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Anticancer Drugs

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Abstract
Plant-derived anticancer drugs play a large role in anticancer pharmaceuticals. Through reviewing the four major types of plant anticancer drugs, namely vinca alkaloids, taxane diterpenoids, podophyllotoxin lignans, and camptothecin quinoline alkaloids, this article illustrates the development process, current status, existing challenges, and future perspective of the plant anticancer drug production. Moreover, this review explains how various biotechnologies, from the mature elicitation strategy to the "omics" techniques that are still undergoing development, can be applied to address the challenges in improving the production of the plant-sourced anticancer drugs.

Keywords
in vitro culture, pathway characterization, heterologous production, metabolic engineering, "omics" techniques

Disciplines
Biochemical and Biomolecular Engineering | Biomaterials | Chemical Actions and Uses | Natural Products Chemistry and Pharmacognosy

Comments
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8
Anticancer Drugs

Le Zhao, Zengyi Shao, and Jacqueline V Shanks

8.1
Natural Products as Anticancer Drugs

Natural products (secondary metabolites) have been a rich repertoire for isolating medical compounds for several millennia. Over 50% of the currently available drugs are natural compounds or their synthetic derivatives with similar chemical structures. Plants, because of their necessity to defend against pathogens and predators, are a rich source of medicinal compounds, especially anticancer compounds. Plant-derived anticancer natural products can be divided into several classes: vinca alkaloids, taxane diterpenoids, podophyllotoxin (PTOX) lignans, camptothecin (CPT) quinoline alkaloids, and others. This review focuses on the recent progress on improving the production of the aforementioned four types of anticancer drugs in various homologous and heterologous culture systems and the biotechnology advancements in *in vitro* culture, metabolic engineering, and pathway elucidation.

8.2
Anticancer Drug Production

8.2.1
Production Systems

There are five potential directions to obtain valuable natural products originating from plants: direct extraction, chemical synthesis, plant cell and tissue culture, endophytic fungi culture, and heterologous synthesis.

As secondary metabolites, natural products usually exist at very low concentrations in native plants, making direct extraction from plants laborious and costly [1]. Some parameters, such as plant growth rate, individual abundance, and seasonal availability, could further weaken the robustness of an extraction process [2]. Even so, current commercial production of most plant-derived

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anticancer natural products is still based on plant extraction because of the lack of other mature approaches. Among the four categories of anticancer drugs, only taxane diterpenoids are produced through plant cell culture, and the other three are all purified from plants in the corresponding commercial production processes.

One possible alternative production method is through chemical synthesis. Examples of the total chemical synthesis of many natural, plant-derived anticancer drugs have been reported in the literature [3–6]. However, in terms of commercial production, the total chemical synthesis is still not economical and sustainable, mainly because it involves many reaction steps, uses harsh solvents, and usually ends up with a low yield of the target product that is often mixed with various structurally similar byproducts. Instead, semi-chemical synthesis procedures are favored for generating derivatives that present better pharmaceutical properties from either extracted natural products or metabolic intermediates [7].

Biotechnology progress in plant *in vitro* cell and tissue culture provides alternatives to produce valuable secondary metabolites [8]. Suspension cells and hairy roots, being amenable to large-scale industrial application, are the two most in-depth studied cultures [9]. Plant suspension cells are biosynthetically totipotent with the potential to produce all the compounds in parent plants. Rao *et al.* have listed the plant species whose cell cultures produce more secondary metabolites than the intact plants [10]. Paclitaxel is the most typical representative of the commercialized anticancer drugs produced in plant cell cultures [11]. The bottlenecks of cell culture development are low product yields, genetic instability, and metabolite variability [12]. Hairy roots in most cases grow with the similar rates as cell culture but with higher biochemical and genetic stability. In addition, since hairy roots are differentiated, compartmentalization of portions of cellular pathways may assist in the production of toxic intermediates and metabolites. They keep most of the properties of the natural roots and contain metabolites synthesized in the roots. This distinction is important since transport of metabolites to and from other plant tissues is not present in a root culture [13].

Plant endophytic fungi play important roles in plant secondary metabolite biosynthesis. Some endophytic fungi produce the same metabolites as their hosts. Corresponding to the four categories of anticancer drugs, a great number of endophytic fungi have been isolated and characterized with the ability to produce these anticancer compounds [14]. Although no fermentation based on plant endophytic fungi has been commercialized because of the low and sometimes unstable yields, fungal fermentation holds a great potential considering fungi have been widely applied in food, pharmaceutical, and agriculture industries for a long time. In order to make fungi-produced anticancer drugs commercially viable, secondary metabolic pathways in endophytic fungi and their dynamic interactions with plant hosts need to be studied well.

Finally, heterologous production in microbes has been of interest for decades. Synthetic biology tools and expanding biochemical and genetic knowledge of

plant medicinal pathways bring a new promise to this option. Some microbial hosts, especially *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (*S. cerevisiae*), have been widely used in the industry because of their fast doubling times, ease of genetic manipulation, and mature large-scale culture technologies. Existing bottlenecks are that some reactions involved in the entire biosynthesis have not been fully identified and sometimes plant-sourced proteins cannot fold and function properly in microbial expression systems. So far, the highest titer of producing a precursor of an anticancer drug in microbes has been demonstrated by the synthesis of taxadiene [15], the precursor of paclitaxel, *in E. coli*, which will be discussed in detail in Section 8.3.2.

8.2.2 Approaches for Improving Production

Elicitation is a common strategy to increase secondary metabolite levels in intact plants, plant tissues, and cell cultures. Among the various elicitors, jasmonic acid, its conjugates, and precursors, collectively known as jasmonates (JAs), are effective in activating the biosynthesis of many plant secondary metabolites through initiating plant defense mechanisms against pathogens and herbivores [16]. In *Taxus* cell suspension cultures, the amounts of paclitaxel and baccatin III (one of the intermediates in the paclitaxel biosynthetic pathway) increased significantly after treatment with methyl jasmonate (MeJA) [17]. Jasmonic acid was found to be the most effective elicitor to increase CPT production in *Camptotheca acuminate* cell suspension cultures in comparison with other biotic and abiotic elicitors [18].

In principle, precursor feeding is another approach to increase the titer of final products; however, since the expression of the involved genes is subjected to strict regulation, increasing precursor concentration does not usually increase the metabolic fluxes proportionally. In a final approach, immobilization of plant cells is a method that enhances cell-to-cell contact. The associated changes in cellular physiology can bring up the levels of certain secondary metabolites [19].

In microbial production systems, various metabolic engineering strategies are often applied to improve production. In contrast, plant metabolic engineering is still in its infancy and many toolkits are needed. Particle bombardment and *Agrobacterium*-mediated transformation are the two frequently used methods to introduce foreign DNA molecules, and protocols have been established for transforming *Catharanthus roseus* (vinca alkaloids producer) [20, 21] and *Taxus* species (taxane diterpenoids producer) [22, 23]. Most recently, *Agrobacterium*-mediated transformation has been applied to *Podophyllum hexandrum* (PTOX lignans producer) successfully, making a further step toward expressing the desired genes in *P. hexandrum* to improve the titer of podophyllotoxin and optimize other traits [24].

Besides the production of anticancer compounds in native organisms, heterologous synthesis, mainly in microbes, brings new opportunities and technical

challenges. The advantages of heterologous synthesis include the following: (i) ease of genetic manipulation and culture processing in heterologous hosts, (ii) lack of complicated regulation belonging to the native organisms, and (iii) a simplified purification process without contamination by structurally similar metabolites that exist in the native organisms. Meanwhile, the challenges are also apparent. First of all, enabling a heterologous host for producing anticancer drugs usually requires the expression of multiple genes, and expressing plant-sourced proteins in microbial systems is sometimes challenging. Second, the accumulation of some intermediates caused by unbalanced gene expression may result in toxicity issues. Furthermore, some metabolites require plant organelles for their synthesis, so that an alternative scaffold in the microbial host will have to be created. Regardless of these challenges, many efforts have been attempted to produce anticancer drugs in heterologous hosts, such as *E. coli* and *S. cerevisiae.* Recently, the rapid development in synthetic biology points out additional directions to tackle the challenges in this area; for example, Martin *et al.* [25] developed *E. coli* strains as platform hosts for the production of terpenoid compounds.

8.2.3 Gene Discovery

One of the prerequisites for applying metabolic engineering strategies is the comprehensive knowledge of a biosynthetic pathway. However, none of the anticancer drug biosynthetic pathways has been fully elucidated. To characterize a pathway, metabolites and the genes involved in the pathway need to be identified. In the post-genomic era, even though the genome sequences of many non-model plants (including the medicinal plants discussed in this review) are still unknown, a large number of expressed sequence tags (ESTs) or cDNA sequences are available in databases, based on which the functions of some plant genes have been predicted through homology search and subsequently been confirmed by experiments. Enzymes belonging to plant cytochrome P450-dependent monooxygenases are the examples that have been identified by this means. They share high sequence similarities and have a well-established subfamily classification [26].

Although none of the genomes of the plant species that produce anticancer drugs has been sequenced and many genes are still not annotated, a great amount of "omics" data including transcriptomics, proteomics, and metabolomics have been generated. Comparative analysis of the "omics" data either individually or in combination from different plant tissues or from different elicitation conditions provides a strategy to narrow down the search scope of the uncharacterized genes. The examples of using "omics" data in gene discovery can be represented by the characterization of five genes in podophyllotoxin biosynthetic pathway, which will be discussed in detail in Section 8.3.3. Similar "omics" efforts for all the plant-derived anticancer drugs will also be introduced in the ensuing sections.

8.3
Important Anticancer Natural Products

8.3.1
Vinca Alkaloids

Vinblastine and vincristine are terpenoid indole alkaloids (TIAs) isolated from the pantropical plant *C. roseus* (Madagascar periwinkle). They have been used for centuries throughout the world as a traditional medicine to treat diseases such as diabetes and eye infections. When researchers began to analyze *C. roseus* in the late 1950s, vincristine and vinblastine were found to be able to lower the number of white cells in blood. Through decades of research, the medicinal mechanism of vinblastine and vincristine has been well studied, and these two compounds are currently widely applied in chemotherapies for many types of cancers [27]. To decrease their toxicity and explore broader pharmacological applications, several semisynthetic derivatives were synthesized, among which vindesine and vinorelbine have been marketed. Together with vinblastine and vincristine, these four compounds have different pharmaceutical profiles and are used to treat different types of cancers [28]. Figure 8.1 shows the structures of these four vinca alkaloids. Semisynthesis of *C. roseus* vinca alkaloid derivatives was summarized by van der Heijden *et al.* [29].

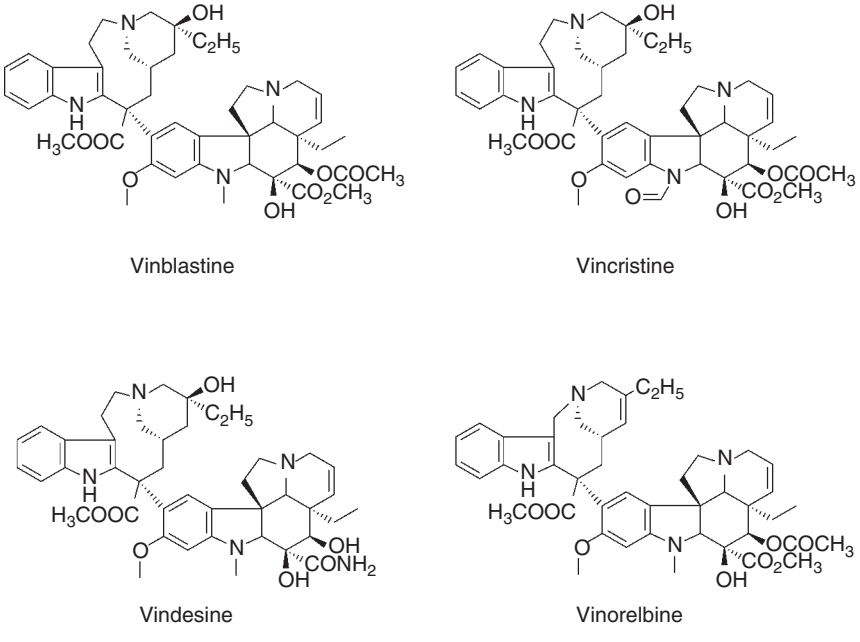

Figure 8.1 Chemical structures of vinblastine, vincristine, vindesine, and vinorelbine.

C. roseus produces more than 130 different TIAs and is the only source for obtaining vinblastine and vincristine. The biosynthetic pathway of TIA in *C. roseus* is complex and highly branched (Figure 8.2). The isolation of vinblastine and vincristine from *C. roseus* is laborious and costly due to their relatively low levels in the plant. Approximately 500 kg of dried leaves are needed in order to isolate 1 g of vinblastine [30]. The low production of vinblastine and vincristine stimulated a great number of research projects aiming at improving productivity. Research has been focusing on production of vinca alkaloids either by fermentation of endophytic fungi in *C. roseus* or by large-scale culturing of plant suspension cells, hairy roots, and other tissues. More than 183 endophytic fungi have been isolated from different tissues of *C. roseus*, and some of them were reported to possess the ability to produce either vinblastine or vincristine. Among the fungi isolated from Chinese *C. roseus*, it was reported that one endophytic fungus, *Fusarium oxysporum*, produced vincristine, and another endophytic fungi species, *Alternaria* sp., produced vinblastine. *F. oxysporum* isolated from the Indian species produced 76 μg vinblastine and 67 μg vincristine in 1 l of fermentation broth, and the dimers were characterized by HPLC (high-performance liquid chromatography), MS (mass spectrometry), and ^{1}H NMR (nuclear magnetic resonance) [31]. For *Fusarium*, the biosynthetic pathways for the production of vinblastine and vincristine from tryptophan and geraniol have not been elucidated.

C. roseus cell suspension and hairy root cultures do not produce the two bisindole alkaloids vinblastine and vincristine, because of the inability to synthesize one of the monoterpene indole alkaloids (MIAs), vindoline (Figure 8.2). Vindoline condenses with another MIA, catharanthine, forming the bisindole alkaloids. In suspension cell cultures, transcripts of *N*-methyltransferase (NMT), desacetoxyvindoline 4-hydroxylase (D4H), and deacetylvindoline acetyltransferase (DAT) are absent, and consequently the biosynthesis of vindoline is blocked [32–34]. Root cultures lack the idioblast and laticifer, which are specialized cell types for the late steps of vindoline synthesis, so vindoline cannot be produced in hairy root cultures as well. A comprehensive RNA-seq data from different tissues of *C. roseus*, including leaf, flower, and root, also indicated that enzymes involved in the vindoline and vinblastine pathways are restricted to aerial tissues [35]. The intermediate metabolite tabersonine in hairy root culture accumulates, and is converted via the lochnericine and hörhammericine branch, instead of the vindoline branch [36]. The high flux through tabersonine in hairy roots indicates the potential of this culture type for the production of vindoline if an active tabersonine to vindoline pathway could be engineered into the roots. In multiple shoot cultures, vindoline and catharanthine levels are comparable to those in intact plants, and vinblastine level is greater than that in callus culture but less than that in the parent plant [37].

Various strategies to increase alkaloids levels, including adjusting the medium, temperature, light, and aeration, have been reviewed [38, 39]. Many plant phytohormones and elicitors, both biotic and abiotic, have been applied to increase alkaloids accumulation [29, 39]. Several studies showed that MeJA, ethylene, and fungal elicitors individually or conjointly promoted vindoline accumulation in

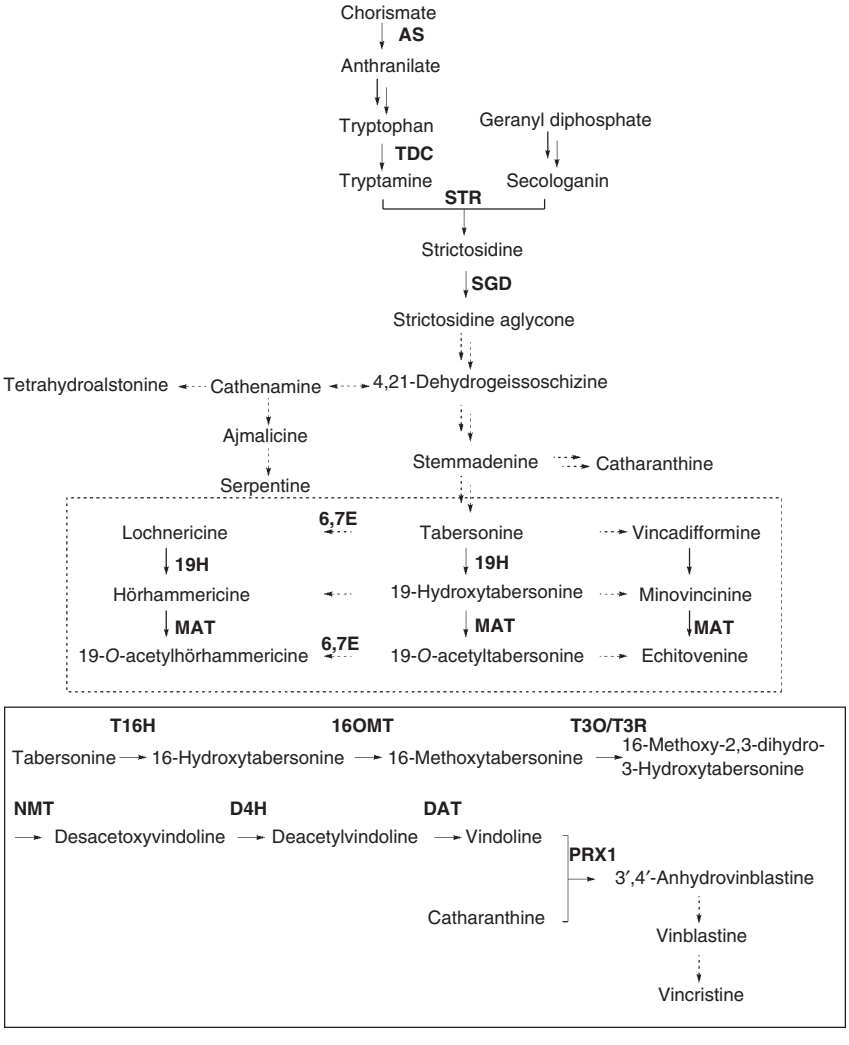

Figure 8.2 Terpenoid indole alkaloid biosynthetic pathway. AS: anthranilate synthase; TDC: tryptophan decarboxylase; STR: strictosidine synthase; SGD: strictosidine β-D-glucosidase; 6,7E: 6,7-epoxidase; 19H: 19-hydroxylase; MAT: minovincinine 19-hydroxy-*O*-acetyltransferase; T16H: tabersonine 16-hydroxylase; 16OMT: 16-hydroxytabersonine-*O*-methyltransferase; T3O/T3R: tabersonine 3-oxygenase and tabersonine 3-reductase; NMT: *N*-methyltransferase; D4H: desacetoxyvindoline 4-hydroxylase; DAT: deacetylvindoline 4-*O*-acetyltransferase; PRX1: peroxidase 1. The double arrows indicate multiple reactions, and the dashed arrows represent uncharacterized reactions. Dashed box encloses the active pathway in root. Solid box encloses the active pathway in leaf.

either callus culture or shoot culture [32, 40], and transcript analysis further indicated that several genes in the vindoline biosynthetic pathway were upregulated by these elicitors.

As mentioned earlier, *C. roseus* suspension cells and hairy roots do not produce anticancer drugs mainly because the vindoline pathway is blocked in those two systems. Through intricate manipulation of several elicitors of the vindoline pathway and inhibitors of the bypass, vindoline and vinblastine were produced in suspension cells as determined by HPLC [41]. The highest vinblastine yield of $810\,\mu g\,g^{-1}$ DW was reported, which was much higher than that in the mature plant ($12\,\mu g\,g^{-1}$ DW). However, this report would be strengthened if additional evidence could be provided to support that the vindoline biosynthetic pathway is active in suspension cell cultures, such as the NMR and MS/MS data of vindoline and transcription levels of all the enzymes in the vindoline pathway.

In general, suspension cell cultures are derived from dedifferentiation-derived calluses (DDCs). However, recent studies suggest that the DDC cells are not real totipotent stem cells because the callus formation might not entail a simple backward reprogramming of the dedifferentiation process [42] and some deleterious genetic or epigenetic changes could occur [43, 44]. In 2015, an undifferentiated cambial meristematic cell (CMC) of *C. roseus* was isolated and a comparison between CMCs and DDCs was made [45]. One of the conclusions of that study was that CMCs are more stable than DDCs. CMCs remained white and transparent after 8–10 rounds of subculturing, while DDCs showed yellow or brown color after two rounds. Interestingly, the transcriptome profile of the genes and metabolites involved in the TIA pathway are also different between CMCs and DDCs. Vindoline was detected in CMCs with several types of culture media but not in any of the DDC media. Zhou *et al.* reported the transcriptome and metabolite profiles of a 2-year-old CMC line from *C. roseus* [46]. The CMCs grew very stable and could accumulate vindoline and several other MIAs. The highest titer of vindoline ($7.45\,mg\,l^{-1}$) was achieved in a 5-l stirred hybrid airlift bioreactor fermentation treated with 10 mM β-cyclodextrin and 150 μM MeJA. Nonetheless, vindoline was measured via HPLC analysis only, and the transcription analysis for the key enzyme, NMT, was not performed; therefore, their (promising) results should be interpreted with caution.

Metabolic engineering of *C. roseus* cell suspensions, hairy root cultures, and even intact plants has been studied with the aim of increasing TIA accumulation. Overexpression of transcription factors or overexpression of a TIA enzyme or a combination of enzymes has been the main strategy to date. Precursor feeding strategies are often combined with these experiments in order to determine rate-limiting steps. These efforts often only result in modest improvement of TIA accumulation (Table 8.1) since transcriptional regulation is hierarchical and a large portion of the regulatory network is still unclear.

Heterologous expression of the TIA synthetic pathway in non-native plant hosts and microbial hosts is under development and will potentially pave the way for producing interesting TIA from hosts that grow faster and are easier to be cultured. Most of the heterologous expression efforts are focused on

Table 8.1 Examples of recent metabolic engineering efforts in the TIA pathway.

Hosts	Engineering strategies	Role in TIA pathway	Expression system	Main observations
Native host	Overexpression of WRKY1 [47]	Transcription activator	Hairy root	Threefold increase in serpentine; 10-fold increase in ajmalicine; Twofold decrease in catheranthine
	Overexpression of ORCA3 [48]	Transcription activator	Hairy root	Threefold increase in hörhammericine; Threefold increase in lochnericine
	Overexpression of DAT [36]	Pathway enzyme	Hairy root	Fourfold increase in hörhammericine
	Overexpression of Asα and Asβ and feeding of 10-deoxy-D-xylulose, loganin, and secologanin [49]	Pathway enzyme	Hairy root	2.3-fold increase in hörhammericine; 1.5-fold increase in cathenamine; 1.3-fold increase in catharanthine; 1.8-fold increase in ajmalicine; 2.1-fold increase in lochnericine
	Overexpression of ORCA3 and feeding of loganin [50]	Transcription activator	Suspension cell	3.2-fold increase in TIA; Detectable strictosidine and ajmalicine
	Overexpression of DAT [51]	Pathway enzyme	Plant	Twofold increase in vindoline
	Overexpression of ORCA2 [52]	Transcription activator	Hairy root	Detectable 19-hydroxytabersonine after induction
	Co-overexpression of ORCA3 and SGD [53]	Transcription activator and pathway enzyme	Hairy root	0.5-fold increase in TIA including serpentine, ajmalicine, catharanthine, tabersonine, lochnericine, and hörhammericine

(*continued overleaf*)

Table 8.1 *(Continued)*

Hosts	Engineering strategies	Role in TIA pathway	Expression system	Main observations
Nonnative hosts	Co-expression of TDC and STR, and feeding of secologanin [54]	Pathway enzyme	Tobacco suspension cell	Strictosidine can be detected
	Co-expression of TDC and STR [55]	Pathway enzyme	*Cinchona officinalis* hairy root	1.2 mg g^{-1} DW of tryptamine; 2.0 mg g^{-1} DW of strictosidine; Not a stable trait
	Co-expression of STR and SGD, and feeding of tryptamine and secologanin [56]	Pathway enzyme	*Saccharomyces cerevisiae*	Strictosidine (major product, 2 g l^{-1}) and cathenamine can be detected
	Expression of most genes in strictosidine biosynthetic pathway and other genetic modifications in expression host [57]	Pathway enzyme	*Saccharomyces cerevisiae*	Strictosidine can be detected without any feeding
	Expression of the pathway from tabersonine to vindoline and feeding of tabersonine [58]	Pathway enzyme	*Saccharomyces cerevisiae*	Vindoline can be detected

tryptophan decarboxylase (TDC), strictosidine synthase (STR), and strictosidine β-D-glucosidase (SGD), the three enzymes in the upstream of the TIA pathway (Figure 8.2). These three genes, individually or in combination, were expressed in tobacco suspension cells, tobacco plants, *Cinchona officinalis* hairy root culture, other plant species tissue cultures, yeast, and *E. coli*, with the precursor fed to the culture [29]. Table 8.1 lists some examples of genetic engineering in non-native hosts. In 2015, as the last two unknown genes in vindoline biosynthetic pathway branch, tabersonine 3-oxygenase (T3O) and tabersonine 3-reductase (T3R), catalyzing the "hydration" reaction from 16-methoxytabersonine to 16-methoxy-2,3-dihydro-3-hydroxytabersonine were isolated, De Luca *et al.* assembled the seven-step pathway from tabersonine to vindoline in yeast [58]. By feeding with tabersonine or one of the intermediates, namely 16-methoxytabersonine, the engineered yeast strains were able to accumulate vindoline and other intermediates and secreted 95% of the MIAs to the medium. In another example, as the genes involved in the upstream secologanin synthetic pathway were all characterized in succession, O'Connor's group introduced the whole pathways in *S. cerevisiae* together with other genetic manipulations to produce the central intermediate strictosidine, without any precursor feeding [57].

Great efforts have been made in the metabolic engineering of the upstream pathways in vinblastine and vincristine biosynthesis, especially in the past 2 years. However, the pathway from the central intermediate strictosidine to tabersonine is still unclear. In addition, it would be more challenging and complex to produce bisindole alkaloids than MIAs in heterologous systems. To further explore the unknown genes, pathways, and regulators using methods other than the classical biochemical approaches that heavily rely on protein purification and enzyme assay, information provided by EST, transcriptome, proteome, and metabolome could be very insightful for predicting and characterizing unknown genes in the TIA pathway.

Very recently, in-depth proteome mining of *C. roseus* suspension cell was conducted. Among the total 1663 identified proteins, 63 enzymes were identified to be potentially involved in secondary metabolism, of which 22 were involved in the TIA biosynthetic pathway and 16 were predicted as putative transporters [59]. In this study, four known enzymes in TIA pathway were confirmed, and gene candidates in other seven reaction steps, mainly in the secologanin (precursor of TIA) biosynthetic pathway, were proposed based on the proteomics data in combination with analogy with other species.

Bioinformatic techniques to discover new genes have relied more heavily on RNA-Seq data in recent years. One example is the discovery of the last four missing enzymes in the secologanin biosynthetic pathway, also named the *iridoid pathway* [60]. With a huge RNA-Seq dataset including different tissues and induction conditions, the complete linkage hierarchical clustering maps were constructed and genes that had the predicted enzyme activities and carried the same expression patterns with the known genes in the pathway were screened as the candidate genes. CathaCyc, a specific pathway database (PDB) derived from *C. roseus* RNA-Seq datasets, is accessible online (http://www.cathacyc.org).

CathaCyc presents a variety of tools for the visualization and analysis of metabolic networks and "omics" data [61] and will certainly help in discovering missing enzymes, studying metabolic pathway evolution, and, ultimately, improving metabolic pathways originating from *C. roseus.*

Two common strategies to understand gene function in the TIA pathway are "forward genetics" and "reverse genetics". Forward genetics seeks the genetic basis triggering a certain phenotype or trait, while reverse genetics studies which phenotypes arise from particular genetic sequences. Leslie van der Fits *et al.* successfully applied forward genetics to identify the ORCA3 gene, one of the transcription factors in the TIA pathway, using *C. roseus* suspension cells by transferred DNA (T-DNA) activation tagging [50]. With increasing knowledge of gene sequences, reverse genetic techniques are in high demand. For example, RNA interference (RNAi)-based gene silencing is one of the powerful tools in reverse genetics. RNAi has been applied effectively in *C. roseus* hairy root culture and cell suspension culture [62–64] to decipher gene function. The success in introducing the virus-induced gene silencing (VIGS) technique into the *C. roseus* plant [65] facilitated the discovery of the last two enzymes in the vindoline biosynthetic pathway [58].

8.3.2
Taxane Diterpenoids

Paclitaxel (also named *taxol*), (Figure 8.3) is a diterpenoid discovered in 1967 from the bark of the Pacific yew tree, *Taxus brevifolia*, working as a mitotic inhibitor used in cancer chemotherapy. One of paclitaxel analogs, docetaxel (Figure 8.3), is a semisynthetic compound produced from 10-deacetylbaccatine-III. 10-deacetylbaccatine-III is an intermediate in the paclitaxel synthetic pathway (Figure 8.4), which was found in relatively large amounts in the needles of *Taxus baccata* (European yew tree). Various *Taxus* species produce taxol and over 300 related compounds in total. Paclitaxel and docetaxel have been approved by the Food and Drug Administration (FDA) for the treatment of breast, lung, ovarian, and prostate cancers. In the past, paclitaxel was commercially extracted from the barks of yew trees. This production method was labor-intensive and unsustainable because the paclitaxel level in the natural yew tree is quite low. Three or four 150–200-year-old trees were sacrificed in

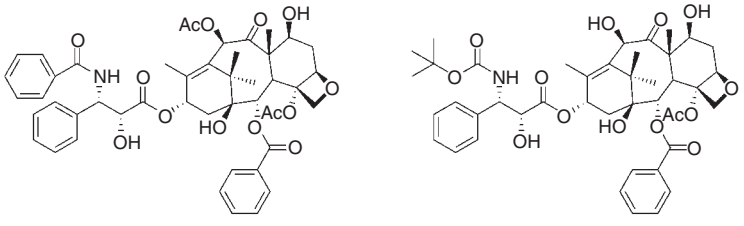

Figure 8.3 Chemical structures of paclitaxel and docetaxel.

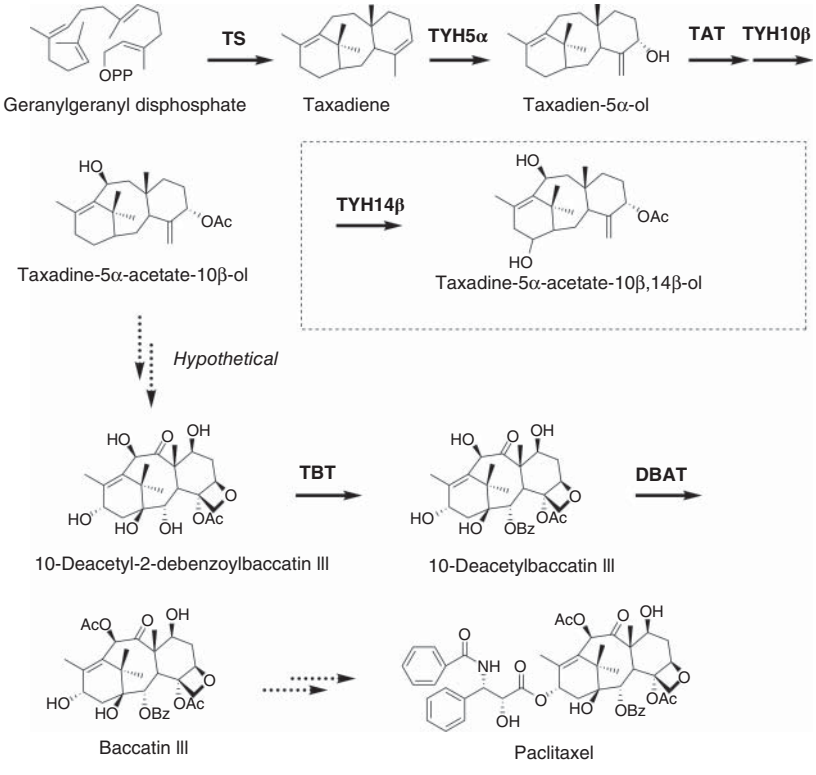

Figure 8.4 Paclitaxel biosynthetic pathway. TS: taxadiene synthase; TYH5α: taxadiene 5α-hydroxylase; TAT: taxadien-5α-ol *O*-acetyl transferase; TYH10β: taxane 10β-hydroxylase; TYH14β: taxoid 14β-hydroxylase; TBT: taxane 2α-*O*-benzoyltransferase; DBAT: 10-deacetylbaccatin III-10-*O*-acetyl transferase. The double dashed arrows indicate multiple reactions including uncharacterized steps. The dashed box represents a bypass of paclitaxel biosynthetic pathway.

order to obtain the amount of paclitaxel needed for one cancer treatment [66]. Later, both paclitaxel and docetaxel could be chemically synthesized from 10-deacetylbaccatine-III, which was isolated from the twigs and needles of the European yew. An even more sustainable approach is to produce paclitaxel via large-scale plant cell suspension culture. Compared to the semisynthesis, the advantage of large-scale plant cell suspension culture is that it eliminates the need for numerous hazardous chemicals and consequently saves on waste disposal. Hundreds of kilograms of paclitaxel can be produced in a 75 000-l bioreactor in a single batch [67].

Although cell suspension cultures (DDC cells) have been used in the industry to produce paclitaxel, the variability in product accumulation is still a key challenge to the regular commercial use [2]. In parallel to the examples shown in Section 8.3.1, CMCs were isolated from *Taxus cuspidate* to produce paclitaxel [68]. The CMCs demonstrated great advantages over the DDCs and overcame

the obstacles associated with the commercial culture of DDCs. First, CMCs grew much faster than DDCs in bioreactors since they were more tolerant of shear stress owing to some molecular properties, such as small and abundant vacuoles, thin cell walls, and less aggregation. Second, CMCs were stable and could keep growing rapidly during 22 months of culture. The biomass of CMCs was fourfold higher than that from DDCs in the same culture condition, and the paclitaxel yield was increased to 7.5-fold compared with the common DDC culture. In all, the development of CMCs has a great potential for paclitaxel's commercial production in the future.

To decrease the cost of paclitaxel production, alternative production approaches have been intensively explored. In 1993, the first endophytic fungus *Taxomyces andreanae*, which is able to produce paclitaxel, was isolated [69]. From then on, over 50 paclitaxel-producing fungi have been isolated and studied worldwide [70]. The reported highest titer of paclitaxel from fungi fermentation is 478 $\mu g\,l^{-1}$ [71], which is much lower than that obtained through large-scale plant suspension culture (900 $mg\,l^{-1}$). Two important issues need to be solved in the fungal system: the biosynthetic mechanism of paclitaxel is unknown, and most fungi lose the capability of paclitaxel synthesis after being cultured for several generations [72].

In addition, hairy root culture was attempted by two groups for paclitaxel production. One of the hairy root lines, established using seedlings of *T. cuspidate* (Korean yew), accumulated 52.5 $mg\,l^{-1}$ paclitaxel after 2 weeks in a 20-l bioreactor operating under MeJA induction [73]. The other hairy root culture generated from the plantlets of *Taxus* × *media* (a hybrid species between *T. cuspidate* and *T. baccata*) accumulated 210 $\mu g\,g^{-1}$ DW paclitaxel following MeJA induction [74].

Applying metabolic engineering strategies in *Taxus* species is difficult because of the extremely low gene transformation efficiency. The total amount of taxanes, especially the level of paclitaxel, has not increased significantly via overexpressing the pathway-related enzymes, knocking out the side-route enzymes, or overexpressing the enzymes in the elicitor pathway (Table 8.2). Clearly, more knowledge of the genetic and biochemical regulation of the pathway is needed.

Much effort has been made to express the paclitaxel synthesis genes in non-native hosts, particularly in two microbial production hosts, *S. cerevisiae* and *E. coli* (Table 8.2). In 2001, Huang *et al.* [87] expressed three genes in *E. coli* and produced taxadiene, which is the first terpenoid intermediate in the paclitaxel synthetic pathway, at a titer of 1.3 $mg\,l^{-1}$. In 2006, DeJong *et al.* [85] expressed eight genes in *S. cerevisiae* with the goal to produce one of the intermediates, taxadien-5α-acetate-10β-ol (Figure 8.4). Analysis of the metabolites showed 1 $mg\,l^{-1}$ taxadiene, a trace amount of taxadine-5α-ol (~25 $\mu g\,l^{-1}$), and no taxadien-5α-acetate-10β-ol, indicating that the first cytochrome P450 hydroxylation reaction had become a limitation in this expression system. In 2008, Engels *et al.* [86] improved the taxadiene titer in *S. cerevisiae* to 9 $mg\,l^{-1}$. In 2010, Gregory Stephanopoulos' group [15] optimized an engineered *E. coli* strain via sophisticated metabolic engineering approaches, improving the taxadiene titer to 1 $g\,l^{-1}$, which is the highest taxadiene level reported in non-native

Table 8.2 Recent examples of using metabolic engineering strategies to improve the production of paclitaxel.

Hosts	Engineering strategies	Role in the paclitaxel pathway	Expression system	Main observations
Native hosts	Overexpression of DBAT with MeJA elicitation [75]	Pathway enzyme	*T. mairei* cell suspension	Increase in baccatin III and paclitaxel
	Overexpression of DBAT [76]	Pathway enzyme	*T. chinensis* cell suspension	1.7-fold increase in paclitaxel
	Overexpression of TXS with MeJA elicitation [77]	Pathway enzyme	*T. media* cell suspension	2.65-fold increase in total taxanes
	Knockout of taxoid 14β-hydroxylase (T14OH) [78]	Side-route pathway enzyme	*T. media* cell suspension	Decrease in the three byproducts of taxanes; no report about paclitaxel level
	Overexpression of 9-*cis*-epoxycarotenoid dioxygenase [79]	Enzyme in the abscisic acid (ABC) biosynthetic pathway	*T. chinensis* cell suspension	1.2-fold increase in abscisic acid 2.8-fold increase in paclitaxel
Non-native hosts	Expression of TXS [80]	Pathway enzyme	*Arabidopsis thaliana* plant	60 µg taxadiene per kilogram of dry weight; a lethal phenotype
	Expression of TXS [81]	Pathway enzyme	Tomato plant	160 mg taxadiene per kilogram fruit; a slow growth phenotype
	Expression of TXS [82]	Pathway enzyme	Moss *Physcomitrella patens*	0.05% fresh weight of tissue
	Expression of TXS with MeJA elicitation [83]	Pathway enzyme	Ginseng root	15.9 µg taxadiene per gram of dry weight
	Expression of TXS with MeJA elicitation [84]	Pathway enzyme	*Nicotiana benthamiana* plant	~40 µg taxadiene per gram of dry weight
	Expression of eight genes in the early part of paclitaxel biosynthetic pathway [85]	Pathway enzyme	*Saccharomyces cerevisiae*	1 mg l^{-1} of taxadiene ~25 µg l^{-1} of taxadine-5α-ol

(*continued overleaf*)

Table 8.2 *(Continued)*

Hosts	Engineering strategies	Role in the paclitaxel pathway	Expression system	Main observations
	Expression of TXS, an increase in precursor pool by gene overexpression and manipulation in feedback inhibition and regulators [86]	Pathway enzyme and regulator	*Saccharomyces cerevisiae*	9 mg l^{-1} of taxadiene
	Expression of TXS and two genes in the geranylgeranyl disphosphate biosynthetic pathway [87]	Pathway enzyme	*Escherichia coli*	1.3 mg l^{-1} of taxadiene
	Expression of TXS and other genes in upstream pathways with a "multivariate-modular" approach [15]	Pathway enzyme	*Escherichia coli*	1 g l^{-1} of taxadiene
	Expression of TYH5α, TAT, TYH10β, and other genes in *S. cerevisiae* with a stable mutualistic *E. coli* and *S. cerevisiae* consortium being constructed [88]	Pathway enzyme	*Escherichia coli* and *Saccharomyces cerevisiae* consortium	33 mg l^{-1} of taxadine-5α-ol 1 mg l^{-1} of taxadien-5α-acetate-10β-ol

hosts. Later, the same group expressed several genes catalyzing taxadiene into subsequent intermediates in the paclitaxel pathway in *S. cerevisiae* and built a stable mutualistic *E. coli* and *S. cerevisiae* consortium that produced 33 mg l^{-1} of taxadine-5α-ol and 1 mg l^{-1} of taxadien-5α-acetate-10β-ol [88].

To further increase the taxadiene level in *E. coli,* "omics" techniques were used to elucidate the potential enzymes needed to be modified. Results from a transcriptomics study suggested that differences in pyruvate metabolism might lead to the disparity in taxadiene biosynthesis between a high-producing *E. coli* strain and a low-producing one. [89]. An overexpression algorithm was employed to identify the overexpression that could increase the taxadiene production flux, based on a transgenic *E. coli* genome-scale metabolic model with taxadiene biosynthetic reactions included [90]. The following experiments confirmed the predicted results to a certain degree. Overexpression of the three predicted genes, *ppk* (polyphosphate kinase), *sthA* (pyridine nucleotide transhydrogenase), and *purN* (phosphoribosylglycinamide formyltransferase), with one key gene in MEP pathway, *idi* (isopentenyl diphosphate isomerase), improved taxadiene titer over 12-fold as compared to the parent strain.

Since the expression of some of the genes originating from plants requires specialized environments or compartments, the host strain *E. coli* alone is not competent to produce subsequent metabolites after taxadiene in the paclitaxel pathway. The production of taxadine-5α-ol and taxadien-5α-acetate-10β-ol in *E. coli* and *S. cerevisiae* consortium brings some inspirations in the heterologous production of natural products: (i) we can take full usage of the properties in different species; And (ii) spatial segregation the whole pathway into different microbes helps to prevent some internal regulation in the pathway, and pathway modules can be separately optimized.

"Omics" technologies have also been widely applied in *Taxus* sp. cell cultures, tissues, and plants to find the potential genes involved in paclitaxel synthesis. Polymerase-chain reaction (PCR)-based suppression subtractive hybridization (SSH) on a *T. cuspidata* cell line identified the genes upregulated or downregulated by MeJA [91]. Since the *T. cuspidata* cell line could produce higher amounts of taxanes after MeJA elicitation, the upregulated cDNA library would presumably include genes related to paclitaxel biosynthesis. Several genes previously known to be involved in the paclitaxel pathway were confirmed to exist in the upregulated library. Four putative taxadiene synthases, five novel putative taxoid hydroxylases, and two newly identified candidate acyl transferases were proposed in the analysis, which further enhanced the understanding of paclitaxel biosynthesis. Hao *et al.* [92] *de novo* assembled the *Taxus mairei* transcriptome using Illumina paired-end sequencing (RNA-seq) and investigated the transcriptome differences among three *T. mairei* tissues: root, leaf, and stem. From the Illumina dataset, 211 candidate genes involved in the paclitaxel biosynthetic pathway were proposed. Transcriptome differences among plant tissues, as well as the subsequent metabolome profiling, suggested that the expression of the taxane biosynthetic genes remained highest in roots, which also had a relatively simple metabolome profile with large quantities of various taxanes, including paclitaxel.

8.3.3
Podophyllotoxin Lignans

PTOX (Figure 8.5), an aryl tetralin lactone, is extracted mainly from the roots and the rhizomes of *Podophyllum* species. PTOX occupies a special place among lignin natural products, considering that its glucopyranoside derivatives have antitumor functions. However, PTOX is too toxic to be applied in humans. Several semisynthetic derivatives of PTOX (Figure 8.5) with lower toxicity, such as etoposide, etopophos, and teniposide, are widely applied as anticancer drugs to treat lung cancer, a variety of leukemia, and genital tumors [93–95].

P. hexandrum (in India and Nepal) and *P. peltatum* (in America) are the two main *Podophyllum* species that produce PTOX. The rhizome of *P. hexandrum* yields the highest amount of PTOX (4.3% of total dry weight), while the rhizome of *P. peltatum* contains 0.3–1.0% PTOX by mass.

Over 40 plant and fungus species have been shown to produce PTOX. Callus, cell, hairy root, and mycelial *in vitro* cultures of these species have been generated and tested for their potential for PTOX production. The few *in vitro* cultures producing significant amounts of PTOX are derived from *Podophyllum* sp., *Callitris drummondii*, and the Iranian *Linum* species, mainly including *L. album* and *L. persicum* [96]. Although the *in vitro* cultures of *Podophyllum* sp. can produce considerable amounts of PTOX (up to 0.3% of DW), they grow very slowly and are stressed, signified by the level of browning observed. Therefore, the *in vitro* cultures of *L. album* have become more attractive because of their relatively fast growth rate and abundance of PTOX.

The approaches that are commonly used to improve the *in vitro* production of PTOX in plant organ cultures include optimization of cultivation, precursor feeding, and elicitation [96]. The highest amount of PTOX (2.26% DW) was achieved in the coculture of *L. album* suspension cells with the live fungus *Sebacina vermifera* [97]. In addition, several endophytic fungi cultures have the capacity to produce PTOX, such as *Fusarium solani* from *P. hexandrum* [98], *Phialocephala fortinii* from *P. peltatum* [99], *Trametes hirsuta* from *P. hexandrum* [100], and

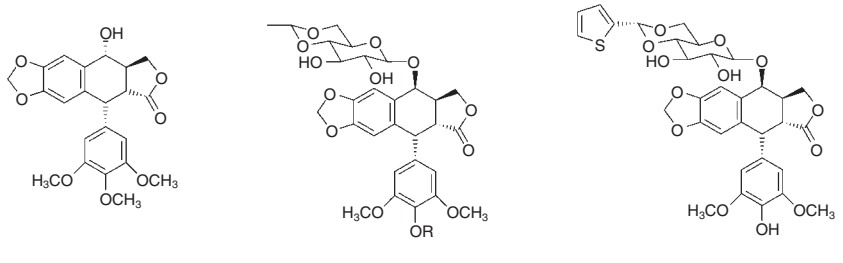

Figure 8.5 Chemical structures of podophyllotoxin, etoposide, etopophos, and teniposide.

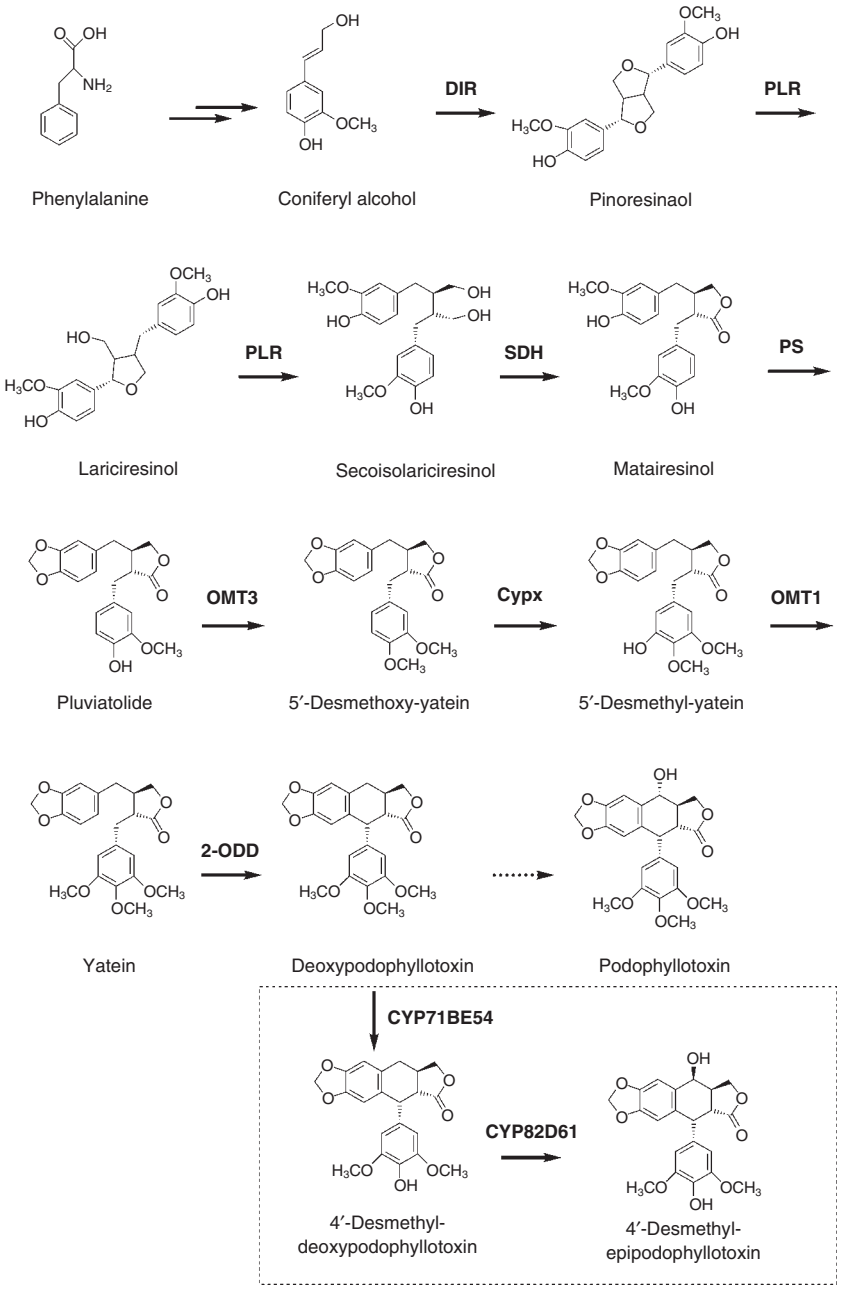

Figure 8.6 Podophyllotoxin biosynthetic pathway. DIR, dirigent protein; PLR, pinoresinol-lariciresinol reductase; SDH, secoisolariciresinol dehydrogenase; PS, pluviatolide synthase; OMT3, *O*-methyltransferase 3; CYPx, cytochromes P450 (CYP71CU1); OMT1, *O*-methyltransferase 1; and 2-ODD, 2-oxoglutarate/Fe(II)-dependent dioxygenase. The double arrows indicate multiple reactions; the dashed arrow represents uncharacterized reactions, and the dashed box encloses a bypass of the podophyllotoxin biosynthetic pathway.

F. oxysporum from *Juniperus recurva* [101]. The accumulation of PTOX in these fungi is approximately 30 μg g^{-1} DW.

Presently, no metabolic engineering effort of PTOX biosynthesis has been reported. Rajesh *et al.* [24] developed an efficient transformation protocol into *P. hexandrum* embryogenic cells, providing a basis for future gene transfer into PTOX-producing plants to optimize the production systems.

After the genes encoding the P450 enzyme pluviatolide synthase were discovered in both *P. hexandrum* and *P. peltatum* via transcriptome analysis in 2013 [102], four subsequent genes in the PTOX biosynthetic pathway in *P. hexandrum* were identified by Lau and Sattely in 2015 [103]. So far, only the last enzyme catalyzing the conversion of deoxypodophyllotoxin to PTOX in the PTOX biosynthesis is missing. In addition, Lau and Sattely also found two more genes in the natural host, catalyzing the step from deoxypodophyllotoxin to 4′-desmethyl-epipodophyllotoxin, which is a precursor to the semisynthetic, clinically used reagent etoposide. The gene identification process relied heavily on analyzing the RNA-seq data or the transcriptome data. During the discovery of pluviatolide synthase, sequence homology search was applied using double criteria as query sequences; the first one was to use nine different known P450s in plant secondary metabolism, and the second one was to use the enzymes with the same catalytic function (the methylenedioxy bridge formation) in lignin and alkaloid biosynthesis. In Lau and Sattely's work, they compared the RNA-seq data from wounded and unwounded leaf tissues to pinpoint the upregulated genes since the PTOX pathway is more active in the wounded condition and further narrowed down the range of gene candidates with the four predicted enzyme types for the reactions in the PTOX pathway. With heterologous expression of the gene candidates in *Nicotiana benthamiana* and the untargeted metabolite profiling using mass spectrometry, six enzymes either in the original PTOX pathway or in the other metabolite branches were identified (Figure 8.6).

8.3.4 Camptothecin Quinoline Alkaloids

CPT (Figure 8.7) is a modified MIA with the indole moiety changed to a quinolone moiety during the CPT biosynthesis. CPT was first isolated in 1958 from the extracts of the tree *C. acuminate.* In preclinical studies, CPT was found effective in curing colonic and gastric tumors [104]. The water-soluble CPT semisynthetic derivatives irinotecan and topotecan (Figure 8.7) were approved by FDA in 1996 to mainly treat ovarian, colon, and lung cancers. CPT has been isolated from at least 16 different angiosperms, and the current commercial production of CPT is through extraction from the bark and seeds of *C. acuminate* and *Nothapodytes foetida.* All plant organs of *C. acuminate* contain certain amounts of CPT, among which young leaves have the highest level (~0.4% DW, 50% and 250% higher than that in seeds and bark, respectively). Thus, repeated harvest of *C. acuminate* young leaves without destroying the trees is a potentially sustainable approach for CPT production [105].

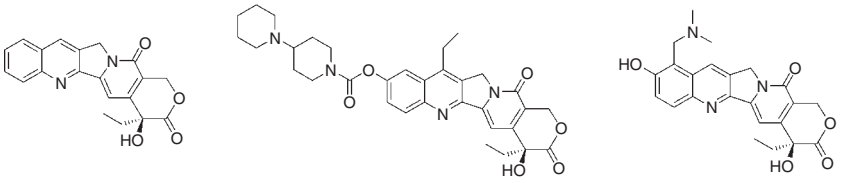

Figure 8.7 Chemical structures of camptothecin, irinotecan, and topotecan.

Numerous studies on the CPT production from *C. acuminate*, *Ophiorrhiza pumila*, or *N. foetida* callus cell suspension, and hairy root cultures have been reported. Generally, CPT levels in *in vitro* plant tissue cultures are 100- to 1000-fold lower than that *in planta* [106, 107]. To date, the highest reported yield of CPT (1.3% DW) is from optimized callus cultures that were derived from cotyledons of *N. foetida* [108]. The highest titers of secreted CPT (0.035 mg ml^{-1}) and the analog 9-methoxycamptothecin (0.026 mg ml^{-1}) were observed in *N. foetida* cell suspension culture medium [109]. Hairy root cultures induced from three *Ophiorrhiza* species produced CPT, in which *O. pumila* hairy roots accumulated the highest specific yield of CPT (0.08% DW) [110]. As in cell suspension culture, the partially secreted CPT in hairy root culture facilitated product enrichment by adding an absorbent resin [111]. Later, *O. pumila* hairy root culture in a 3-l bioreactor was established with approximately 7 mg l^{-1} total CPT titer including 17% secreted CPT [112].

Entrophospora infrequens, one of the endophytic fungi isolated from the twigs of *N. foetida*, was found to produce a relatively high specific yield of CPT, 4.96 ± 0.73 mg 100 g^{-1} DW, in a bioreactor at 48 h [113]. Two endophytic fungi strains of *F. solani*, isolated from *Apodytes dimidiata*, produced CPT (maximum 53 μg 100 g^{-1} DW) and its analogs 10-hydroxycamptothecin (maximum 8.2 μg 100 g^{-1} DW) and 9-methoxycamptothecin (maximum 44.9 μg 100 g^{-1} DW) after 4 days of incubation in broth culture [114]. Other fungi isolated either from *C. acuminate* [115] or from *N. foetida* [116] also produced CPT and its analogs but in small amounts (Figure 8.8).

Since vinca alkaloid-producing plants and CPT-producing plants share almost the same strictosidine synthetic pathway, it would be possible to enhance CPT production by introducing *C. roseus* genes encoding enzymes in the upstream of the strictosidine pathway or encoding transcriptional activators of the whole TIA pathway into CPT-producing plants. Geraniol-10-hydroxylase (G10H) has been reported as a highly regulated enzyme in the upstream TIA pathway in *C. roseus.* Co-overexpression of G10H and STR in *O. pumila* hairy root line led to an increase of 56% on the CPT yield (1.77 mg g^{-1} DW) compared with the non-engineered hairy root line [117]. ORCA3 is a transcription factor from *C. roseus* that activates several genes in the whole TIA biosynthetic pathway. Ni *et al.* overexpressed the ORCA3 gene in *C. acuminate* hairy root to study the effect of ORCA3 regulator on

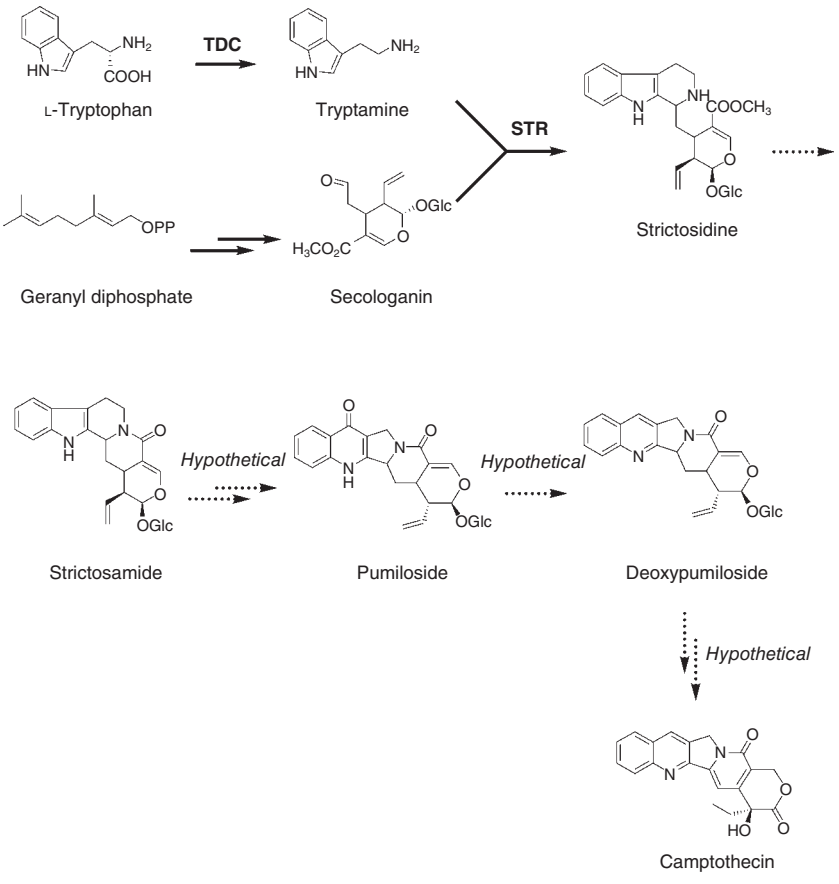

Figure 8.8 Camptothecin biosynthetic pathway. TDC: tryptophan decarboxylase; STR: strictosidine synthase. The double arrows indicate multiple reactions, and the dashed arrows represent uncharacterized reactions.

CPT biosynthesis [118]. The specific yield of CPT in ORCA3 transgenic hairy root lines increased 1.5-fold compared with the control, indicating that the ORCA3 regulator positively affected CPT synthesis.

Several "omics" works have been conducted to further characterize the CPT biosynthetic pathway. The transcriptomic datasets for three medicinal plants, *Camptotheca acuminata* (producer of CPT), *C. roseus*, and *Rauvolfia serpentina*, that produce various MIAs were obtained via next-generation sequencing technology [119]. Mining of these datasets will enable the discovery of new genes involved in MIAs pathway in the three species or even other species. The combined analysis of transcriptomic datasets with untargeted metabolic profiling datasets in *O. pumila* has further enhanced the understanding of the CPT biosynthesis [120]. The untargeted metabolic profiling by Fourier transform

ion cyclotron resonance-mass spectrometry (FTICR-MS) provided evidence for identifying the possible intermediates during the reactions from strictosamide to CPT. Metabolic profiling showed that CPT accumulated in hairy root culture but not in suspension cells. Thus, the classification of the P450s enzymes and glycosidase-like proteins in the upregulated unigenes in hairy roots compared with that in the cell suspension culture provided candidates of the P450s enzymes and the glycosidase-like enzymes in the post-strictosamide CPT biosynthetic pathway. Among all the candidate genes, the deglycosylation gene candidate was proposed according to the sequence similarly to the reported analogous gene from *R. serpentina* [121].

8.4 Prospects

8.4.1 Identification of Intermediates in the Biosynthetic Pathways of Anticancer Drugs

Metabolomics or metabolite profiling is widely applied in drug discovery and identification of intermediates. Anticancer drugs are usually produced through secondary metabolic pathways in plants, most of which have not been well characterized because of the low levels of secondary metabolite concentrations. In particular, the major part of the CPT biosynthetic pathway is still undefined. Typically, when referring to analytical instruments, MS coupled with HPLC or gas chromatography (GC) is effective for most of the plant metabolic profiling and metabolomics, especially when high-resolution MS and tandem MS techniques are put into practice. However, the MS technique, even the tandem MS technique, cannot provide sufficient information to identify the chemical structures of isomers. For instance, the accurate molecular weights acquired by FTICR-MS helped to determine the elemental composition of unknown metabolites in the proposed CPT biosynthetic pathway, but no further structure analysis could be provided by MS [120]. NMR is complementary to the MS-based metabolomics platform. In recent years, HPLC-NMR has assisted in natural product profiling, especially of marine natural products [122–124], which paves the way for metabolite identification and profiling in other plants. Secondly, metabolic differential accumulation in diverse plant tissues, in different *in vitro* cultures or in various environments, facilitates the search for target metabolites. Finally, new plant reference biochemical databases and metabolite profile databases are needed in the future. Until recently, metabolite profile databases were scarce, whereas several microarray databases, including ArrayExpress and Gene Expression Omnibus (GEO), have stored gene expression data [125]. Archiving metabolite profile data in a specified format would undoubtedly facilitate data sharing.

8.4.2 Discovery of Unknown Genes in Biosynthetic Pathways

Among the four categories of anticancer drugs, most of the genes in the biosynthetic pathways of vinca alkaloids, taxane diterpenoids, and PTOX lignans have been identified from extensive research studies using diverse gene characterization techniques. However, there are still several uncharacterized reactions in the pathways that block the strategy of importing the entire pathways into heterologous hosts. In order to further discover unknown genes, deeper "omics" techniques, especially proteomics and transcriptomics differential expression in diverse explant types, different growth stages, or various induction conditions, are needed to facilitate the determination of putative gene candidates. Alex *et al.* not only built the CathaCyc database but also integrated the transcriptome expression levels of the known genes in secondary metabolism in 23 different tissues and treatments to establish a hierarchical cluster of these genes [61]. This research can be extended to collect all the RNA-seq data of different tissues and treatments in *C. roseus* and build a more accurate hierarchical cluster based on the transcriptome differential expression. This approach would provide a more reliable and accurate means to further pinpoint the target genes. A similar strategy can also be applied to proteomics. Even without the aforementioned "omics" analysis, screening a cDNA library to characterize genes in either plant hosts or microbial hosts is a feasible technique, especially for the genes involved in the plant stress response pathways [126, 127].

8.4.3 Production of Anticancer Drugs in Microbial Hosts

Besides producing anticancer drugs in homologous and heterologous plant tissue cultures, production of plant-derived secondary metabolites in microbial hosts is a promising avenue despite several challenges. Taxane diterpenoids could become the first case among all the plant-derived anticancer drugs that are produced in microbial hosts because of their relatively well-characterized pathways. The biggest issue is how to efficiently express plant cytochrome P450 enzymes in microbes, such as *E. coli* and *S. cerevisiae.* Ajikumar *et al.* have applied the transmembrane engineering strategy and generated chimera enzymes composed of P450 and CYP450-reductase (CPR) to convert taxadiene to taxadien-5α-ol [15]. Cytochrome b_5 reportedly enhances the activity of certain cytochrome P450s [128]. Therefore, coexpression of P450s together with CPR and cytochrome b_5 may increase the enzyme activity. On the other hand, how to maximize and balance the flux toward the target product and minimize the flux into byproducts is the key to heterologous production. Here, the substantial body of work in microbial metabolic pathway engineering, including integration of computation analysis with flux measurements and a genome-scale model, can shorten the development time for strain design [129].

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