway or by increasing their catabolism [2]. Antisense, co-supression, and RNA interference (RNAi) methods are used to block, to reduce or to eliminate levels of undesirable compounds. This so called silencing can be targeted to specific plant tissues and organs with minimal interference of the normal plant life cycle, by using tissue or organ-specific RNAi vectors. Mutants with the RNAi effect have been shown to be stable for at least 20 generations [101].

19.5
Challenges in Plant Metabolic Engineering

19.5.1
Unexplored Regulation of Secondary Metabolism

The lack of complete understanding of the regulation of secondary metabolism, especially in the complex alkaloid biosynthesis, hinders the determination of an effective metabolic engineering strategy to achieve a specific production phenotype. The complexities comprise a pathway compartmentalization, the existence of multiple alkaloid biosynthetic pathways and the regulatory control mechanisms [102]. To date, only four biosynthetic routes of alkaloid subclasses have been partially characterized, in particular the benzylisoquinoline, monoterpenoid indole,

purine, and tropane alkaloids [102]. This could be ascribed to the limited genome/cDNA sequence information of medicinal plants [5].

19.5.2 Pathways Are Often Species Specific

A number of genes encoding enzymes, which control key steps of secondary metabolic pathways, have been cloned from a number of a medicinal plant species using classical and modern genomics approaches [5]. However, this represents a small fraction of a total of about 1000 plant genes known to function in secondary metabolism [103]. The progress in isolating genes involved in secondary metabolism is limited due to species specificity, the difficulty in producing large numbers of mutants, their intermediate precursor availability, their analysis, and to the instability of secondary metabolites caused by environmental factors [104]. The major bottleneck for secondary metabolism will remain, as per definition, species specific. Only early parts of the pathways are common to most plants, for example in the flavonoid and terpenoid biosynthetic pathways, thus homology between genes can be used for strategies to clone genes from other plants [104]. The genes encoding enzymes involved in the more specific "decoration" of the basic skeletons can only be studied at the level of the producing plant [105].

19.5.3 Cell Compartmentalization and Tissue Differentiation

Plant cells have a complicated intercellular organization with metabolite flow between compartments highly regulated and orchestrated depending on the biosynthetic needs of the plants [106]. They have numerous organelles of which some are not found in mammalian or yeast cells [51]. The highly compartmentalized nature of enzymes, substrate precursors, and metabolic intermediates also contributes to the complexity of secondary metabolites production, which is regulated at a different level [2].

Plants also have numerous specialized and differentiated organs in which physiological processes and gene expression may differ substantially. Next to organelles, the compartmentalization of secondary metabolite pathways also occurs at the subcellular level [107]. Furthermore, temporal and developmental processes can profoundly influence whether and when a transgene is active. Thus, the issues of compartmentalization complicate the targeting gene strategy. Moreover, if the engineered plants are going to be propagated as crops, environmental effects may add to the level of variability and unpredictability, which is not encountered in a fermentor based system [51].

There is increasing evidence that intra- and intercellular translocation of enzymes are key elements in secondary metabolite production. Localization of enzymes to diverse cellular compartments showed the importance of protein targeting in the assembly of the alkaloid pathway [2]. Alkaloids are generally stored in specific types of compartments due to their cytotoxicity and probable role in

plant defense responses. The subcellular compartmentalization of alkaloid pathway enzymes is extremely diverse and complex because of the cell type-specific localization of the gene transcripts, enzymes, and metabolites [108].

Other examples are phenylpropanoid derivatives. Their biosynthesis occurs in the cytoplasm, but the precursors are derived from metabolism in other organelles, including the chloroplasts and mitochondria [106].

19.5.4
Unpredicted or Unexpected Outcome

The use of metabolic engineering approaches in medicinal plant species to improve the yield of pharmaceutical products has been, and still is, a challenge. There are several limitations such as gene silencing, unpredictable results due to complex network genes, and no increase in concentration of desirable metabolites up to the level of commercialization [5]. Techniques used to introduce new genes into plants also do not allow a prediction about the site of integration and the level of gene expression, even when a strong promoter is used [65].

Single-enzyme perturbations of alkaloid pathways resulted in unexpected metabolic consequences, suggesting the existence of key rate-limiting steps, potential multi-enzyme complexes, or unsuspected compartmentalization [108]. Overexpression of COR1 (codeinone reductase), the final enzyme in morphine biosynthesis, increased the morphine and codeine contents in transgenic poppy [109]. However, thebaine, an upstream metabolite in the 23 branch pathways, was also unexpectedly significantly increased. The knock down of COR1 with RNAi technology would expect to suppress 23 upstream biosynthetic steps and the accumulation of codeinone and morphinone, the immediate precursor of COR. The amount of morphinan alkaloids decreased, while the biosynthesis of (*S*)-reticuline, an early upstream metabolite in the pathway, was increased instead of the target compounds codeinone and morphinone [110]. The complexity and redundancy of many biosynthetic pathways coupled with incomplete knowledge of their regulation could lead to an unpredictable outcome from a targeted metabolic engineering strategy [24].

Selected case studies using different approaches and strategies in metabolic engineering are discussed in the next paragraph.

19.6
Metabolic Engineering Applications in Medicinal Plant Biotechnology

19.6.1
Case Study: Podophyllotoxin Production in *Anthriscus sylvestris*

Anthriscus sylvestris (L.) Hoffm. (Apiaceae) is a common wild plant in Northwest Europe that accumulates considerable amounts of lignans. Deoxypodophyllotoxin, an aryltetralin-lignan is the main attractive constituent that is much more

abundant in the plant kingdom than podophyllotoxin, can be used as a precursor for the production of podophyllotoxin. Podophyllotoxin is used as a precursor for the semi-synthesis anticancer drugs: Etoposide phosphate and Teniposide [111]. To date, podophyllotoxin has been obtained by isolation from *Podophyllum* species. In the future, the availability of podophyllotoxin from this source is likely to become a major bottleneck. *Podophyllum* species are on the endangered species list, proving that the increasing demand of podophyllotoxin is a serious threat to the plant [112]. An alternative source of podophyllotoxin may be obtained by (biotechnological) hydroxylation of deoxypodophyllotoxin at the C7 position (see Figure 19.1). Human cytochrome P450 3A4 in *E. coli* DH5α selectively hydroxylates deoxypodophyllotoxin at the C7 position yielding podophyllotoxin [113]. Studies to transform *A. sylvestris* with this cytochrome are in progress.

19.6.2
Case Study: Scopolamine Biosynthesis in *Nicotiana tabacum*

Scopolamine and hyoscyamine are tropane alkaloids. They form an important class of plant derived anticholinergic compounds occurring in several genera of the Solanaceae, such as *Hysoscyamus*, *Atropa*, *Duboisia*, *Scopolia*, and *Datura* [12, 108]. Scopolamine has a higher commercial market value than hyoscyamine but has a lower yield from plants than hyoscyamine [12]. The world demand for scopolamine is estimated to be about ten times higher than hysocyamine and its racemic form atropine. The main sources of raw material worldwide are *Duboisia* leaves containing 2–4% of total alkaloids, with more than 60% scopolamine and 30% hyoscyamine [15]. Up to 6% of scopolamine has been achieved by conventional cultivation of selected varieties in Australia, Equador, and Brazil, producing 1 t/ha of plant material for industrial alkaloid extraction [15].

The heterologous expression of PMT (putrescine *N*-methyltranferase) from *Nicotiana tabacum* in *Scopolia parviflora* yielded an 8-fold increase in scopolamine and a 4.2-fold increase in hyoscyamine production [114]. A similar effect has been achieved in *Hyoscyamus muticus* and *Datura metel* [115]. Surprisingly, this PMT expression has no effect on alkaloid production when it is expressed in other tropane alkaloid producing hairy root cultures of *Hyoscyamine niger*, *Atropa belladonna*, and *Duboisiana hybrid* [16, 116, 117]. It was suggested that PMT expression in the roots was insufficient to boost the tropane alkaloid synthesis of these plants. Overexpression seems to be species related due to a different, specific post-translational regulation of the endogenous enzyme with respect to the foreign one [117].

The constitutive expression of H6H (hyoscyamine 6β-hydroxylase) from *Hyoscyamus niger* in *Atropa belladonna*, a plant that normally accumulates hyoscyamine, converts hyoscyamine into scopolamine up to 1.2% dry weight [118]. The alkaloid composition of aerial parts of mature plants changed from over 90% hyoscyamine in controls and wild type plants, to almost exclusively scopolamine in transgenics [118]. In transgenic hairy roots of *Atropa belladonna*, up to a 5-fold scopolamine

increase was observed [119]. In *Hyoscyamus muticus* hairy root, expressing the H6H gene, up to a 100-fold increase of scopolamine was found, while the hyoscyamine content remained unaltered [120].

Transgenic tobacco plants expressing constitutively H6H were fed with hyoscyamine and 6β-hydroxyhyoscyamine. These precursors were converted into scopolamine in the leaves of the plants [118]. The hairy root cultures of *Nicotina tabacum*, which do not produce hyoscyamine, were used to express the H6H gene from *Hyoscymanus niger*. The cultures successfully converted added hyoscyamine into scopolamine. They showed efficient uptake of hyoscyamine (average of 95%) from the culture medium and a higher rate of bioconversion of hyoscyamine into scopolamine (10–45%). Up to 85% of the total scopolamine was released into the culture medium [121]. This was in contrast to the normal metabolic behavior of tropane alkaloid-producing hairy roots in which the scopolamine remained accumulated in the root tissues [122]. Feeding exogenous hyoscyamine to cell suspension cultures, which were obtained from the hairy root, showed considerable capacity to convert hysocyamine into scopolamine and the product was secreted into the culture medium [123]. The scaling up of the transgenic cells grown in a 5l turbine stirred tank reactor in a batch mode yielded scopolamine up to a 1.6-fold higher than the small-scale cultures. Almost 18% of the hyoscyamine added to the medium was transformed into scopolamine, which showed a 65% increase with respect to the same alkaloid obtained by bioconversion in shakes flasks [15].

The constitutive co-expression of genes encoding the rate-limiting upstream enzyme PMT and the downstream enzyme H6H of scopolamine biosynthesis yielded only a modest increase in alkaloid accumulation when it was expressed alone, but exhibited a synergistic effect on alkaloid levels when expressed together [16]. It resulted in the highest production of scopolamine in hairy root culture reported of 411 mg/l. It is a 10-fold increase over control cultures and a 2–3-fold increase over cultures that expressed only H6H [16].

19.6.3
Case Study: Genistein Production in Transgenic *Arabidopsis*, Tobacco, Lettuce, Corn, Petunia, and Tomato

Genistein is a common precursor of the isoflavonoid biosynthesis, occurring in particular in the subfamily Papilionoideae of the Fabaceae [124]. Isoflanoids are interesting because of their pharmaceutical and nutraceutical activity that attract considerable interest with the prospect of introducing them into vegetables, grains, and fruits for dietary disease prevention [125].

Genistein production in nonlegume plants has been performed but as yet with unsatisfactory yields. This might be due to the competitive use of narigenin between isoflavon syntase (IFS) and the endogenous flavonoid pathway [125–127].

Soy products are the major dietary sources of isoflavonoids (genistein) for humans. The IFS isolated from soybean has been introduced into *Arabidopsis*

thaliana, corn (*Zea mays*), and tobacco (*Nicotiana tabacum*) [127]. There was no accumulation of free genistein in *Arabidopsis*, but genistein was glycosylated with glucose-rhamnose-genistein and rhamnose-genistein [125].

The overexpression of soybean IFS in tobacco, petunia (*Petunia hybrida* Vilm), and lettuce (*Lactuca sativa* L.) resulted in genistein accumulation in transgenic plants [128]. Another approach was the introduction of a heterogeneous phenylalanine ammonia-lyase (PAL) and IFS into genetically manipulated plants. This increased the genistein content in tobacco petals (1.80-fold) and lettuce leaves (1.5-fold) [128]. The overexpression of IFS soybean in tomato (*Solanum lycopersicum* L) resulted in the presence of genistein 7-*O*-glucoside as the major isoflavone metabolite in the transgenic plants [129].

19.6.4
Case Study: Expression of Spearmint Limone Synthase in Lavender

Essential oil quantity and quality can be regulated by metabolic engineering [92]. In principle, it is possible to engineer the biosynthesis of monoterpenes in order to increase or to modify the essential oil profiles in the target plant [130]. For example, the expression of a sense of the 1-deoxy-D-xylulose-5-phosphate (DXP) reductoisomerase cDNA, and with an antisense of menthofuran synthase cDNA under the control of CaMV 35S promoter, resulted in up to 50% more essential oil in *Mentha* × *piperita* L. without changing the composition of the monoterpenes as compared with the wild-type [92]. Meanwhile the expression of DXP synthase in *Lavandula latifolia* increased the essential oil up to 3.5-fold in leaves and up to 7-fold in flowers as compared with the control, without obvious deleterious effects on plant development and fitness [93].

Until now, studies on the expression of monoterpene synthase in transgenic aromatic plants have been scarce and have only been focused on mint species transformed with limonene synthase (LS). LS catalyzes the stereo-specific cyclization of geranyl diphosphate to yield the monocyclic monoterpene limonene [130].

Spike lavender (*Lavandula laftifolia* Med.) is an aromatic shrub that is cultivated worldwide for oil production, which has limonene as a minor constituent (0.5–2%). Overexpression of the LS gene from spearmint (*Mentha spicata*) in spike lavender under the regulation of the CaMV35S constitutive promoter showed more than 450% increase of limonene content in developing leaves as compared with the control [130].

19.6.5
Case Study: Artemisinin Biosynthesis in *Artemisia annua*

In the early 1980s, efforts began to establish *Artemisia annua* L. cultures that produced artemisinin [131]. A range of variable but always low artimisinin levels were found in callus, shoot, and root cultures but no artemisinin in cell suspension cultures, suggesting that some degree of differentiation is required for the production [132]. Transformation of *Artemisia annua* with *Agrobacterium*

rhizogenes resulted in hairy root cultures that produced artemisinin, and currently many efforts are directed toward optimizing production in hairy root cultures [133].

Several key genes involved in the biosynthesis of artemisinin have been introduced in *A. annua*. Approaches with genetic engineering have been focused on the overexpression of cloned key enzymes involved in the biosynthesis, such as farnesyl diphosphate synthase (FDS) [134] and amorpha-4,11-diene synthase (AMS) [135].

Genetic transformation and regeneration of *A. annua* has been established to introduce genes of interest via *A. tumefaciens* [136]. *A. annua* expressing FPS from *Gossypium arboreum* accumulated higher levels of artemisinin compared with *A. annua* expressing the FPS from *A. annua*. *A. annua* expressing FPS accumulated up to 10.08 mg/g DW artemisinin [136–138].

Hairy root cultures of *A. annua* were established by transforming it via *A. rhizogenes* carrying the farnesyl diphosphate synthase (FDS) gene. The artemisinin content in the transgenic plants, which were regenerated from the hairy root cultures, was significantly higher than in the control plant [96].

Despite all the genetic engineering attempts, the mean of production of artemisinin is still mainly from the plant itself. Recently, the FDA has approved Coartem® (Novartis) as the first artemisinin-based combination treatment (ACT) for malaria in the United States [139]. Novartis has stimulated the cultivation of *Artemisia annua* in more than 1000 hectares in Kenya, Tanzania, and Uganda. In addition, it has also cultivated in China, where, in total, it reaches up to 10 000 hectares [140].

19.6.6
Case Study: Morphine Biosynthesis in *Papaver somniferum*

Papaver somniferum remains the sole source of morphine. The commercial chemical synthesis of morphine, codeine, and other benzylisoquinoline alkaloids is not economically feasible due to the complexity of the molecule and multiple chiral centers [12].

Reticuline is an essential precursor leading to the biosynthesis of benzylisoquinoline alkaloids such as codeine, berberine, and morphine. One of the strategies to increase the flux into morphinan alkaloid is by blocking the BBE (berberine bridge enzyme). Blocking the BBE will increase the (*S*)-reticuline concentration. The expression of an antisense-BBE construct in transgenic opium poppy plants indeed showed increased flux into the morphinan and the tetrahydrobenzylisoquinoline branch pathway [141].

Another strategy is to increase the precursor pool leading to the formation of (*S*)-reticuline, which is (*S*)-*N*-methylcoclaurine 3′-hydrolase. The overexpression of cytochrome P450 monooxygenase (*S*)-*N*-methylcoclaurine 3′-hydrolase (CYP80B3) resulted in an up to 450% increase of total morphinan alkaloids [73]. The suppression of this gene by an antisense construct led to a reduced total alkaloid content in the transgenic poppy [73].

The existence of multi-enzyme complexes has been proposed for flavonoid [142–144] and polyamine metabolism [145]. The occurrence of multi-enzyme complexes also seems to exist in the morphine biosynthesis [108], therefore the metabolic engineering strategies have to be developed carefully. However, not all enzymes of the morphinan branch are necessarily involved in such a macromolecular complex [146].

A further approach is to increase salutaridinol, a precursor of thebaine, by overexpressing salutaridinol 7-*O*-acetyltransferase (SaIAT) and salutaridine reductase (SaIR). RNAi-silenced SalAT in opium poppy plants showed an accumulation of salutaridine instead of salutaridinol, which is normally not abundant in the plants [82]. Salutaridine may be channeled to thebaine through an enzyme complex that includes SaIR and SaIAT. Recent results showed that there is an interaction between SaIR and SaIAT [146]. Morphine, codeine, and thebaine levels were increased in both SaIAT overexpressing and SaIAT RNAi plants [82].

The codeinone reductase (COR) converts codeinone into codeine. Hypothetically, the morphine production can be increased by blocking this enzyme using the RNAi technique. On the contrary, there was an accumulation of (*S*)-reticuline instead of morphine, codeine, oripavine, and thebain [110]. The reasons are unknown, but there were some speculations. It was suggested that there was a feedback mechanism preventing intermediates from the general benzylisoquinoline synthesis entering the morphine-specific branch [2]. The impairment of a required metabolic channel composed of morphinan branch pathway enzymes resulted in accumulation of alkaloid intermediates produced by enzymes that were not part of the same complex [108]. The COR could be part of a multienzyme complex, which cannot function if one of the enzymes is removed [12]. It might be that the side effect of silencing COR was the suppression of 1,2-dehydroreticuline reductase [108]. The potential homology between the two reductases could lead to cosilencing [108]. The COR seems to be an important target for metabolic engineering. The overexpression of COR in *Papaver somniferum* yielded a 15% increase of benzylisoquinoline alkaloids as compared with the high-yielding control genotypes and a 30% increased as compared with the non-transgenic control [72].

19.6.7
Case Study: Gossypol Reduction in Cottonseeds by Blocking δ-Cadinene Synthase

Cotton (*Gossypium hirsutum* L.) could become, apart from its existing production, a nutritionally important crop, not only in developed countries but also in many developing countries where malnutrition and starvation are widespread and it is mainly used for fiber production. The plant produces approximately 1.65 kg of seed for every 1 kg of fiber [83].

After fiber extraction, the cottonseed could be used extensively as a source of proteins and calories, but it is hampered by the presence of the toxic gossypol. Gossypol is a cardiotoxic and hepatotoxic terpenoid and is unsafe for human and

monogastric animal consumption [147]. Gossypol and related terpenoids are present in the glands of foliage, floral organs, and bolls, as well as in the roots [83]. It protects the plant from both insects and pathogens [148, 149].

Gossypol and other sesquiterpenoids are derived from (+)-δ-cadinene. An RNAi-silencing approach on δ-cadinene synthase, coupled with a highly seed-specific α-globulin B gene promoter from cotton, showed significant and selective reduction of gossypol content from cottonseed, without diminishing its content and related defensive terpenoids in non-seed tissues of the plant usually attacked by insects [83]. If the gossypol level in the seeds can be reduced under the safety limit set by United Nations, Food and Agricultural Organization, and World Health Organization, it might be safe for human consumption [83]. Their limit of free gossypol in edible cottonseed products is less than 0.6 g/mg (600 ppm) [150].

19.7 Crossing Borders – Heterologous Production of Plant Compounds in Microorganisms

19.7.1 Artemisinic Acid

One of the success stories of using the synthetic biology approach is related to artemisinic acid. It is a naturally occurring precursor of artemisinin, used as an antimalarial drug. Malaria causes nearly a million deaths each year, mostly of children below 5 years old. The World Health Organization (WHO) estimated 247 million malaria cases among 3.3 billion people who were at risk in 2006 [151]. This leads to a demand to supply artimisinin in an economically attractive and environmental friendly way. The relatively low yield (0.01–0.6%) of artemisinin from *Artemisia annua* is unable to supply the world demand [136]. The total chemical synthesis of artemisinin is difficult and costly [152]. However, the semi-synthesis of artemisinin or any derivatives from microbial sourced artemisinic acid and its immediate precursor gives an alternative for availability and economic feasibility [153]. Using the synthetic biology approach with the use of appropriate promoters and an expression vector resulted in the production of artemisinic acid of up to 300 mg/l in the yeast *Saccharomyces cerevisiae* [68, 154].

19.7.2 Stilbenes

Stilbenes are polyketides, produced by plants. Resveratrol is a representative of stilbenes and is known as a constituent of red wine, which has possible interesting biological activities as an anti-cancer agent [155], inhibitor of inflammation, tumor promotion, angiogenesis and metastasis, and regulation of cell cycle progression [156].

The biosynthetic pathway and the enzymes have been characterized and metabolic engineering has been achieved in plants, microbes, and animals [157]. *E. coli* cells carrying PAL (phenylalanine ammonia-lyase), 4CL (4-coumarate:CoA ligase), STS (stilbene synthase), and ACC (acetyl CoA carboxylase) produced 40 mg/l resveratrol (1 h) from tyrosine. The PAL is from the yeast *Rhodotorula rubra*, 4CL is from actinomycete *S. coelicolor* A3 [2], and STS is from *Arachis hypogaea* [158, 159]. Resveratrol yields were >100 mg/l in *E. coli* expressing 4CL and STS [160].

Stilbenes are rapidly absorbed and metabolized when given orally. The modification of the resveratrol scaffold by hydroxylation and methylation enhanced its bioactivities. The recombinant *E. coli* carrying PAL, 4CL, STS, ACC, and OsPMT (pinosylvin methyltransferase in rice) with the addition of tyrosine resulted in the production of 18 mg/l pinostilbene and 6 mg/l pterostilbene. Addition of phenylalanine resulted in production of pinosylvin monomethyl ether and pinosylvin dimethyl ether almost in the same yield of 27 mg/l [161].

19.7.3 Curcuminoids

Curcumin, bisdemethoxycurcumin, and dicinnamoylmethane are known as curcuminoids [162]. Curcumin is the active ingredient of turmeric (*Curcuma longa*), which has a surprisingly wide range of beneficial claims, but not yet clinically proven. Its use is related to traditional medicine as an anti-inflammatory, antioxidant, anti-HIV, chemopreventive, and chemotherapeutic agent. These actions are partly supported by preclinical pharmacology [163, 164].

Horinouchi [161] discovered a type III polyketides synthase (PKS) in *Oryza sativa* (rice) that can synthesize curcuminods via *p*-courmaroyl-CoA. This PKS, named CUS (curcuminoid synthase), is part of an artificial biosynthetic pathway for production of curcuminoids in *E. coli* [165]. The *E. coli* expressing PAL, 4CL, CUS, and ACC with the additional supply of 1 mM each of the phenylpropanoid acid (*p*-coumaric acid, cinnamic acid or ferulic acid) yielded about 100 mg/l of curcumin or dicinnamoylmethane, and bisdemethoxycurcumin, respectively [161]. *E. coli* carrying 4CL, ACC, and CUS with the addition of ferulic acid isolated from 1 g of rice bran pitch yielded 60 mg of curcumin. Rice bran pitch is a dark and viscous oil, which is a waste from the production of rice edible oil from rice bran. Rice bran pitch (1 g) contains about 22 mg of ferulic acid [162].

19.7.4 Flavonoids

For the first time the complete flavonoid pathway from a plant has been successfully transferred into a microorganism [161]. Genes from various organisms were assembled in *E. coli* on a single pET plasmid for the production of flavanones. They are PAL from the yeast *Rhodotorula rubra*, 4CL or ScCCL from the

actinomycete *S. coelicolor A3* [2], CHS from the plant *Glycyrrhiza echinata*, and CHI from the plant *Pueraria lobata* [166, 167]. The construction proved to be optimal using isopropyl β-D-thiogalactopyranoside (IPTG) inducible T7-promoter and a synthetic ribosome-binding sequence in front of each of the four genes in a *recA*-host [167]. The yield of pinocembrin from 3 mM phenylalanine exogenously added, and naringenin from 3 mM tyrosine were both 60 mg/l [158]. Flavanone-3β-hydroxylase (F3H), flavonol synthase (FLS) and flavone synthase (FNS) were introduced into *E. coli* to modify flavanones into flavonols (kaempferol and galangin). The expression of the genes led to the production of kaempferol (15.1 mg/l) from 3 mM tyrosine and galangin (1.1 mg/l) from 3 mM phenylalanine [168]. Cloning of an FNS gene from *Petroselinum crispum* into pACYC in the *E. coli* host led to production of flavones: apigenin (13 mg/l) from tyrosine and chrysin (9.4 mg/l) from phenylalanine [168].

19.7.5 Vanillin

An example of "white biotechnology" is the production of vanillin as one of the most important aromatic flavor compounds used in foods, beverages, perfumes, and pharmaceuticals. The production scale is more than 10 000 tons per year by chemical synthesis [13]. The increasing demand of customers for natural flavors has shifted the interest of the flavor industry to produce vanillin from natural sources by biotransformation instead of organic synthesis [13]. The aim of the biotransformation of vanillin is to avoid toxic and mutagenic solvents such as phenol and dimethyl sulfate and to avoid corrosive compounds such as hydrogen peroxide, which are used for the organic synthesis [169].

Many different possibilities have been investigated for the biotechnological production of vanillin using different types of bacteria and fungi and different precursors [13]. The transformed *E. coli* BL21(DE3) cells carrying the isoeugenol monooxygenase gene of *Pseudomonas putida* IE27 produced up to 28.3 g/l of vanillin from 230 mM isoeugenol, with a molar conversion yield of 81% at 20 °C after 6 h [170]. The growing knowledge regarding enzymes involved in biosynthetic pathways as well as the identification and characterization of the corresponding genes offers new opportunities for metabolic engineering and for the construction of genetically engineered production strains [13].

19.8 Conclusion and Future Prospects

Plants definitely play an essential role in modern pharmacy and medicine. Efforts to obtain the desired natural compounds to be used as drugs in an efficient way are ongoing and include various approaches.

Metabolic engineering has been applied to both plants and plant cell cultures. Plant cell cultures have been shown to be feasible for industrial production only

to a limited extent, as shown for paclitaxel. Understanding secondary metabolism within cells and cell cultures is essential to use them as a means to supply natural products. The characteristics and metabolic capacities of plant cell/tissue and microbial systems are inherently different; therefore they can serve as complementary unit operations in order to solve the long-standing problem of robust secondary metabolites production [102].

The lack of complete information about the genomes of most medicinal plants is still an immense challenge for applying the appropriate metabolic engineering strategy. To date, only a few plant genomes (e.g., *Oryza sativa*, *Zea mays*, and *Arabidopsis thaliana*), but none of the medicinal plants, have been fully sequenced. The challenges of unravelling the unknown biosynthetic pathways, the encoding genes, and the transcription factors are still there. However, with the progress of sequencing techniques, it will likely be feasible to fully sequence medicinal plants in a shorter time. The only main constraint will, however, be the funding.

Conventional breeding of medicinal plants is another way to enhance the concentration of the desired compounds. Breeding and genetic engineering essentially go hand in hand and are necessary to ensure the availability of the desired compounds.

Until now, it seems that there has been limited success in engineering medicinal plants in which the product could be commercialized based on the economic feasibility. However, genetic engineering strategies have been applied to crop plants such as rice, maize, soybean, and cotton with great and significant success. The genetically modified crop plants with Bt (*Bacillus thuringiensis*) toxin for pest resistance have been grown commercially in approximately 42 million hectares worldwide [171]. In addition, Bt transgenic rice varieties are in field tests and are close to approval for commercialization [172].

The advance of technology holds great promise for the future of plant metabolic engineering. Genomics approaches may lead to the identification of regulatory genes and proteomic approaches may explain why the expression level of some biosynthetic genes do not correlate with the metabolites profile [108].

Finding alternative ways to produce originally plant-derived compounds are still continuing. Microorganisms such as endophytes may serve as an alternative host for production of bioactive substances as reviewed in reference [173]. The success of transferring the biosynthetic pathway from plants into microorganisms or other hosts for the production of artemisinic acid and flavonoids showed that it is feasible to engineer the entire pathway into microorganisms.

The latest promising approach is through synthetic biology for optimizing the biotechnological production of the plant-derived compounds. However, well-characterized biological components, such as the knowledge of the biosynthetic pathways, the genes involved, the promoters, and the precursors, are essential to build the system. An integrated approach to synthetic biology and metabolic engineering will be necessary in the near future. For successful engineering to enhance and optimize the production of the desired metabolites, crossing borders of different disciplines will be needed.

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