Chapter 1
Metabolic Engineering: New Approaches in Pharmaceutical Production

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Abstract Natural products, natural products-derived, and natural products-inspired compounds represent a major sector of drugs and drug leads. Nature has the potential to produce compounds built to interact with biological systems. Mostly are produced as secondary metabolites by a living organism for different purposes such as defense and communication. Natural compounds usually contain multiple chiral centers within strictly specific molecular architecture that are very difficult to be obtained by combinatorial synthesis for economic supply of drugs. Meanwhile, natural compounds serving as drugs or precursors for synthesis are having several drawbacks with insufficient and fluctuating supply as the most serious problems. For these reasons metabolic engineering exploiting advanced biotechnology techniques such as sequencing, recombinant DNA, and protein engineering can add new incentives to produce secondary metabolites. Metabolic engineering is based on manipulating metabolic networks/pathways within prokaryotic organisms or cultured eukaryotes. Both organisms have common features, including a cytoplasmic membrane, DNA that codes for genetic information, and RNA that is subsequently translated into proteins. Successful study of the biosynthetic pathways responsible for the production of natural products together with advances in fermentation technologies and expression systems, variation in tool kits, and mutation tools allowed metabolic engineering to be a promising approach for the targeted production of a wide variety of natural compounds (taxol, camptothecin, vincristine, artemisinin, silymarin) with bacterial, fungal, and plant origins.

Keywords Metabolic engineering · Plant · Pathway · Secondary metabolites · Taxol · Camptothecin · Vincristine · Artemisinin · Silymarin

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Introduction

Metabolic engineering (ME) is defined as the directed development and improvement for the production of biochemical, biofuels, pharmaceuticals, and medicinal products by modifying and bioengineering a particular metabolic pathway(s). This method includes functional alteration of metabolic pathways toward better understanding and utilizing cellular/biological pathways. In addition, metabolic engineering is driven to advance different commercial applications by which we can enhance the strain's efficiency for producing valuable metabolites. The standard metabolic engineering method requires controlling the expression of a specific protein(s) either in overexpression or downregulation manner within a metabolic pathway, which promotes the biological cell to produce a new product (Yadav et al., 2018).

Directionality is the core of a successful metabolic engineering system, but scaling, stability, and productivity are essential factors. Unfortunately, many trials have failed during the last decade due to problems confronted in scaling up or other critical factors. Notably, meanwhile, the plant production systems are generally slower than microbial systems; plants are more suitable for commercial production due to their autotrophic and scale-up features, in addition to their low cost, especially when agricultural infrastructure is available. However, for plant production systems to be effective, using either fermentation or agriculture, stability and high yields of the bioengineered metabolites must be ensured (Nielsen, 2001).

Since Bailey's formal recognition of metabolic engineering in 1991, it demonstrated its powerful capability as a modern approach in engineering numerous microbial strains to produce hundreds of chemicals and bioproducts from raw substances (Nielsen, 2001; Bailey, 1991; Birchfield & McIntosh, 2020).This production is classically achieved by introducing targeted genetic changes through recombinant DNA technologies and analyzing all possible consequences of these changes at the cellular/biological levels. However, the number of bioproducts touching industrial-scale production is limited due to the high production costs, including raw materials, fermentation process, and purification. Thus, although immense efforts have been paid in the metabolic engineering field, it is still needed to enhance strain performance in order to create competitive bioprocesses compared to traditional chemical processes. Also, ME projects at their beginning stages frequently fail due to many critical practical issues occurring during industrial scaling production (Choi et al., 2019). Moreover, inaccurate trial-and-error cycles to enhance strain/plant performance usually resulted from a poor understanding of the metabolic and/or genetic mechanisms controlling a particular pathway in the host organisms, especially in the laboratory (Courdavault et al., 2021).

In a global sense, metabolic engineering is not considerably different from genetic or cellular engineering since they all intended to manipulate genes to generate a product of interest. However, metabolic engineering is focused explicitly on understanding the complex metabolic pathways/networks; meanwhile, genetic and cellular engineering focuses on particular enzymatic reactions inside the cell. Thus, a systemic approach is required for the straightforward and precise design of

complex pathways through remodeling the optimal cell pathways, which can divert cell supplies to achieving more beneficial fitness (Choi et al., 2019; Courdavault et al., 2021; Liu & Nielsen, 2019). Accordingly, metabolic engineering represents a robust framework for dissecting differential gene expression at a genome-wide level, protein production, and the levels of a wide range of intracellular metabolic fluxes. Therefore, in order to increase the metabolic engineering approach productivity of certain products, many questions need to be carefully answered: (1) which host/organism must be chosen, (2) what commodity could be produced, and (3) which ME approach could be applied (Liu & Nielsen, 2019). Therefore, this chapter highlighted the different biological systems that can be used in the ME, examples of current commodities produced, and lastly, the various engineering strategies that can be employed to engineer and improve cellular production capabilities of specific metabolites that are in clinical use and are facing difficulties in commercial production hindering stable market supplies.

Strategies

Several approaches are adopted for secondary metabolites development or enhancement using bacteria, such as (1) overexpressing of a gene family or set of transcription factors to simultaneously stimulate/initiate diverse pathway-related endogenous genes to improve the synthesis of particular metabolites, (2) engineering of regulatory networks controlling a particular pathway through delicate tuning of many enzymes within the target pathway to assure an adequate accumulation of precursors and consequently boost metabolic flux through the targeted pathway, (3) gene deletion (knocking out) or/and insertion of essential enzyme genes regulating or controlling the pathway of the product of interest, (4) stimulation using specific substrates/elicitors, and (5) combining one or more of the above approaches to maximize the synthesis of the target metabolites. Recently, gene editing via CRISPR/Cas9-based activation and/or repression schemes can also be used in metabolic engineering development and improvement. Therefore, metabolic engineering represents potential cell factories to produce pharmaceuticals, amino acids, bioplastics, biofuels, new bioproducts, silk proteins, etc. (Liu & Nielsen, 2019; Zhu et al., 2020).

Many designed tools were implemented in metabolic engineering during the last decade for more precise pathway construction and optimized pathway development. These include selecting hosts with well-known and knowledgeable omics databases, computational pathway simulations resources/tools paired with custom enzyme designs, and guided evolution approaches that attempt to maximize the output of the desired product (Zhu et al., 2020). In addition, advancements in microbial engineering have enabled a wide range of plant metabolites to be manufactured (Fu et al., 2018).

Host Selection

Metabolic engineering is based on manipulating metabolic networks/pathways within prokaryotic organisms or cultured eukaryotes. Both organisms have common features, including a cytoplasmic membrane, DNA that codes for genetic information, and RNA that is subsequently translated into proteins. However, the distinguishing factors between the two kinds of organisms are revealed intracellular when comparing their genomes or organelles. For example, prokaryotes, including the Bacteria kingdom, include mostly stable chromosomal DNA with one inherent single loop and lacks membrane-bound organelles. In contrast, eukaryotes, such as Fungi, Plantae, and Animalia kingdoms, comprise DNA molecules organized in tightly bound chromosomes and contain membrane-bound organelles (e.g., chloroplasts, mitochondria, and the Golgi apparatus). The bacteria, fungi, plant, and animal cells are currently used in different metabolic engineering applications to manufacture products, and accurate analysis is required to decide the most fitting host for a particular target (Nabavi et al., 2020).

The selection of an organism that will be used in the production for an industrial biotechnology process is mainly driven by its potential to manufacture the product of interest efficiently. Genetically engineered cells are usually used to make two main kinds of products, proteins, and nonproteins (Zhu et al., 2020). Protein products comprise those used for human therapeutics, food processing, and industrial catalysts, and are generated by introducing their encoding genes into the host genome. Besides, nonprotein products, including metabolites such as amino acids, biofuels, antioxidants, and vitamins, are created by inserting genes coding for necessary enzymes that manipulate precursors into desired metabolites. Cultures of complex eukaryotes (yeast, plant cells, mammalian cells) are used to produce proteins, whereas prokaryotes and cultures of simple eukaryotes (yeast, filamentous fungi, and plants) are used more frequently in the production of nonprotein metabolites (Nabavi et al., 2020).

For long decades, bacteria, fungi, and plants have been acknowledged as excellent sources of natural compounds, especially those with multiple bioactive attributes and associated applications in the domains of nutrition and human health. Among the common valuable plant natural products (PNPs), scientists have identified those usually used to treat different pathologies such as cancers, infectious diseases, or cardiovascular diseases. Additionally, selected PNPs have been further processed into various active pharmaceutical constituents at an industrial scale. Examples of the most famous PNPs, and their associated applications, include tropane alkaloids from Solanaceae (mostly anticholinergics), wormwood sesquiterpene lactones (antimalarial drugs), monoterpene indole alkaloids of Apocynaceae (antihypertensive and anticancer drugs), poppy isoquinoline alkaloids (antitussives, antimicrobials, analgesics, vasodilators), yew taxane-type terpenoids (anticancer drugs), and mayapple lignans (anticancer and immunosuppressive drugs) (García-Granados et al., 2019).In the following pages, we will throw the light on the engineering of some vital metabolites that are used in the treatment of some vital

diseases such as cancer, malaria, and hepatitis. The drug markets need these metabolites, and the demand for them is increasing day by day. These metabolites as arranged in the text are taxol, camptothecin, vincristine, artemisinin, and silymarin.

Examples of Engineered Metabolites

Taxol (TXL)

Paclitaxel, commercially known as taxol, is a terpenoidal pseudo-alkaloid that has potent antitumor activity (Sabzehzari & Naghavi, 2019). Paclitaxel is one of the strong plant-derived anticancer drugs that was first isolated from the park of Pacific yew. It was first isolated from the bark of the slow-growing coniferous tree *Taxus parvifolia* known as Pacific yew or western yew (Mansukhlal et al., 1971) in a very low yield 0.01–0.05 % (Cragg, 1988). Being only isolated from the bark, it is very hard to meet the increasing clinical consumption of taxol. One tree yields 18 kg of green bark, which becomes about 9 kg after drying (Connolly, 1988). 27,700 kg of dried bark yield about 4 kg of taxol (Cragg, 1988). Despite its clinical success, limited supply of paclitaxel for clinical trials is a very serious drawback. Meanwhile, combinatorial synthesis is very hard due to multiple chirality. Meanwhile, consumption of taxol is expected to show a yearly growth of 8.2% between 2020 and 2025 (Ning et al., 2020).

Biosynthetic Background

Biosynthesis of paclitaxel is divided into three stages (Fig. 1.1). The first is the MVA/MEA pathway-dependent synthesis of isopentenyl pyrophosphate (IPP) as terpene precursor which is the rate-limiting step (Soliman & Tang, 2015), the second is the synthesis of baccatin III as taxol's main carbon framework, and the third is the synthesis of nitrogenous side chain originated from the amino acid phenylalanine (Sabzehzari & Naghavi, 2019; Croteau et al., 2006).

Conventional Strategies to Increase TXL Yields

Due to its enormously increasing clinical importance, many attempts have been made to increase the production of paclitaxel including chemical synthesis that mainly depended on the exploitation of commercially abundant biosynthetic precursors as starting materials (Denis et al., 1988; Holton et al., 1994; Borah et al., 2007), searching for microbial sources that can be bioengineered to increase the production such as *Taxomyces andreanae* isolated from *Taxus chinensis* (Stierle

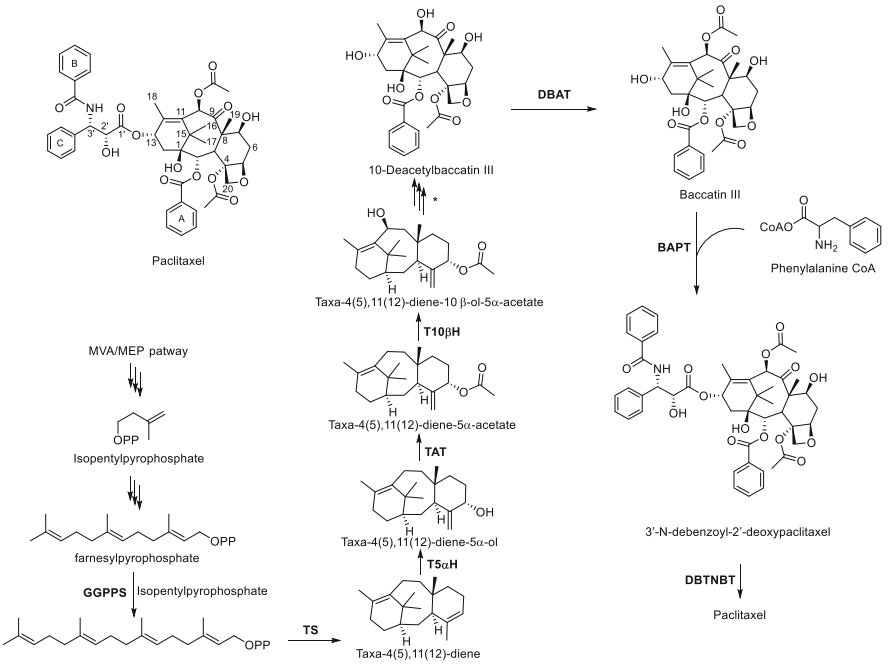

Fig. 1.1 Biosynthetic pathway for taxol [abbreviations: *MVA* mevalonic acid, *MEP* 2-C-methyl-D-erythritol-4-phosphate, *GGPPS* geranylgeranyl pyrophosphate synthase, *TS* taxa-4(5),11(12)-diene synthase (committed step), *T5αH* taxa-4(5),11(12)-diene-5α-hydroxylase, *TAT* taxa-4(5),11(12)-diene-5α-ol-O-acetyltransferase, *T10βH* taxane-10β-hydroxylase; *oxetane ring formation and branch migration enzymes taxane 2α-O-benzoyltransferase (*T2BT* or *DBBT* debenzoyltaxane-2/-α-O-benzoyltransferase) and C-13 hydroxylation; *DBAT* 10-deacetylbaccatin III-O-acetyltransferase, *BAPT* baccatin III 13-O-(3-amino-3-phenylpropanoyl)transferase, *DBTNBT* 3/-N-debenzoyl-2/-deoxytaxol-N-benzoyltransferase, after side chain hydroxylation by unknown enzyme; multiple arrows mean more than one step]

et al., 1993), and finding other plant species that can produce paclitaxel such as *T. chinensis*, *T. wallichiana*, *T. brevifolia*, *T. canadensis*, *T. floridana*, *T. cuspidata*, *T. baccata*, and *T. globosa* (Liu et al., 2016).

Biotechnological Strategies

On the other hand, biotechnology and metabolic engineering were extensively used to increase the production of paclitaxel.

Cell Culture

The first report of *Taxus* cell culture was in a patent application and an abstract (Christen et al., 1989, 1991). Back in 1992, Fett-Neto and coworkers managed to obtain an in vitro culture of the taxol-producing plant *T. cuspidata* (Fett-Neto et al., 1992). With the development of tissue culture technologies via optimization of culture conditions such as media composition procedure, specific metals, light, pH, temperature, osmotic pressure, gas contents, and elicitors exploitation (Howat et al., 2014), higher amounts of taxol could be produced from a variety of species such as *T. cuspidate*, *T. canadensis*, *T. baccata*, *T. globosa*, *T. yunnanensis*, and *T. chinensis*, with taxol production improved from ~1–3 mg/L to ~77.5–153 mg/L (Liu et al., 2016; Onrubia et al., 2013; Bringi et al., 1995).

Heterologous Expression Systems

Another biotechnological approach for steady and enhanced production of taxol is the use of heterologous expression systems. In these systems a part of genome encoding the biosynthetic pathway of taxol is transferred to another type of cells through a suitable transformation vector. New cells will start the expression of enzymes necessary for taxol production, whose speed and yield could be engineered by controlling the fermentation conditions. Some of the successful heterologous expression systems are *Saccharomyces cerevisiae* yeast model (Dejong et al., 2006), *Escherichia coli* bacteria model (Ajikumar et al., 2010), and *Arabidopsis thaliana* plant model (Besumbes et al., 2004). The highest taxol content from *E. coli* was 570 mg/L (Biggs et al., 2016). Taxadiene is the first diterpene intermediate involved in taxol biosynthesis through baccatin III. Its production exhibited 40-fold increase in *S. cerevisiae* via retarding the competition between steroid and taxol biosynthesis in favor to taxol. That was done by the expression of GGPPS, tHMGR1, and flux control by transcription factor UPC2-1 (Engels et al., 2008). Moreover, acetyl-CoA accumulation in *S. cerevisiae* via acetaldehyde dehydrogenase and acetyl-CoA synthetize overexpression was found to increase the terpenoid content over steroid content in transgenic yeasts which can help in increasing taxol production (Shiba et al., 2007). Recently, Abdallah and coworkers managed to engineer the first *B. subtilis* strain that can produce taxadiene. They expressed the plant-derived enzyme taxadiene synthetize and overexpressed the eight key biosynthetic enzymes of MEV pathway to enhance geranylgeranyl pyrophosphate (GGPP) flux that led to a ~82-fold increase in taxadiene content when compared to wild-type *B. subtilis*. Taxadiene content reached ~17.7 mg/L (Abdallah et al., 2019). In the transgenic plant heterologous expression model *Arabidopsis thaliana*, Besumbes and coworkers managed to produce taxadiene in a yield of 600 ng/g of dry weight by overexpressing TASY and GGPP synthase. However, growth retardation resulted from taxadiene accumulation (Besumbes et al., 2004). On the other hand, TASY

overexpression in another model, *Physcomitrella patens* resulted in taxadiene production in a yield of 0.05% from fresh weight without growth retardation (Anterola et al., 2009).

IPP and GGPP are very important targets in taxol biosynthesis since they are the rate-limiting intermediates for this process. While trying to enhance IPP-mediated terpenoids biosynthesis, isopentenol utilization pathway (IUP) was transformed to *E. coli*, resulting in continuous phosphorylation of isopentenol (prenol or isoprenol) isomers and increased IPP production (Chatzivasileiou et al., 2019). IUP pathway optimization only needs the cofactor adenosine triphosphate, and it occurs within two reactions. Thus, IUP pathway is far much simpler than MEP or MVA pathways (Chatzivasileiou et al., 2019).

Overexpression Positive Effectors

Overexpression positive effectors can also play an important role in taxol biosynthesis. In 2017 Yu *et al*. suggested that manipulating the expression of some candidate genes in taxol biosynthetic pathway can affect taxol contents. Overexpression of the hydroxylation and acetylation enzymes (i.e., 2-debenzoyl-7, 13-diacetylbaccatin III-2-O-benzoyl-transferase (DBBT), 5-alpha-taxadienol-10-beta-hydroxylase (T10OH), taxadiene 5-alpha-hydroxylase (T5H), and taxadienol acetyltransferase (TAT)) increased taxol content. Later on, Wang and coworkers confirmed this approach when they revealed a ~ threefold increase in taxol content in *T. wallichiana* with overexpression of the genes WRKY, bHLH, MYB, and ERF (Wang et al., 2019). Taxadiene synthase (TS) is a pivotal enzyme in taxol biosynthesis (Köksal et al., 2011).

Knock-Outing Negative Effectors

Knock-outing negative effectors together with overexpression of positive effector genes can result in dramatic increase in taxol levels via blocking the competing downstream metabolic pathways. Sabzehzari and Naghavi (2018) used CRISPR/Cas9 and miRNA technology to achieve this strategy, in order to silence the genes involved in sterol biosynthesis that encode lanosterol/squalene synthase (Do et al., 2009) competing with terpene pathway for farnesyl pyrophosphate (FPP) (Sabzehzari & Naghavi, 2018).

Moreover, miR171 and miR164 were found to decrease paclitaxel biosynthesis in *T. baccata* (Hao et al., 2012). MiR171 and miR164 inhibit taxane 2α-obenzoyl-transferase and taxane 13α-hydroxylase, which are very important for the biosynthesis path of taxol (Ramírez-Estrada et al., 2016). Therefore, silencing MiR171 and miR164 using CRISPR/Cas9 or miRNA can promote paclitaxel production.

Camptothecin (CPT)

Camptothecin (CPT) is a modified monoterpene indole alkaloid (Fig. 1.2) possessing potent antitumor activities. FDA approved some camptothecin (CPT) derivatives, such as topotecan and irinotecan, which were developed to overcome poor water solubility and severe side effect, for clinical treatment of some human tumors such as ovarian cancer, colorectal cancer, and leukemia via binding DNA topoisomerase I and DNA leading to ternary structure inhibiting DNA relegation and DNA damage (Venditto & Simanek, 2010; Hsiang et al., 1985; Sirikantaramas et al., 2007). The main commercial source of CPT, which is the main starting material for the clinically used synthetic derivative, is some woody plant species such as *Camptotheca acuminata* and *Nothapodytes foetida* (Wall et al., 1966; Kai et al., 2015). The slow growth of such species, the low yield of CPT (average, 1 mg/g) (Lopez-Meyer et al., 1994), and environmental concerns of overharvesting woody plants cannot fulfill the increasing market demand that recently reached 2.2 billion US dollars in 2008 in the form of topotecan and irinotecan sales with high probability of increase (Kai et al., 2014). Meanwhile, combinatorial synthesis is yet not effective (Kai et al., 2015). Hence, improvement of CPT production could be achieved through the CPT production through biotechnology techniques such as introducing biosynthetic genes encoding enzymes responsible of its production to rapidly growing vector cells such as hairy roots which are rapidly growing, with genetic stability, easily elicited, and hormone free (Shi et al., 2020).

Biosynthetic Background

The biosynthetic pathway for CPT production is similar to several other terpenoid indole alkaloids (TIA), originating from strictosidine which is a condensed product of both the amino acid derivative tryptamine (nitrogen bearing moiety) and the monoterpene secologanin (terpenoid moiety). CPT biosynthesis (Fig. 1.3) was extensively studied over the last three decades (Kai et al., 2015). Knowledge of biosynthetic genes encoding enzymes for TIA and CPT enabled the production of transgenic cell suspension, hairy roots or seedlings with increased CPT yields, and

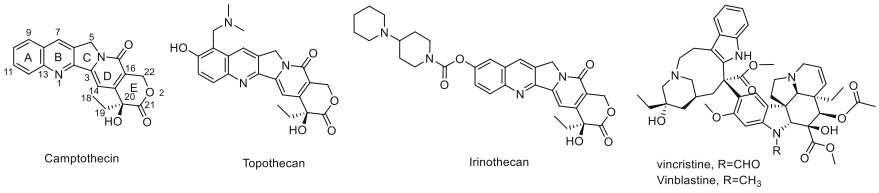

Fig. 1.2 Structures of some clinically important indole alkaloids

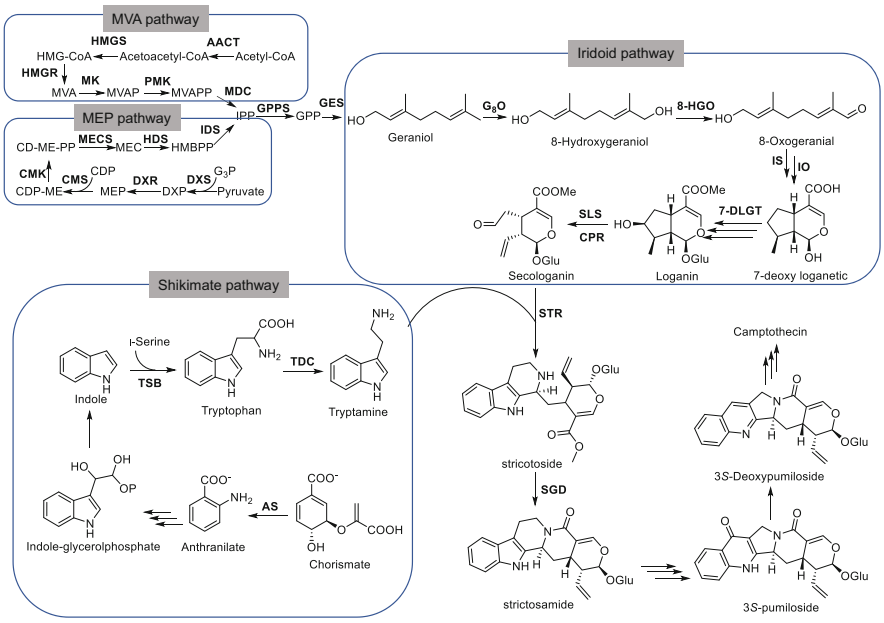

Fig. 1.3 Biosynthetic pathway for camptothecin [*CMS* 4-(cytidine 5-diphospho)-2-C-methylerythritol synthase, *CMK* 4-(cytidine 5-diphospho)-2-C-methylerythritolkinase, *MECS* 2-C-methylerythritol-2,4-cyclodiphosphate synthase, *HDS* hydroxymethylbutenyl 4-diphosphate synthase, *IDS* IPP/DMAPP synthase, *GPPS* geranyl pyrophosphate synthase, *CPR* NADPH-cytochrome P450 reductase, *GES* geraniol synthase, *G8O* geraniol-8-hydroxylase, *8-HGO* 8-hydroxy-geraniol oxidoreductase, *IS* iridoid synthase, *IO* iridoid oxidase (CYP76A26), *DLGT* 7-deoxyloganetic acid UDP-glucosyltransferase, *SLS* secologanin synthetase, *AACT* acetyl-CoA C-acetyltransferase, *HMGS* 3-hydroxy-3-methylglutaryl-CoA synthase, *HMGR* 3-hydroxy-3-methylglutaryl-CoA reductase, *MK* mevalonate kinase, *PMK* phosphomevalonate kinase, *MDC* mevalonate 5-diphosphate decarboxylase, *AS* anthranilic acid synthetase, *TSB* tryptophan synthase beta, *TDC* tryptophan decarboxylase, *STR* strictosidine synthase, *SGD* strictosidine beta-glucosidase; multiple arrows indicate multiple steps]

stable rapid production and that can overcome the difficulties of finding a stable transformation system for woody plants (Ni et al., 2011).

Hairy Root Culture

Interestingly in 2001, Saito and coworkers established a successful hairy root culture system of *Ophiorrhiza pumila* (Rubiaceae) transformed by *Agrobacterium rhizogenes* strain 15834, which carries the PGSGluc1 gene plasmid. The hairy roots grew in liquid culture media with 16-fold increase in the first 5 weeks. It produced CPT as the main alkaloid with 0.1% yield of dry weight. Meanwhile, CPT was found to accumulate in culture media and its production was increased by

adding the polystyrene resin (Diaion HP-20) to the culture media to absorb CPT and facilitate its regeneration and later release from the resin, rendering this method more suitable for commercial production (Saito et al., 2001).

Moreover, the jasmonate-responsive APETALA2 domain transcription factor called ORCA3 isolated from *C. roseus* strongly upregulated the expression of several TIA biosynthetic genes (van der Fits & Memelink, 2000). *C. acuminata* transgenic hairy root lines with overexpressed ORCA3 produced CPT with 1.5-fold increase (1.12 mg/g dw) (Ni et al., 2011).

In 2020 Shi and coworkers managed to increase the production of CPT from the hairy roots of *Ophiorrhiza pumila* which originally produce CPT in higher amounts compared to the wild-type plantlets and cell suspension culture that did not accumulate CPT. Comparative transcriptome analysis revealed that hairy roots of *Ophiorrhiza pumila* contained genes encoding key enzymes involved in CPT biosynthesis, which were mainly identified as *OpG10H* and *OpSLS*. The knockout of these genes by CRISPR-Cas9 editing resulted in CPT-free hairy roots whereas individual overexpression of both genes resulted in two transgenic hairy root lines, G line for *OpG10H* and S line for *OpSLS*, showing increased CPT contents up to 2.40 mg/g and 3.28 mg/g, respectively. On the other hand, SG line with overexpression of both genes exhibited CPT contents up to 3.50 mg/g (Shi et al., 2020).

Endophytes

On the other hand, several reports stated the isolation of CPT from plant endophytic fungi such as *Entrophospora infrequens*, isolated from the inner bark of *Nothapodytes foetida* (Puri et al., 2005; Amna et al., 2006), a novel endophytic fungus from the bark of *C. acuminata* (Kusari et al., 2009), and *Fusarium solani* from *Apodytes dimidiata* (Shweta et al., 2010). Although the yield of CPT from endophytic fungi was very low, it provides incentives for stable production of CPT through the improvement of culture conditions or metabolic engineering of the fungi via identification and manipulation of its biosynthetic gene clusters (BGCs).

Vinca Alkaloids (Vincristine (VCT) and Vinblastine (VBL)

Other anticancer alkaloids produced through TIA biosynthetic pathway and have increased market demand and clinical use are *Vinca* alkaloids, vincristine (VCT) and vinblastine (VBL) produced by *Catharanthus roseus* (Jacobs et al., 2004).

Such alkaloids are produced in very low yield and difficult to meet commercial pharmaceutical production. Meanwhile, they have multiple chiral centers, rendering their synthesis very difficult (O'Connor & Maresh, 2006).

Biosynthetic Background

They are formed from strictosidine through a stereoselective Pictet-Spengler reaction between tryptamine and secologanin (Kutchan, 1993; Stöckigt & Zenk, 1977; Maresh et al., 2008), catalyzed by the enzyme STR1 that is highly substrate-specific, limiting precursor-directed biosynthesis in plant cell culture to produce natural product analogs (McCoy & O'Connor, 2006).

Several attempts have been made to produce bis-indole *Vinca* alkaloids through biochemical engineering and cell suspension cultures without any successful result. Therefore, other trials have been directed toward metabolic engineering and manipulation of TIA pathway (Zhao & Verpoorte, 2007).

In 2007 Peebles and coworkers exploited ethanol-inducible promoter system to increase the biomass of *C. roseus* hairy roots to enable the production of higher amounts of the target *Vinca* alkaloids. Ethanol concentration greatly affected ethanol-inducible promoter at very low concentration of 0.005% that resulted in a sixfold increase in chloramphenicol acetyltransferase (CAT) reporter activity after 24 h and 24-fold after 72 h with 0.5% ethanol. Being commercially available and biodegradable, ethanol induction can be promising tool to increase *Vinca* alkaloids production (Peebles et al., 2007).

Sharma and coworkers found that overexpression of two upstream TIA pathway genes tryptophan decarboxylase (CrTDC) and strictosidine synthase (CrSTR) enhanced terpenoid indole alkaloid (TIA) pathway activity and antineoplastic vinblastine biosynthesis in *Catharanthus roseus* in the transgenic whole plant produced via transformation with *Agrobacterium tumefaciens* LBA1119. The produced transgenic plant exhibited twofold increase alkaloid production, ninefold increase in vindoline and catharanthine, and fivefold increase in vinblastine production (Sharma et al., 2018).

Recently, McGehee and coworkers found that carbon-based nanomaterials (CBNs) such as multiwalled carbon nanotubes (MWCNT) and graphene enhanced biomass production of *C. roseus* cell culture under dark conditions. It also doubled the production of total alkaloids under both dark and light conditions. Further HPLC analysis revealed that vincristine and vinblastine production was dramatically increased in CBN-treated cell cultures (McGehee et al., 2019)

Artemisinin (ATN)

Another example of clinically relevant natural compound with increasing demand for pharmaceutical industry is the sesquiterpene lactone artemisinin.

Artemisinin-based combination therapies (ACTs) are the frontline treatments for malaria (Dalrymple, 2013). Moreover, both artemisinin and its semisynthetic congener artesunate proved to be cytotoxic against several cancer cell lines and *Schistosoma* (Efferth, 2006; Utzinger et al., 2007; Efferth et al., 2011). Artesunate

exhibited strong activity against ganciclovir-resistant human *Cytomegalovirus* (Shapira et al., 2008). According to the published statistics of WHO, 229 million people are infected with malaria in 2019 and 409,000 people died of malaria in 2019 (World Health Organization, 2020). However, patients in endemic regions cannot benefit these therapies due to low yield of natural sources (White, 2008). Given the large number of cases, world demand for artemisinin is 130 tons per year (Artepal, 2010).

Biosynthetic Background

Artemisinin is a sesquiterpene lactone endoperoxide assembled from three isoprene units. The common sesquiterpene precursor is farnesyl pyrophosphate (FPP) that is generated via either the mevalonate-dependent or mevalonate-independent (MEP/DXP) pathway (Lange et al., 2000; Hunter, 2007). The committed step of artemisinin biosynthesis is the cyclization of FPP to amorpha-4,11-diene (amorphadiene) as shown in Fig. 1.4. This step is carried out by the enzyme amorphadiene synthase (ADS) (Bouwmeester et al., 1999). Amorphadiene is then oxidized by the cytochrome P450 monooxygenase, CYP71AV1, to artemisinic aldehyde (Ro et al., 2006; Teoh et al., 2009). Its reduction to dihydroartemisinic aldehyde is catalyzed by artemisinic aldehyde Δ11 (13) reductase, BDR2 (Zhang et al., 2008). Artemisinin is mainly produced in the leaves in glandular hairs. Artemisinin biosynthesis is mediated by three key enzymes exclusively expressed in apical cells of glandular trichomes. Although both apical trichome cells and

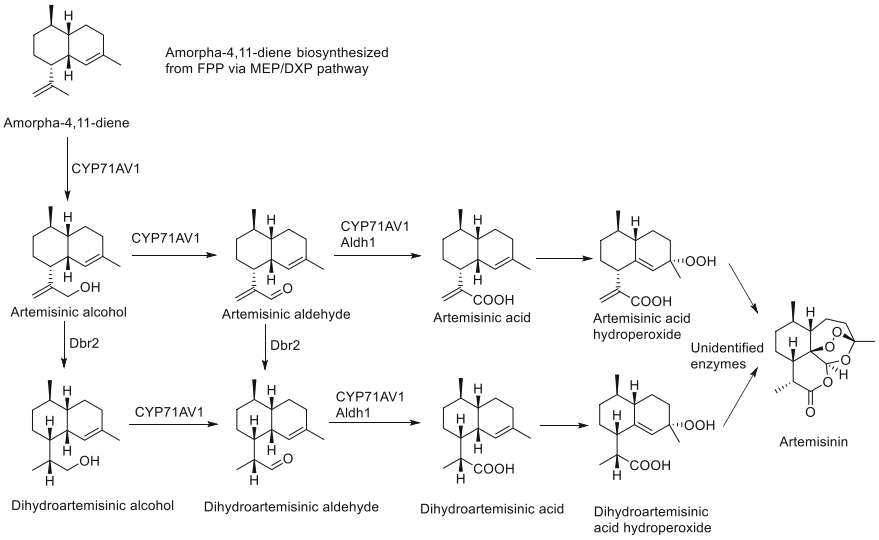

Fig. 1.4 Biosynthesis of artemisinin

subapical chloroplast-containing cells express farnesyl pyrophosphate (FPP) synthase, only glandular trichomes can produce artemisinin (Olsson et al., 2009). Artemisinin production is sensitive to variation in many factors such as genetic variation, cultivation processes, and harvesting time. Moreover, biosynthesis is affected by biotic and abiotic conditions (Omer et al., 2013, 2014, 2016; Ferreira et al., 1995; Davies et al., 2009, 2011). Artemisinin yield from *Artemisia annua* is ~0.5% of dry weight (Brisibe & Chukwurah, 2014), whereas the high-yield variety "CIM-Arogya," developed for marker-assisted breeding, has maximum yields of ~1% (Khanuja et al., 2008) which are very low yields.

All the aforementioned reasons render the development of transgenic production platforms for artemisinin, including microbes and plants essential to lower artemisinin prices and stabilize supply for millions of people who depend on the drug.

Heterologous Expression Systems

Engineering *E. coli* to produce a high level of artemisinin precursor was exploited. Instead of using the native MEP/DXP pathway, Martin and coworkers expressed the heterologous mevalonate pathway from *S. cerevisiae* in *E. coli* to improve isoprenoid flux (Martin et al., 2003). When coupled with the expression of ADS, amorphadiene (amorpha-4,11-diene) was produced, demonstrating the usefulness of this approach. However, a high level of expression of the mevalonate pathway resulted in accumulation of pathway intermediates and inhibition of cell growth (Pitera et al., 2007). Metabolite analysis revealed the accumulation of HMG-CoA, suggesting that the bottleneck was HMG-CoA reductase (HMGR). By modulating the expression level of this enzyme via addition of another copy of the truncated HMGR (tHMGR), the growth rate was restored. To further alleviate this rate-limiting step, bacterial HMGR genes, mvaA from *S. aureus* and mvaE from *Enterococcus faecalis*, were investigated. The strain containing mvaA from *S. aureus* produced the highest titer, compared to those containing the yeast or *E. faecalis* HMGR gene. In light of this success, another strain was constructed to replace the yeast HMG synthase (HMGS) gene with the *S. aureus* HMGS gene mvaS. The strain containing both mvaS and mvaA had an even higher amorphadiene titer. Under fermentation conditions where carbon and nitrogen were strictly controlled, this strain had a high titer with commercial potential (27.4 g/L) (Tsuruta et al., 2009).

Metabolic engineering of *S. cerevisiae* for artemisinic acid production was adopted as an approach to increase the production of an important artemisinin production in transgenic yeast model. As demonstrated in *E. coli*, the key to a high titer is to optimize the isoprenoid pathway flux. In yeast, this was accomplished by the upregulation of several key genes in the mevalonate pathway and the downregulation of genes for sterol biosynthesis (Ro et al., 2006). The HMG-CoA reductase in the mevalonate pathway is the principal target of complex regulation. Deletion of the N-terminal regulatory region of HMG-CoA reductase increases the

carbon flux to isopentenyl diphosphate (Donald et al., 1997). Overexpression of tHMGR increased amorphadiene production by fivefold in yeast. When tHMGR overexpression was combined with reduction in the expression of squalene synthase using the methionine-repressible MET3 promoter, another twofold increase in titer was obtained. In yeast, the sterol biosynthesis competes for the common precursor FPP. To further downregulate the sterol pathway, the upc2-1 mutant allele was overexpressed. The transcriptional factor UPC2 is a key regulator of yeast steroid uptake (Vik & Rine, 2001). Expression of the mutant upc2-1 allele allows uptake of exogenous steroids, which inhibits endogenous sterol biosynthesis. After integration of another copy of the tHMGR gene, the final strain produced 150 mg L-1 amorphadiene, nearly 500-fold higher than previously reported levels.

Overexpression of Key Biosynthetic Genes

Efforts are underway to increase artemisinin level in *A. annua* through metabolic engineering. Similar to strategies used in yeast, attempts have been made to increase carbon flux through the isoprenoid pathway for artemisinin biosynthesis in *A. annua*. One example is the expression of HMGCoA reductase gene. The HMGR gene from *Catharanthus roseus* (L.) G. Don was integrated into *A. annua* using *Agrobacterium*-mediated transformation (Aquil et al., 2009).

Based on information gathered from *N. benthamiana*, Plant Research International and Dafra Pharma International NV are collaborating to engineer chicory (*Cichorium intybus*) to produce the artemisinin precursor dihydroartemisinic acid in the roots (Brisibe et al., 2008; Dafra Pharma, n.d.). Dihydroartemisinic acid, like artemisinic acid, can be chemically converted to artemisinin at low cost. As a member of the Asteraceae family, chicory already produces considerable amounts of sesquiterpene lactones through the isoprenoid pathway. Chicory is a well-established plant for industrial nonfood applications, and the entire chain of large-scale agricultural production is already in place. Experiments are underway to evaluate the expression of HMGR, FPS, and ADS in chicory (Ye & Bhatia, 2012).

Silymarin

Phenolic compounds are among the most ubiquitous plant secondary metabolites that have diverse structures and biological activities (Ammar et al., 2012; El-Desoky et al., 2018). They could be found in almost all plant genera. Phenolic compounds vary in their structure complexity from simple phenolic acids to highly complex tannins (Elkhateeb et al., 2019). Among phenolic compounds with high medicinal value is silymarin. Silymarin is a flavolignan mixture consisting of silybin (SB), isosilybin (ISB), silydianin (SD), silychristin (SC), and taxifolin (TXF) as shown in Fig. 1.5, extractable from milk thistle's (*Silybum marianum* L. Gaertn.) seeds and

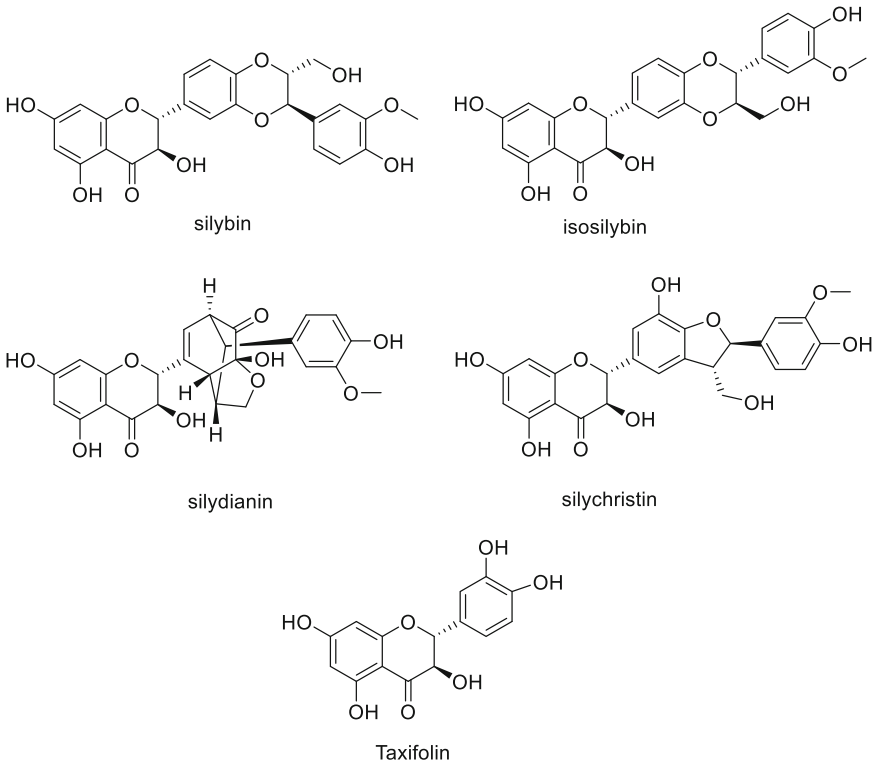

Fig. 1.5 The main compounds of silymarin (flavolignan mixture)

fruits (Khalili et al., 2009a, 2009b; Elyasi, 2021; AbouZid & Ahmed, 2013). Several research projects and studies have been conducted to increase the silymarin content of *Silybum marianum* seeds. A large part of these studies were concerned with environmental factors, whether edaphically or non-edaphically, and agricultural treatments such as irrigation, fertilization, planting dates, and harvesting in order to increase the content of silymarin in seeds (Elsayed et al., 1993; Omer et al., 1993). Silymarin is used worldwide as liver support treatment for several hepatic dysfunctions (Crocenzi & Roma, 2006). Being the main active component (60–70%), silybin reserves the main potential in silymarin hepatoprotective activities (Negi et al., 2007).

Chalcone synthase (CHS, EC 2.3.1.74) is a key enzyme for the naringenin chalcone biosynthesis catalyzing sequential addition of three acetate units from malonyl-CoA to p-coumaroyl-CoA (Spribille & Forkman, 1982). Then naringenin is produced by cyclization and hydroxylated to taxifolin and then silybin is produced by other steps. Therefore, CHS overexpression is a promising target to increase silybin production.

After detailed bioinformatics study, Rahnama and coworkers overexpressed chalcone synthase (chsA) of *Petunia hybrid* L. petals in the hairy root of *Silybum marianum*. ChsA gene was transformed to *S. marianum* hairy roots by *A. rhizogenes* AR15834 strain harboring pCamCHS vectors. That resulted in a sevenfold overexpression of chsA gene in transgenic hairy roots. And subsequent ten times increase in silybin content in transgenic hairy roots when compared to non-transgenic ones (Rahnama et al., 2013).

Some other factors were found to enhance the production of silymarin in the hairy root cultures of *S. marianum* such as the use of elicitors. Among these elicitors is the abiotic elicitor salicylic acid, which enhanced silymarin production (1.89 mg/g dry weight) in hairy roots of *S. marianum*. Salicylic acid (6 mg/50 ml culture) after 24 h increased silymarin content 2.42 times compared to control (0.78 mg/g dry weight). The content of silybin, isosilybin, silychristin, silydianin, and taxifolin were 0.703, 0.017, 0.289, 0.02, and 0.863 mg/g dry weight, respectively. Lipoxygenase activity was increased by ~1.57-fold (Khalili et al., 2009a, 2009b). Similarly, the same group studied the effect of silver ion $[Ag]^+$. Ag^+ was found to increase the activity of lipoxygenase that mediates signal transduction pathway for silymarin production. That resulted in a fourfold increase in silymarin production at 2 mM Ag^+ and after 96 h after elicitation, when compared to non-treated hairy roots (Khalili et al., 2010).

Another type of elicitors is biotic elicitors. Hasanloo and coworkers studied the effect of three fungal species, namely, *Fusarium proliferatum*, *Aspergillus niger*, and *Rhizoctonia solani*, as elicitors for hairy root cultures of *Silybum marianum* (L.) Gaertn. The maximum production for each of the fungal treatment was different: 10 mg *A. niger*/50 ml culture produced 0.18 mg/g dry weight of silymarin after 48 h while 20 mg *F. proliferatum*/50 ml culture produced 0.34 mg/g dry weight of silymarin. And 20 mg *R. solani*/50 ml produced 0.22 mg/g dry weight of silymarin after 72 h. The best profile owed to *F. proliferatum* treated hairy roots that contained taxifolin (0.068 mg/g dry weight), silydianin (0.11 mg/g dry weight), silybin (0.021 mg/g dry weight), and isosilybin (0.015 mg/g dry weight) which were 1.94-, 1.19-, 2.62-, and 1.25-fold greater than untreated hairy roots, respectively. The results indicated that the type of fungi, their concentrations, and their exposure time have significant effect on the stimulation of silymarin production (Hasanloo et al., 2013).

Conclusion

The abovementioned engineered metabolites highlight important aspects to be considered for such objective. Specifications of the host organism have a high impact on final commercial success. These specifications include metabolic capabilities, genetic systems, and scale-up potential. The limitations of the genetic system for plants or even microbes could present challenges. As the optimal host is chosen, the strategy for metabolic engineering may involve determining a specific biosynthetic pathway and enzymes to target. The full understanding of the biochemistry and gene regulation networks is critical for pathway construction. Optimization of

pathways involves balancing the upstream and downstream steps as well as exclusion or decrease of challenging reactions.

Biotechnology and metabolic engineering are extensively used to increase the production of paclitaxel.

The production of CPT from the hairy roots of *Ophiorrhiza pumila* which originally produce CPT in higher amounts compared to the wild-type plantlets and cell suspension culture that did not accumulate CPT was managed to increase its production. Comparative transcriptome analysis revealed that hairy roots of *Ophiorrhiza pumila* contained genes encoding key enzymes involved in CPT biosynthesis, which were mainly identified as *OpG10H* and *OpSLS*. Knockout of these genes by CRISPR-Cas9 editing resulted in CPT-free hairy roots. Overexpression of two upstream TIA pathway genes tryptophan decarboxylase (CrTDC) and strictosidine synthase (CrSTR) enhanced terpenoid indole alkaloid (TIA) pathway activity and antineoplastic vinblastine biosynthesis in *Catharanthus roseus* in the transgenic whole plant produced via transformation with *Agrobacterium tumefaciens* LBA1119.

Engineering *E. coli* to produce a high level of artemisinin precursor was exploited. Instead of using the native MEP/DXP pathway, the heterologous mevalonate pathway from *S. cerevisiae* in *E. coli* to improve isoprenoid flux is used.

Efforts are underway to increase artemisinin level in *A. annua* through metabolic engineering. Similar to strategies used in yeast, attempts have been made to increase carbon flux through the isoprenoid pathway for artemisinin biosynthesis in *A. annua*. One example is the expression of HMGCoA reductase gene.

The studies with hairy roots indicated that the type of fungi, their concentrations, and their exposure time have significant effect on the stimulation of silymarin production.

Mechanisms of metabolite engineering are the most promising ways to contribute to the provision of many important entities in the field of pharmaceuticals and medicines, especially high-cost treatments, such as cancers, liver diseases, and viruses.

References

Abdallah, I. I., Pramastya, H., & Van Merkerk, R. (2019). Metabolic engineering of *Bacillus subtilis* toward taxadiene biosynthesis as the first committed step for Taxol production. *Frontiers in Microbiology, 10*, 218.

AbouZid, S., & Ahmed, O. M. (2013). Chapter 14 - Silymarin Flavonolignans: Structure–activity relationship and biosynthesis. *Studies in Natural Products Chemistry, 40*, 469–484.

Ajikumar, P. K., Xiao, W. H., Tyo, K. E. J., Wang, Y., Simeon, F., Leonard, E., Mucha, O., Phon, T. H., Pfeifer, B., & Stephanopoulos, G. (2010). Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. *Science, 330*, 70–74.

Ammar, N. M., El-Hawary, S. S. E. D., El-Anssary, A. A., Othman, N., Galal, M., & El-Desoky, A. H. (2012). Phytochemical and clinical studies of the bioactive extract of *Glycyrrhiza glabra* L. Family Leguminosae. *International Journal of Phytomedicine, 4*(3), 429.

Amna, T., Puri, S. C., Verma, V., Sharma, J. P., Khajuria, R. K., Musarrat, J., Spiteller, M., & Qazi, G. N. (2006). Bioreactor studies on the endophytic fungus *Entrophospora infrequens* for the production of an anticancer alkaloid camptothecin. *Canadian Journal of Microbiology, 52*(3), 189–196.

Anterola, A., Shanle, E., Perroud, P. F., & Quatrano, R. (2009). Production of taxa-4(5),11(12)-diene by transgenic *Physcomitrella patens*. *Transgenic Research, 18*, 655–660.

Aquil, S., Husaini, A. M., Abdin, M. Z., & Rather, G. M. (2009). Overexpression of the HMG-CoA reductase gene leads to enhanced artemisinin biosynthesis in transgenic *Artemisia annua* plants. *Planta Medica, 75*, 1453–1458.

Artepal, J. P. (2010). *Artemisinin market: Quantities and pricing*. Artemisinin Conference, Antananarivo, Madagascar.

Bailey, J. E. (1991). Toward a science of metabolic engineering. *Science, 252*(5013), 1668–1675.

Besumbes, O., Sauret-Güeto, S., Phillips, M. A., Imperial, S., Rodríguez-Concepción, M., & Boronat, A. (2004). Metabolic engineering of isoprenoid biosynthesis in Arabidopsis for the production of taxadiene, the first committed precursor of Taxol. *Biotechnology and Bioengineering, 88*, 168–175.

Biggs, B. W., Lim, C. G., Sagliani, K., Shankar, S., Gregory, S., De Marjan, M., et al. (2016). Overcoming heterologous protein interdependency to optimize P450-mediated Taxol precursor synthesis in *Escherichia coli*. *PNAS, 113*, 3209–3214.

Birchfield, A. S., & McIntosh, C. A. (2020). Metabolic engineering and synthetic biology of plant natural products – a mini review. *Current Plant Biology*, 100163.

Borah, J. C., Boruwa, J., & Barua, N. C. (2007). Synthesis of the C-13 side-chain of taxol. *Current Organic Synthesis, 4*, 175–199.

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

Bringi, V., Kadkade, P.G., Prince, C.L., Schubmehl, B.F., Kane, E.J., Roach, B., (1995). Enhanced production of taxol and taxanes by cell cultures of *Taxus* species. U.S. Patent No. 5,407,816.

Brisibe, E. A., & Chukwurah, P. N. (2014). Production of artemisinin in planta and in microbial systems need not be mutually exclusive. In *Artemisia annua—Pharmacology and biotechnology* (pp. 269–292). Springer.

Brisibe, E. A., Uyoh, E. A., Brisibe, F., Magalhaes, P. M., & Ferreira, J. F. S. (2008). Building a golden triangle for the production and use of artemisinin derivatives against falciparum malaria in Africa. *African Journal of Biotechnology, 7*, 4884–4896.

Chatzivasileiou, A. O., Ward, V., McBride Edgar, S., & Stephanopoulos, G. (2019). Two-step pathway for isoprenoid synthesis. *Proceedings of the National Academy of Sciences of the United States of America, 116*(2), 506–511.

Choi, K. R., Jang, W. D., Yang, D., Cho, J. S., Park, D., & Lee, S. Y. (2019). Systems metabolic engineering strategies: integrating systems and synthetic biology with metabolic engineering. *Trends in Biotechnology, 37*(8), 817–837.

Christen, A. A., Bland, J., & Gibson, D. (1989). Cell cultures as a means to produce taxol. In *Proceedings of the American Association for Cancer Research* (p. 566).

Christen, A.A., Gibson, D.M., Bland, J., (1991). Production of taxol or taxol-like compounds in cell culture. *U.S. Patent* No. 5,019,504.

Connolly, Patrick (1988–1989) . *Personal communications*. Yew Wood Industries, Portland, OR. (April 1988, December 1988, January 1989).

Courdavault, V., O'Connor, S. E., Jensen, M. K., & Papon, N. (2021). Metabolic engineering for plant natural products biosynthesis: new procedures, concrete achievements and remaining limits. *Natural Product Reports*.

Cragg, G. (1988). *Personal communications*. National Cancer Institute. (March, April, July 1988).

Crocenzi, F. A., & Roma, M. G. (2006). Silymarin as a new hepatoprotective agent in experimental cholestasis: new possibilities for an ancient medication. *Current Medicinal Chemistry, 13*, 1055–1074.

Croteau, R., Ketchum, R. E. B., Long, R. M., Kaspera, R., & Wildung, M. R. (2006). Taxol biosynthesis and molecular genetics. *Phytochemistry Reviews, 5*(1), 75–97.

Dafra Pharma. (n.d.) Chicory Project (http://www.dafra.be/content/chicory-project).

Dalrymple, D. G. (2013). *Artemisia annua, Artemisinin, ACTs and malaria control in Africa: Tradition, science and public policy*. American Journal of Agricultural Economics.

Davies, M., Atkinson, C., Burns, C., Arroo, R., & Woolley, J. (2011). Increases in leaf artemisinin concentration in *Artemisia annua* in response to the application of phosphorus and boron. *Industrial Crops and Products, 34*, 1465–1473.

Davies, M. J., Atkinson, C. J., Burns, C., Woolley, J. G., Hipps, N. A., Arroo, R. R., Dungey, N., Robinson, T., Brown, P., & Flockart, I. (2009). Enhancement of artemisinin concentration and yield in response to optimization of nitrogen and potassium supply to *Artemisia annua*. *Annals of Botany, 104*, 315–323.

Dejong, J. M., Liu, Y., Bollon, A. P., Long, R. M., Jennewein, S., Williams, D., & Croteau, R. B. (2006). Genetic engineering of taxol biosynthetic genes in *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering, 93*(2), 212–224.

Denis, J. N., Greene, A. E., Guenard, D., Gueritte-Voegelein, F., Mangatal, L., & Potier, P. (1988). Highly efficient, practical approach to natural Taxol. *Journal of the American Chemical Society, 110*, 5917–5919.

Do, R., Kiss, R. S., Gaudet, D., & Engert, J. C. (2009). Squalene synthase: A critical enzyme in the cholesterol biosynthesis pathway. *Clinical Genetics, 75*, 19–29.

Donald, K. A., Hampton, R. Y., & Fritz, I. B. (1997). Effects of overproduction of the catalytic domain of 3-hydroxy-3-methylglutaryl coenzyme A reductase on squalene synthesis in *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology, 63*, 3341–3344.

Efferth, T. (2006). Molecular pharmacology and pharmaco-genomics of artemisinin and its derivatives in cancer cells. *Current Drug Targets, 7*, 407–421.

Efferth, T., Herrmann, F., Tahrani, A., & Wink, M. (2011). Cytotoxic activity of secondary metabolites derived from *Artemisia annua* L. towards cancer cells in comparison to its designated active constituent artemisinin. *Phytomedicine, 18*, 959–969.

El-Desoky, A. H., Abdel-Rahman, R. F., Ahmed, O. K., El-Beltagi, H. S., & Hattori, M. (2018). Anti-inflammatory and antioxidant activities of naringin isolated from *Carissa carandas* L.: *In vitro* and *in vivo* evidence. *Phytomedicine, 42*, 126–134.

Elkhateeb, A., El-Shabrawy, M., Abdel-Rahman, R. F., Marzouk, M. M., El-Desoky, A. H., Abdel-Hameed, E. S. S., & Hussein, S. R. (2019). LC-MS-based metabolomic profiling of *Lepidium coronopus* water extract, anti-inflammatory and analgesic activities, and chemosystematic significance. *Medicinal Chemistry Research, 28*(4), 505–514.

Elsayed, A. A., Razin, A. M., Omer, E. A., & Ahmed, S. S. (1993). *Use of micronutrients in increasing yield of milk thistle (Silybum marianum, Gaertn) in a desert area under irrigation* (pp. 693–696). Kluwer Academic Publishers, Dordrecht.

Elyasi, S. (2021). Chapter 43 – *Silybum marianum*, antioxidant activity, and cancer patients. In *Cancer* (*Oxidative stress and dietary antioxidants*) (2nd ed., pp. 483–493).

Engels, B., Dahm, P., & Jennewein, S. (2008). Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. *Metabolic Engineering, 10*, 201–206.

Ferreira, J., Simon, J., & Janick, J. (1995). Relationship of artemisinin content of tissue-cultured, greenhouse-grown, and field-grown plants of *Artemisia annua*. *Planta Medica, 61*, 351–355.

Fett-Neto, A. G., DiCosmo, F., Reynolds, W. F., & Sakata, K. (1992). Cell culture of *Taxus* as a source of the antineoplastic drug taxol and related taxanes. *Biotechnology (N Y), 10*(12), 1572–1575.

Fu, R., Martin, C., & Zhang, Y. (2018). Next-generation plant metabolic engineering, inspired by an ancient Chinese irrigation system. *Molecular Plant, 11*(1), 47–57.

García-Granados, R., Lerma-Escalera, J. A., & Morones-Ramírez, J. R. (2019). Metabolic engineering and synthetic biology: Synergies, future, and challenges. *Frontiers in Bioengineering and Biotechnology, 7*, 36.

Hao, D. C., Yang, L., Xiao, P. G., & Liu, M. (2012). Identification of Taxus microRNAs and their targets with high-throughput sequencing and degradome analysis. *Physiologia Plantarum, 146*, 388–403.

Hasanloo, T., Ahmadi, M., Khayyam Nekoei, S. M., & Salehi Jouzani, G. H. (2013). Improvement of silymarin production in hairy root cultures of *Silybum marianum* (L.) Gaertn using fungal elicitors. *Romanian Biotechnology Letters, 18*(3), 8221–8232.

Holton, R. A., Somoza, C., Kim, H. B., Liang, F., Biediger, R. J., Boatman, P. D., et al. (1994). First total synthesis of Taxol. 1. Functionalization of the B ring. *Physiological Chemistry and Physics and Medical NMR, 34*(2), 103–117.

Howat, S., Park, B., Oh, I. S., Jin, Y. W., Lee, E. K., & Loake, G. J. (2014). Paclitaxel: Biosynthesis, production and future prospects. *New Biotechnology, 31*, 242–245.

Hsiang, Y. H., Hertzberg, R., Hecht, S., & Liu, L. F. (1985). Camptothecin induces protein linked DNA breaks via mammalian DNA topoisomerase I. *The Journal of Biological Chemistry, 260*, 14873–14878.

Hunter, W. N. (2007). The non-mevalonate pathway of isoprenoid precursor biosynthesis. *The Journal of Biological Chemistry, 282*, 21573–21577.

Kai, G., Wu, C., Geng, L., Zhang, L., Cui, L., & Ni, X. (2015). Biosynthesis and biotechnological production of anti-cancer drug camptothecin. *Phytochemistry Reviews, 14*, 525–539.

Kai, G. Y., Teng, X. J., Cui, L. J., Li, S. S., Hao, X. L., Shi, M., & Yan, B. (2014). Effect of three plant hormone elicitors on the camptothecin accumulation and gene transcript profiling in *Camptotheca acuminata* seedlings. *International Journal of Science, 3*(1), 86–95.

Khalili, M., Hasanloo, T., & Kazemi Tabar, S. K. (2010). Ag {+} enhanced silymarin production in hairy root cultures of *'Silybum marianum'* (L.) Gaertn. *Plant Omics, 3*(4), 109–114.

Khalili, M., Hasanloo, T., KazemiTabar, S. K., & Rahnama, H. (2009a). Influence of exogenous salicylic acid on flavonolignans and lipoxygenase activity in the hairy root cultures of *Silybum marianum*. *Cell Biology International, 33*, 988–994.

Khalili, M., Hasanloo, T., Tabar, S. K. K., & Rahnama, H. (2009b). Influence of exogenous salicylic acid on flavonolignans and lipoxygenase activity in the hairy root cultures of *Silybum marianum*. *Cell Biology International, 33*(9), 988–994.

Khanuja, S.P.S., Paul, S., Shasany, A.K., Gupta, A.K., Darokar, M.P.,Gupta, M.M., Verma, R.K., Ram, G., Kumar, A., & Lal, R.K.(2008). High Artemisinin Yielding Artemisia Plant Named 'CIMArogya': U.S. Patent 7, 375,260.

Köksal, M., Jin, Y., Coates, R. M., Croteau, R., & Christianson, D. W. (2011). Taxadiene synthase structure and evolution of modular architecture in terpene biosynthesis. *Nature, 469*(7328), 116–120.

Kusari, S., Zühlke, S., & Spiteller, M. (2009). An endophytic fungus from *Camptotheca acuminata* that produces camptothecin and analogues. *Journal of Natural Products, 72*(1), 2–7.

Kutchan, T. M. (1993). Strictosidine: from alkaloid to enzyme to gene. *Phytochemistry, 32*, 493–506.

Lange, B. M., Rujan, T., Martin, W., & Croteau, R. (2000). Isoprenoid biosynthesis: The evolution of two ancient and distinct pathways across genomes. *Proceedings of the National Academy of Sciences of the United States of America, 97*, 13172–13177.

Liu, W. C., Gong, T., & Zhu, P. (2016). Advances in exploring alternative Taxol sources. *Journal of RSC Advances Issue, 54*, 48272–49288.

Liu, Y., & Nielsen, J. (2019). Recent trends in metabolic engineering of microbial chemical factories. *Current Opinion in Biotechnology, 60*, 188–197.

Lopez-Meyer, M., Nessler, C. L., & McKnight, T. D. (1994). Sites of accumulation of the antitumor alkaloid camptothecin in *Camptotheca acuminata*. *Planta Medica, 60*(7), 558–560.

Mansukhlal, C. W., Harold, L. T., Monroe, E. W., Philip, C., & Andrew, T. M. (1971). Plant anticancer agents. VI. The isolation and structure of Taxol, a novel antileukemic and anticancer agent from *Taxus brevifolia*. *Journal of the American Chemical Society, 93*, 2325–2327.

Maresh, J., Giddings, L. A., Friedrich, A., Loris, E. A., Panjikar, S., Stockigt, J., Peters, B., & O'Connor, S. E. (2008). Strictosidine synthase: Mechanism of a Pictet-Spengler catalyzing enzyme. *Journal of the American Chemical Society, 130*, 710–723.

Martin, V. J., Pitera, D. J., Withers, S. T., Newman, J. D., & Keasling, J. D. (2003). Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nature Biotechnology, 21*, 796–802.

McCoy, E., & O'Connor, S. E. (2006). Directed biosynthesis of alkaloid analogs in the medicinal plant *Catharanthus roseus*. *Journal of the American Chemical Society, 128*, 14276–14277.

McGehee, D. L., Alimohammadi, M., & Khodakovskaya, M. V. (2019). Carbon-based nanomaterials as stimulators of production of pharmaceutically active alkaloids in cell culture of *Catharanthus roseus*. *Nanotechnology, 30*(27), 275102.

Nabavi, S. M., Šamec, D., Tomczyk, M., Milella, L., Russo, D., Habtemariam, S., Suntar, I., Rastrelli, L., Daglia, M., Xiao, J., & Giampieri, F. (2020). Flavonoid biosynthetic pathways in plants: Versatile targets for metabolic engineering. *Biotechnology Advances, 38*, 107316.

Negi, A. S., Kumar, J. K., Luqman, S., Shanker, K., Gupta, M. M., & Kbanuja, S. P. S. (2007). Recent advance in plant hepatoprotective: A chemical and biological profile of some important leads. *Medicinal Research Reviews, 28*, 746–772.

Ni, X. L., Wen, S. Q., Wang, W., Wang, X. Y., Xu, H., & Kai, G. Y. (2011). Enhancement of camptothecin production in *Camptotheca acuminata* hairy roots by overexpressing ORCA3 gene. *Journal of Applied Pharmaceutical Science, 1*(8), 85–88.

Nielsen, J. (2001). Metabolic engineering. *Applied Microbiology and Biotechnology, 55*(3), 263–283.

Ning, L., You, C., Zhang, Y., Li, X., & Wang, F. (2020). Synthesis and biological evaluation ofsurface-modified nanocellulose hydrogel loaded with paclitaxel. *Life Science, 241*, 117137.

O'Connor, S. E., & Maresh, J. J. (2006). Chemistry and biology of monoterpene indole alkaloid biosynthesis. *Natural Product Reports, 23*(4), 532–547.

Olsson, M. E., Olofsson, L. M., Lindahl, A.-L., Lundgren, A., Brodelius, M., & Brodelius, P. E. (2009). Localization of enzymes of artemisinin biosynthesis to the apical cells of glandular secretory trichomes of *Artemisia annua* L. *Phytochemistry, 70*, 1123–1128.

Omer, E. A., Abou Hussein, E. A., Hendawy, S. F., El-din, E., Azza, A., & El-Gendy, A. G. (2014). Introduction of three different *Artemisia* species into the Egyptian cultivation. *Journal of Essential Oil-Bearing Plants, 2*(2), 11–20.

Omer, E. A., Abou, H. E. A., Hendawy, S. F., Ezz El-din, A. A., & El-Gendy, A. G. (2013). Effect of soil type and seasonal variation on growth, yield, essential oil and artemisinin content of *Artemisia annua* L. *International Research Journal of Horticulture, 1*(1), 15–27.

Omer, E. A., Hendawy, S. F., Hussein, E. A. A., Gendy, A. G. E., & Ezz El-din, A. A. (2016). Introduction of *Artemisia* species into the Egyptian cultivation. *Journal of Essential Oil-Bearing Plants, 19*(4), 793–805.

Omer, E. A., Refaat, A. M., Ahmed, S. S., Kamel, A., & Hammouda, F. M. (1993). Effect of spacing and fertilization on the yield and active constituents of *Silybum marianum*. *Journal of Herbs Spices & Medicinal Plants, 1*(4), 17–23.

Onrubia, M., Cusido, R. M., Ramirez, K., Hernandez-Vazquez, L., Moyano, E., Bonfill, M., & Palazon, J. (2013). Bioprocessing of plant *in vitro* systems for the mass production of pharmaceutically important metabolites: paclitaxel and its derivatives. *Current Medicinal Chemistry, 20*, 880–891.

Peebles, C. A., Gibson, S. I., Shanks, J. V., & San, K. Y. (2007). Characterization of an ethanol-inducible promoter system in *Catharanthus roseus* hairy roots. *Biotechnology Progress, 23*(5), 1258–1260.

Pitera, D. J., Paddon, C. J., Newman, J. D., & Keasling, J. D. (2007). Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. *Metabolic Engineering, 9*, 193–207.

Puri, S. C., Verma, V., Amna, T., Qazi, G. N., & Spiteller, M. (2005). An endophytic fungus from *Nothapodytes foetida* that produces camptothecin. *Journal of Natural Products, 68*(12), 1717–1719.

Rahnama, H., Razi, Z., Dadgar, M. N., & Hasanloo, T. (2013). Enhanced production of flavonolignans in hairy root cultures of *Silybum marianum* by over-expression of chalcone synthase gene. *Journal of Plant Biochemistry and Biotechnology, 22*(1), 138–143.

Ramírez-Estrada, K., Altabella, T., Onrubia, M., Moyano, E., Notredame, C., Osuna, L., VandenBossche, R., Goossens, A., Cusido, R. M., & Palazon, J. (2016). Transcript profiling of jasmonate-elicited Taxus cells reveals a β-phenylalanine-CoA ligase. *Plant Biotechnology Journal, 14*(1), 85–96.

Ro, D. K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Ho, K. A., Eachus, R. A., Ham, T. S., Kirby, J., Chang, M. C. Y., Withers, S. T., Shiba, Y., Sarpong, R., & Keasling, J. D. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature, 440*, 940–943.

Sabzehzari, M., & Naghavi, M. R. (2018). Phyto-miRNA: A molecule with beneficial abilities for plant biotechnology. *Gene, 683*, 28–23.

Sabzehzari, M., & Naghavi, M. R. (2019). Phyto-miRNAs-based regulation of metabolites biosynthesis in medicinal plants. *Gene, 682*, 13–24.

Saito, K., Sudo, H., Yamazaki, M., Koseki-Nakamura, M., Kitajima, M., Takayama, H., & Aimi, N. (2001). Feasible production of camptothecin by hairy root culture of *Ophiorrhiza pumila*. *Plant Cell Reports, 20*(3), 267–271.

Shapira, M. Y., Resnick, I. B., Chou, S., Neumann, A. U., Lurain, N. S., Stamminger, T., Caplan, O., Saleh, N., Efferth, T., & Marschall, M. (2008). Artesunate as a potent antiviral agent in a patient with late drug-resistant cytomegalovirus infection after hematopoietic stem cell transplantation. *Clinical Infectious Diseases, 46*, 1455–1457.

Sharma, A., Verma, P., Mathur, A., & Mathur, A. K. (2018). Overexpression of tryptophan decarboxylase and strictosidine synthase enhanced terpenoid indole alkaloid pathway activity and antineoplastic vinblastine biosynthesis in *Catharanthus roseus*. *Protoplasma, 255*(5), 1281–1294.

Shi, M., Gong, H., Cui, L., Wang, Q., Wang, C., Wang, Y., & Kai, G. (2020). Targeted metabolic engineering of committed steps improves anti-cancer drug camptothecin production in *Ophiorrhiza pumila* hairy roots. *Industrial Crops and Products, 148*, 112277.

Shiba, Y., Paradise, E. M., Kirby, J., Ro, D. K., & Keasling, J. D. (2007). Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. *Metabolic Engineering, 9*(2), 160–168.

Shweta, S., Zuehlke, S., Ramesha, B. T., Priti, V., Mohana Kumar, P., Ravikanth, G., Spiteller, M., Vasudeva, R., & Uma Shaanker, R. (2010). Endophytic fungal strains of *Fusarium solani*, from *Apodytes dimidiata* E. Mey. ex Arn (Icacinaceae) produce camptothecin, 10-hydroxycamptothecin and 9-methoxycamptothecin. *Phytochemistry, 71*(1), 117–122.

Sirikantaramas, S., Asano, T., Sudo, H., Yamazaki, M., & Saito, K. (2007). Camptothecin: Therapeutic potential and biotechnology. *Current Pharmaceutical Biotechnology, 8*, 196–202.

Soliman, S., & Tang, Y. (2015). Natural and engineered production of taxadiene with taxadiene synthase. *Biotechnology and Bioengineering, 112*(2), 229–235.

Spribille, R., & Forkman, G. (1982). Genetic control of chalcone synthase activity in flowers of *Antirrhinum majus*. *Phyotochemistry, 21*, 2231–2234.

Stierle, A., Strobel, G., & Stierle, D. (1993). Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of pacific yew. *Science, 260*(9), 214–216.

Stöckigt, J., & Zenk, M. H. (1977). Strictosidine (isovincoside): the key intermediate in the biosynthesis of monoterpenoid indole alkaloids. *Journal of the Chemical Society, Chemical Communications, 18*, 646–648.

Teoh, K. H., Polichuk, D. R., Reed, D. W., & Covello, P. S. (2009). Molecular cloning of an aldehyde dehydrogenase implicated in artemisinin biosynthesis in *Artemisia annua*. *Botany, 87*, 635–642.

Tsuruta, H., Paddon, C. J., Eng, D., Lenihan, J. R., Horning, T., Anthony, L. C., Regentin, R., Keasling, J. D., Renninger, N. S., & Newman, J. D. (2009). High-level production of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin, in *Escherichia coli*. *PLoS One, 4* (2), e4489. https://doi.org/10.1371/journal.pone.0004489

Utzinger, J., Xiao, S.-H., Tanner, M., & Keiser, J. (2007). Artemisinins for *schistosomiasis* and beyond. *Current Opinion in Investigational Drugs, 8*, 105–116.

van der Fits, L., & Memelink, J. (2000). ORCA3, a jasmonate responsive transcriptional regulator of plant primary and secondary metabolism. *Science, 289*, 295–297.

van Der Heijden, R., Jacobs, D. I., Snoeijer, W., Hallard, D., & Verpoorte, R. (2004). The Catharanthus alkaloids: Pharmacognosy and biotechnology. *Current Medicinal Chemistry, 11*(5), 607–628.

Venditto, V. J., & Simanek, E. E. (2010). Cancer therapies utilizing the camptothecins: A review of the in vivo literature. *Molecular Pharmaceutics, 7*, 307–349.

Vik, A., & Rine, J. (2001). Upc2p and Ecm22p, dual regulators of sterol biosynthesis in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology, 21*, 6395–6405.

Wall, M. E., Wani, M. C., Cook, C. E., Palmer, K. H., McPhail, A. T., & Sim, G. A. (1966). Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*. *Journal of the American Chemical Society, 88*, 3888–3890.

Wang, T., Chen, Y., Zhuang, W., Zhang, F., Shu, X., Wang, Z., & Yang, Q. (2019). Transcriptome sequencing reveals regulatory mechanisms of Taxol synthesis in *Taxus wallichiana* var. Mairei. *International Journal of Genomics, 2019*, 1596895.

White, N. J. (2008). Qinghaosu (Artemisinin): The price of success. *Science, 18*, 330–334.

World Health Organization. (2020, November 30). *World malaria report 2020*. Featured Publication.

Yadav, D., Tanveer, A., Malviya, N., & Yadav, S. (2018). Overview and principles of bioengineering: the drivers of omics technologies. In *Omics technologies and bio-engineering* (pp. 3–23). Academic Press.

Ye, M. V., & Bhatia, S. K. (2012). Metabolic engineering for the production of clinically important molecules: Omega-3 fatty acids, artemisinin, and taxol. *Biotechnology Journal, 7*, 20–33.

Zhang, Y., Teoh, K. H., Reed, D. W., Maes, L., Goossens, A., Olson, D. J. H., Ross, A. R. S., & Covello, P. S. (2008). The molecular cloning of artemisinic aldehyde Delta 11(13) reductase and its role in glandular trichome-dependent biosynthesis of artemisinin in *Artemisia annua*. *Journal of Biological Chemistry, 283*, 21501–21508.

Zhao, J., & Verpoorte, R. (2007). Manipulating indole alkaloid production by *Catharanthus roseus* cell cultures in bioreactors: From biochemical processing to metabolic engineering. *Phytochemistry Reviews, 6*(2), 435–457.

Zhu, Q., Wang, B., Tan, J., Liu, T., Li, L., & Liu, Y. G. (2020). Plant synthetic metabolic engineering for enhancing crop nutritional quality. *Plant Communications, 1*(1), 100017.