Metabolic Engineering for Improving Production of Taxol

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Subrata Kundu, Sumita Jha, and Biswajit Ghosh

Abstract

Taxol (generic name paclitaxel), a complex diterpenoid, is an efficient antineoplastic drug extracted from the plant. It has been approved for the management of several cancers including lungs, breast, and ovary cancers. The bark of several *Taxus* species is the natural source of taxol, but its lower accumulation (0.01–0.04% dry weight) elevated the price of extraction. Its complex structure prohibits the complete chemical synthesis of the compounds in economical approach at the industrial level. Therefore, a plethora of approaches has been implemented by several researchers for alternative and economical production of taxol. The advent of recombinant DNA technologies has resulted in the commencement of metabolic engineering as an effective alternative for the production of pharmaceutically important plant natural products at industrial levels. Plants have emergence as a perfect system for metabolic engineering due to its relatively cheap price and easiness in growing. Plant cell factories provide an alternative source for the scale-up of the production of high added value secondary metabolites including the anticancer drug taxol that is biosynthesized in *Taxus* spp. in very tiny quantity. The demand for taxol and its derivatives has increased enormously owing to its unique antineoplastic activity, lack of the taxane ring in nature and complexity of chemical synthesis. Therefore, countless efforts have been executed in worldwide for the biotechnological production of taxol. In this

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chapter, we have discussed different features of metabolic engineering, including genetic manipulation of plants as well as microbes to increase production of taxol and its precursors.

Keywords

Paclitaxel • Taxol • *Taxus brevifolia* • Plant metabolic engineering • Anticancer drug

Abbreviations

10-DAB	10-Deacetylbaccatin III
BAPT	Baccatin III C-13-phenylpropanoyl-CoA transferase
BMS	Bristol-Myers Squibb
DBAT	10-Deacetylbaccatin III-10-O-acetyltransferase
DBTNBT	3′-N-debenzoyl-2′-deoxytaxol N-benzoyl transferase
DMAPP	Dimethylallyl pyrophosphate
FDA	Food and Drug Administration
FDP	Farnesyl diphosphate
GGPP	Geranylgeranyl diphosphate
GGPPS	Geranylgeranyl diphosphate synthase
IPI	Isopentenyl diphosphate isomerase
IPP	Isopentenyl pyrophosphate
MVA	Mevalonic acid
NCI	National Cancer Institute
PAM	Phenylalanine aminomutase
T10βH	Taxoid 10β-hydroxylase
TBT	Taxane 2α-O-benzoyl transferase
TDAT	Taxadiene-5α-ol-O-acetyltransferase
TXS	Taxadiene synthase
USDA	United States Department of Agriculture

Contents

1 Introduction

Plants as sessile organisms synthesize a wide range of chemical molecules collectively known as secondary metabolites. These versatile chemical compounds are not essential for basic growth and development but are primarily due to environmental adaptation against both biotic and abiotic stresses. Plant secondary metabolites are usually classified according to their biosynthetic pathways and have been categorized into three large molecules families including phenolics, terpenes, and steroids, and alkaloids [1–4]. Alkaloids are the oldest and structurally diverse group of nitrogen-containing secondary metabolites and associated pronounced pharmacological and medicinal importance [5]. Among different types of alkaloids, plants synthesize 20-carbon (C20) polycyclic isoprenoids collectively known as diterpenoids. They are generally confined to restricted groups and found to be the signature molecules [6]. The taxoids are diterpenoids compounds with a unique taxane (pentamethyl [9.3.1.0]^{3,8} tricyclopentadecane) skeleton and generally found in *Taxus* (yew tree) species. There are about 400 taxoids whose structure and function have been characterized [7, 8]. The anticancer drug paclitaxel was found to be most important compared to other taxoids. The taxol was first extracted from the barks of *Taxus brevifolia* (Pacific yew tree), and the work was published in the year 1971 with over 4000 citations [9]. This important diterpene alkaloid is primarily found in the bark of different *Taxus* species, but its extraction cost is extremely high due to its minute quantity (0.01–0.04% dw) [9, 10]. The yew tree grows extremely slow and the mature trees are only suitable for the extraction of taxol. It has been reported that each mature tree could supply only about 2 kg of bark, and for the production of 500 mg taxol, 12 kg of bark was needed [9]. To extract sufficient amount of taxol at pharmaceutical industry level, destruction of the mature tree will also be harmful to nature. Subsequently, the growing demand for taxol greatly surpasses the supply, and alternative sources of the drug are needed. Due of its low yield from a natural source, intensive research has been accompanied to produce taxol more effectively in alternative source [11–13]. Though several groups of researchers have achieved the total chemical synthesis of taxol through sophisticated approaches, the high cost of this synthetic methodology forbids its commercial application [14–16].

Metabolic engineering was originally defined as “the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technologies” [17]. The genetic engineering only focuses on a narrow range of phenotypic improvement through manipulation of target genes. In contrast to other cellular engineering strategies, the approaches of metabolic engineering include modulation of larger cellular metabolic networks. Metabolic engineering is considered as a powerful technique to manipulate the biosynthetic pathway and modulate the production of the natural product as well as novel molecules. The application of state-of-the-art molecular and genetic tool kit has enabled the modulation of enzyme activity either toward increase or decrease of metabolic flux toward desired metabolic pathway [17–22]. Nevertheless, modern expression and regulation system have allowed the refinement of metabolic pathway

and creation of fusion pathways for the synthesis of novel compounds in the heterologous host [23–25]. The improvement in different features in metabolic engineering exposed the promises for large-scale production of important metabolites at industrial scale.

Plant metabolic engineering is enthusiastically gaining importance as an alternative to other biotechnological approaches for the production of novel compounds, for the industrial products, as well as for the cleaning of the environment through bioremediation. Plants are very attractive for biotechnological manipulation due to the cheap availability and simple growth nature. The engineering of rate-limiting step to induce or reduce metabolic flux toward target compounds, blocking of unwanted pathways, and introduction of shunting to divert metabolic flux in the desired direction are the major goals in plant metabolic engineering. In this chapter, we have emphasized the advancement in the production of taxol by metabolic engineering. The enormous efforts implemented by several researchers in the biotechnological production of taxol and its precursor, including cell culture, metabolic engineering in heterologous plants, as well as in microbial systems were highlighted.

2 Taxol: Most Potent Anticancer Drug from Nature

The classification of taxoid is based on the oxygenation pattern of the carbon skeleton as well as on existence of lateral chains. Among them, taxol and cephalomanine with C-13 lateral chain and baccatin III without lateral chain are most important toxoids. The chemical name of taxol (molecular formula $C_{47}H_{51}NO_{14}$) is 5β, 20-epoxy-1, 2α,4,7β,13α-hexahydroxytax-11-en-9one-4, 10-diacetate-2-benzoate 13 ester with molecular weight of 853.9 Da [26]. A, B, and C rings are present within the taxol. These rings also associated with functional groups including one benzoyl group, two hydroxyl groups, two acetyl groups, and an oxetane ring. The chemical structures of taxol, its important derivatives, and precursors are represented in Fig. 1. Among these Taxotere (semisynthetic analog of taxol) that is synthesized from the precursor 10-deacetylbaccatin III also found to be potent anticancer drug.

2.1 Historical Perspective of Taxol

In the year 1962, the bark of Pacific yew trees was first collected by the scientists of the US Department of Agriculture (USDA) in association with National Cancer Institute (NCI). The research groups of Dr. Monroe E. Wall and Dr. Mansukh Wani of Natural Product Laboratory at the Research Triangle Institute, Triangle Park, NC, discovered that extract of the bark has potent cytotoxic activity. This research group was engaged in identification and purification of the most active component of the extracts. In 1967, they were able to isolate and identified the active principal of the bark of *T. brevifolia* and named it taxol, according to its species of origin and the presence of hydroxyl groups [27, 28]. In 1977 nearly 1500 old yew tree of Pacific

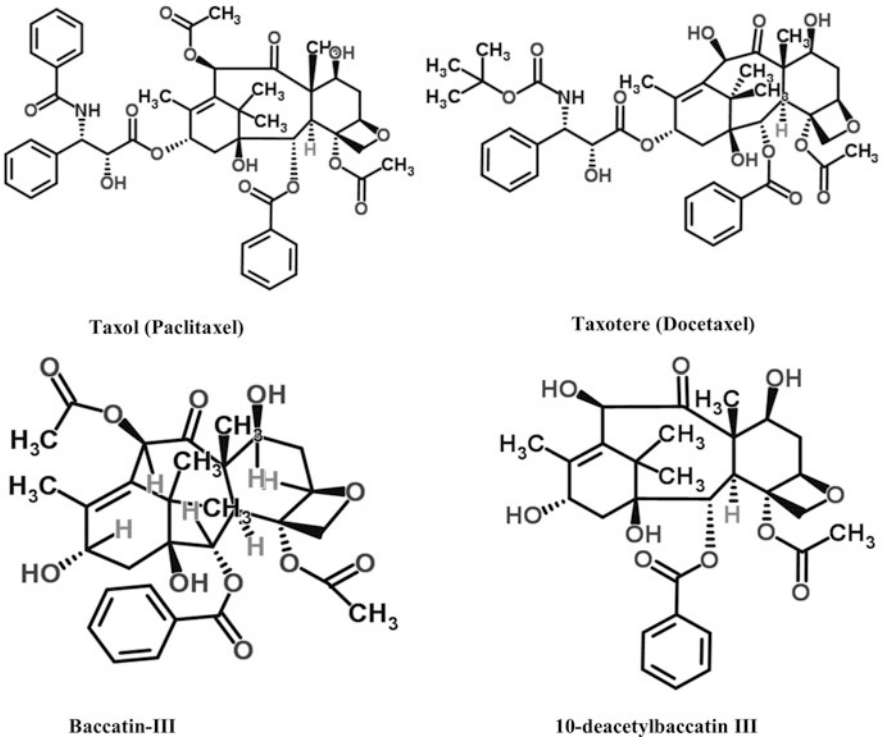

Fig. 1 The chemical structure of taxol, semisynthetic derivative Taxotere, and the two taxol precursors

Northwest forest sacrificed for collection of 700 pounds of bark which are used for taxol extraction. Later, the structure of taxol was published and it was incorporated in the NCI drug development program [9, 29]. Although taxol exhibited varied results in the preclinical trial against the tumor, but it represents efficacy against a subclass of mouse tumor models, including P388 leukemia [30]. Intensive clinical trials were hindered not only due to the shortage of the adequate amount of taxol but also for the inappropriate delivery system. Irrespective of the scarceness, clinical study on ovarian cancer was executed, and it was reported that 30% of patients with advanced ovarian cancer responded significantly [31]. Extensive demands of taxol ultimately resulted in severe depletion of *T. brevifolia* trees. In 1992, *T. brevifolia* was enlisted as endangered species to protect the trees [32]. Due to the limited accessibility of *T. brevifolia* tree for extraction of taxol, as well as its unique cytotoxic potential, a plethora of research group was involved in total chemical synthesis. However due to the complex chemical structure of taxol, about 40 reactions were found to be required for complete synthesis [11, 16]. Therefore, NCI decided to transfer taxol to a pharmaceutical company for its commercialization to obtain a large quantity of taxol. The Bristol-Myers Squibb (BMS) Company developed a cell culture

technique to produce the drug. The bioreactor-based technology has been deployed by the BMS Company for large-scale production. They have trademarked the name "Taxol" and created the new generic name paclitaxel [33, 34]. The FDA approved taxol for the treatment for ovarian as well as for advanced breast cancer in 1990s. Till the date, it is the most lucrative and best-selling chemotherapeutic drug in clinical use identified by the plant screening program.

2.2 Sources of Taxol

Taxol was first obtained by extracting peeled bark of the Pacific yew tree. *Taxus* belongs to the class Pinopsida, the order Taxales, and the family Taxaceae. Different *Taxus* species can easily be separated geographically compared to morphologically. So far eight species of *Taxus* have been recognized including *T. baccata* (European yew), *T. brevifolia* (Pacific yew or Western yew), *T. canadensis* (Canadian yew), *T. chinensis* (Chinese yew), *T. cuspidata* (Japanese yew), *T. floridana* (Florida yew), *T. globosa* (Mexican yew), and *T. wallichiana* (Himalayan yew). The remarkable discovery of antitumor properties of the extract of the bark of *T. brevifolia*, taxol, has also been described from other species of the genus *Taxus* [35–38]. Although the mixture of taxoids produced is variable among different species and tissues of the same species [39, 40]. The discovery of 10-deacetylbaccatin III from needles and leaves of *T. brevifolia* as well as the European yew *T. baccata* provided the precursor for semisynthesis of taxol and its analog, Taxotere (docetaxel) [41–43]. The leaves and bark of *T. brevifolia*, *T. wallichiana*, and *T. baccata* have been extensively used for the extraction of taxol in medicinal purpose. Unfortunately, several *Taxus* species are disappearing at an alarming rate due to overexploitation.

The slow growth of *Taxus* species, low yield, and overwhelming pharmaceutical success lead to the search for alternative source of this important alkaloid. It was found *Taxomyces andreanae*, a novel endophytic fungus isolated from the phloem of the inner bark of Pacific yew, was capable of producing taxol growing in the semisynthetic liquid medium [44]. Another endophyte, *Pestalotiopsis guepinii* isolated from the inner bark of *T. wallichiana*, was also reported to produce taxol [45]. These findings created interest in the screening for alternate endophytes sources of taxol in other *Taxus* species as well as non-*Taxus* species, and several endophytes have been isolated [46–49].

2.3 Anticancer Properties and Mechanism of Action

The natural product taxol is considered as one of the most valuable anticancer drugs of plant origin [11, 50]. It is beneficial against ovarian and breast cancer and showed efficacy for other cancers including the head and neck, lung, bladder, and gastrointestinal [51, 52]. It has been reported that taxol displayed toxicity against 9 KB cancer cell culture, B16 murine melanoma, as well as against different leukemia cell lines [9, 30, 53–56]. Due to its versatility in antitumor activity, FDA in the United

States and other countries has approved taxol as first-line chemotherapeutic agent treatment for cancer [52].

The anticancer properties of taxol are quite unique and novel compared to other antitumor agents [57–59]. In spite of preventing the polymerization of tubulin into microtubule, taxol binds to assembled microtubules and blocks its depolymerization and ultimately inhibits mitosis. It has been reported that at 1–10 nM of concentration, taxol exerts its anticancerous properties through inhibiting with dynamics of the microtubule. The suppression of microtubule dynamics hinders the assemblage of mitotic spindle and separation of the chromosome during meiosis [60–62]. The arrests of the cell cycle are correlated with cytotoxicity that ultimately resulted in the induction of apoptosis through caspase-independent and caspase-dependent pathways particularly caspases 3, 8, and 10 [63–66]. The antitumor activity in taxol is due to side chain, A ring, C2 benzoyl group, and oxetane ring. It has been reported that the activity is sustained due to the C3′ amide-acyl group in the C13 chain and presence of hydroxyl group at C2′ improved its activity [41, 67]. The cytotoxicity and stabilization of microtubule are induced by the interaction of these components with β-tubulin [68].

2.4 Biosynthetic Pathway

In spite of their enormous biological and economic importance, taxol biosynthetic pathway and its regulation are not extensively known (Fig. 2). The enzyme taxadiene synthase (TXS) catalyzes the cyclization of geranylgeranyl diphosphate (GGPP) to the taxa-(4,5, 11,12)-diene. This is the first committed step of taxol biosynthesis pathway [69–71]. Then, oxygen and acyl groups are added to the taxane at different locations catalyzed by cytochrome P450 monooxygenases. The next step of taxol biosynthesis is the construction of taxa-4(20),11(12)-dien-5α-ol by the enzyme cytochrome P450 taxadiene-5α-hydroxylase (T5αH) [72, 73]. Then the enzyme taxadiene-5α-ol-O-acetyltransferase (TDAT) acrylates the taxa-4(20),11(12)-dien-5α-ol at C5 position to form taxa-4(20),11(12)-dien-5α-yl-acetate [74]. The product is then hydroxylated at C10 position by the enzyme taxoid 10β-hydroxylase (T10βH) to form taxadiene-5α-10β-diol-acetate [75]. It was followed by the hydroxylations, oxidation of C9, and epoxidation at the C4C5 double bond that resulted in the formation of 2-debenzolytaxane [40, 76]. Then taxane 2α-O-benzoyl transferase (TBT) catalyzes the alteration of 2-debenzolytaxane to 10-deacetylbaccatin III (10-DAB). It was followed by the hydroxylation at the C10 position of the 10-DAB to form a diterpene intermediate, baccatin III. The reaction is catalyzed by the enzyme 10-deacetylbaccatin III-10-O-acetyltransferase (DBAT). The enzyme phenylalanine aminomutase (PAM) transfers coenzyme A side chain to the amino acid β-phenyalanine that resulted in the formation of β-phenylalanyl-CoA [77]. The C13 hydroxyl group of baccatin III is esterified with β-phenylalanyl-CoA side chain by the enzyme baccatin III C-13-phenylpropanoyl-CoA transferase (BAPT). The product of this reaction, 3′-N-debenzoyl-2′-deoxytaxol (β-phenylalanyl baccatin III), is hydroxylated to form N-debenzoyltaxol by an unknown Cyt P450-dependent

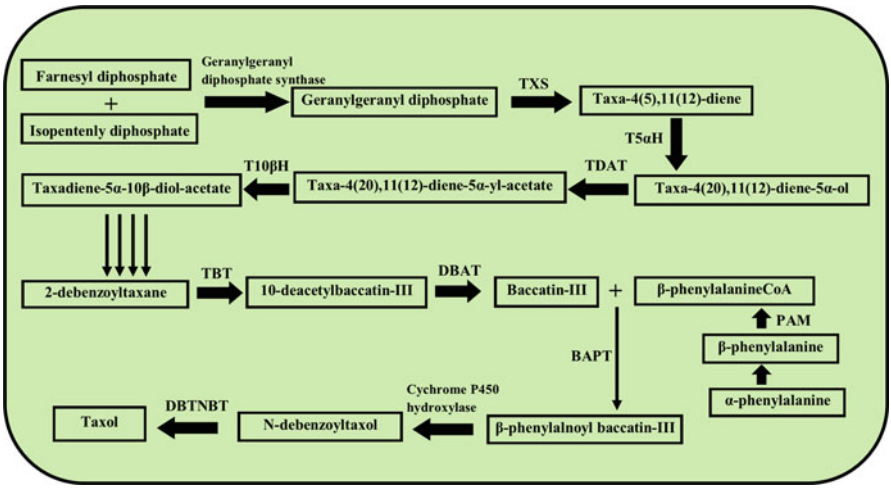

Fig. 2 Taxol biosynthetic pathway (*TXS* taxadiene synthase, *T5αH* cytochrome P450 taxadiene-5α-hydroxylase, *TDAT* taxadiene-5α-ol-O-acetyltransferase, *T10βH* taxoid 10β-hydroxylase, *TBT* taxane 2α-O-benzoyl transferase, *DBAT* 10-deacetylbaccatin III-10-O-acetyltransferase, *PAM* phenylalanine aminomutase, *BAPT* baccatin III C-13-phenylpropanoyl-CoA transferase)

hydroxylase enzyme. Finally, the enzyme 3′-N-debenzoyl-2′-deoxytaxol N-benzoyl transferase (DBTNBT) catalyzes the conjugation of benzoyl-CoA to 3′-N-debenzoyl-2′-deoxytaxol to form taxol. It is the final steps of 19 enzymes catalyzed biosynthetic pathway [77–79].

3 Metabolic Engineering

Metabolic engineering is the rewiring of cellular events through modulation of enzyme activities and regulatory functions of the cell with the implication of state-of-the-art recombinant DNA technologies [17, 22]. It includes leveraging of genetic and regulatory systems of individual cells to produce pharmaceutically or clinically important substances. The accumulation novel or desired compound could be achieved through modifying genes expression of cellular pathways to relocate the metabolic flux toward preferred pathway. The prospective applications of such modern technologies include the production of fuels, foods, and pharmaceuticals. The alteration of cells into effective factories is challenging due to the robust-regulated metabolic networks that prevent its modification. Nevertheless, the advancement in metabolic engineering has explored the potentiality in the production of complex biological molecules. Metabolic engineering can be executed in two approaches including direct and holistic approach. The direct approach is based on the introduction of genes into the host genome to control the outcome of biosynthetic pathway either by upregulation of an intermediate step or inhibit undesirable products (Fig. 3). In a holistic approach, the foreign gene codes transcription factor that

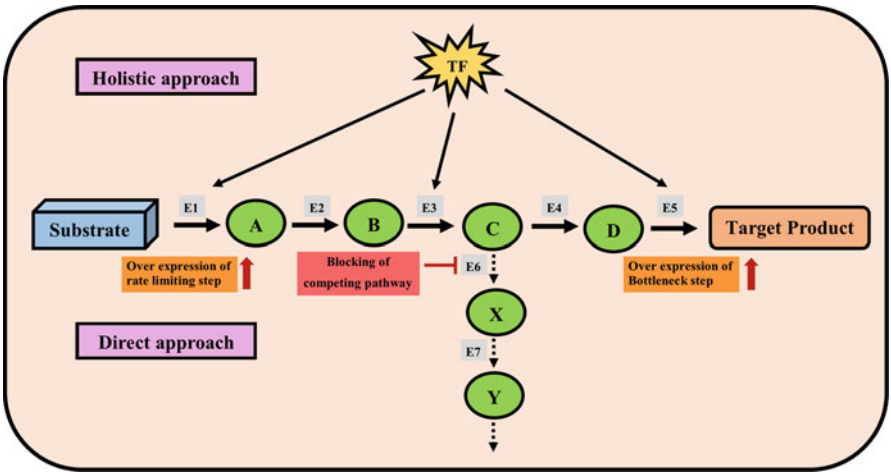

Fig. 3 Schematic representation of two approaches for metabolic engineering techniques to improve the biotechnological production of a target compound

regulates several biosynthetic pathways concurrently [80]. Endowed by the immense developments in molecular biology techniques in "post-genomic" era, the emphasis of metabolic engineering has progressively relocated from disturbing specific pathways toward modulation of the complete cellular activities that ultimately resulted in the generation of the concept of "systems metabolic engineering" [81–83]. The approaches have transformed the concept of metabolic engineering from conventional ideas of upregulation or inhibition of single gene expression toward the regulation and modification of complete cellular network [84–86].

Metabolic engineering is also applicable for transforming microorganisms into efficient cell factories for the synthesis of heterologous metabolites. The concept and techniques of system biology, synthetic biology, and evolutionary engineering have enabled a conceptual and technological framework to scale up the production of novel metabolic enzymes and biosynthetic pathways and modulation of preexisting pathway for optimizing desired product synthesis [87, 88]. The development of genetically modified microorganism capable of synthesizing various chemicals in industrial scale can be executed by systems metabolic engineering. Nevertheless, expansion of the metabolic capacity of the microbial system is a prerequisite for the large-scale production of a medicinally important metabolite. Identification of biochemical gap whose corresponding genes might not present in the host and artificial construction of metabolic pathways can efficiently be executed through the implication of different techniques of systems metabolic engineering including pathway prediction tools, incorporation of promiscuous enzymes that are compatible for of nonnatural reactions.

The pioneering approach in engineering secondary metabolism can also be achieved through multivariate modular metabolic engineering (MMME). The novelty of MMME approach includes assessment and exclusion of regulatory pathways

through the rebuilding of the metabolic network as a collection of discrete interacting modules [25, 89, 90]. Due to its simplicity and wide spectrum adaptability, MMME has the prospective to transform the field of metabolic engineering toward industrial biotechnology. In this technique, the key enzymes of the desired metabolic pathway are rearranged into distinct modules and concurrently altered based on the level of expression to balance flux through a specific pathway. Therefore, by adjusting the concentrations the turnover of the different modules can be balanced to gain maximum production of the desired compound. The transcriptional, posttranscriptional, and translational regulation enable precise control of the expression of a module's enzymes.

3.1 Plant Metabolic Engineering

The metabolic pathways of the plant represent a huge resource of important compounds with pharmacological, biotechnological, and medicinal prominence. Generally, the plant metabolites were isolated from the natural sources or were semisynthesized from intermediates of metabolic pathways. Nevertheless, this process has been outshined due to small harvest and plentiful practical problems. The implementation of modern powerful techniques for the efficient transformation techniques along with the availability of complete genome sequence conveys plant metabolic engineering as a potential alternative to the traditional chemical synthesis of biologically important metabolites [91, 92]. Plant metabolic engineering includes the controlling of present metabolic pathways by either channelizing metabolic flux toward target metabolite or diverting flux from the undesirable compound and the synthesis of the innovative chemical compound through the integration of genes from heterologous organisms into its genome. Metabolic engineering in the plant is executed by the rerouting of the enzymatic pathways to modulate the production of medicinally important compounds that are normally synthesized in small quantities to industrial level [93–95]. The degradation of environmentally toxic compounds or conversion of the plant toward resistance to abiotic or biotic factors is also another important feature of plant metabolic engineering. Nevertheless, the principal objectives of utilizing plant as a green factory in the pharmaceutical industry or agriculture are the stimulation of the manufacture of final secondary metabolites and biosynthesis of precursor molecules of medicinally important compounds.

The engineering of microbial metabolism is established technology, but its position in plants delays due to the existence of large metabolic diversity with the complex metabolic network [96, 97]. Nevertheless, highly compartmentalized cellular structure particularly huge vacuoles and plastids make the plant more complicated compared to another organism. Therefore, in silico model-based methods have recently been implemented in plant engineering. The advancement in systems biology and bioinformatics has initiated to unravel the complication of plant metabolism and enable the construction of effective theoretical models of plant metabolism [98, 99]. The arrival of high-throughput skills of next-generation sequencing and phenotyping tools for phenomics has extended the study of the model as well as

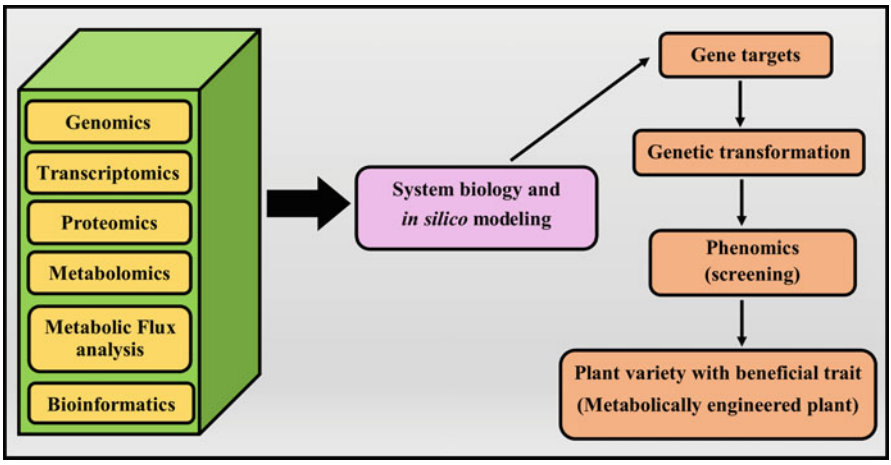

Fig. 4 Schematic representation of the application of "omics" technology for construction of metabolically engineered plants with beneficial traits

nonmodel plants through genome sequencing, transcriptome, proteomics, metabolome, and metabolic flux analysis which has subsequently provided fast, reliable, and dynamic evaluation of metabolically engineered crops with beneficial agricultural traits (Fig. 4).

4 Metabolic Engineering for Taxol Production

Taxol was first isolated from the bark of western yew, *Taxus brevifolia* [9, 54, 55]. It has been reported that the bark of yew is the primary source and it exists only in about 0.02% concentration. Additionally, the *Taxus* species are endangered, grow very slowly in areas of the Pacific Northwest, and peel off its bark which ultimately resulted in complete destruction of the plant. The endorsement of taxol for the treatment of ovarian cancer and due to its novel therapeutic application the demand of taxol is increasing worldwide [100, 101]. The economical way in the chemical synthesis of taxol is challenging due to its complex molecular architecture. Therefore, plethora of approaches including plant cell culture, metabolic engineering in heterologous plant, as well as in microbial system has been implemented from the scientists all over the world in the hunt for alternative approach to protect the limited resource of the yew plant as well as obtaining taxol in relatively inexpensive way to reduce the cost of therapy.

4.1 Plant Cell Culture

Cell culture provides an attractive way for the establishment of uniform conditions to optimize the production of medicinally important phytochemical. It provides the

benefit of growth in minimal space without the necessity for greenhouses. Nevertheless, the culture conditions can be manipulated by the application of elicitors and other stresses in such a way to maximize the accumulation of targeted metabolites [102]. Cell lines can also be transformed with specific genes of metabolic pathway to modulate the metabolic profiles as well as to scale up the production. For harvesting of unusual metabolites from rare plant species, novel drugs or large-scale production cell culture provides a lucrative substitute compared to the plants that as impetuous to genetic manipulation. The metabolic engineering of *Taxus* cell culture to modulate the production of taxol and related toxoids is very challenging due to the difficulty in genetic transformation of the gymnosperm plantlike *Taxus* and due to its slow growing nature. Besides these obstacles, a wild strain of *Agrobacterium tumefaciens* was used to transform *T. brevifolia* and *T. baccata* in 1994 [103]. Later hairy root culture of *Taxus* was successfully established first time using *Agrobacterium rhizogenes* [104]. Unfortunately, the production of taxane was low, and therefore the system was inappropriate for industrial scale production. Hairy root culture of *Taxus* x *media* var. Hicksii was fed with precursors (L-phenylalanine and p-amino benzoic acid), or in combination with methyl jasmonate and accumulation of paclitaxel, baccatin III and 10-deacetylbaccatin III were monitored [105]. The highest amount of paclitaxel (221.8 μg L^{-1}) was accumulated in the medium supplemented with 100 μM of the precursors along with methyl jasmonate. The successful application of metabolic engineering toward the production of taxol using a direct approach was first successfully executed in 2005 [106]. Transgenic *T. marei* cell culture overexpressing 10-deacetylbaccatin III-10-O-acetyltransferase (DBAT) and taxadiene synthase (TXS) genes produce the highest level of taxol. *Taxus media* cell cultures with same genetic characteristics compared to the parent were established using *A. rhizogenes* LBA 9402 and the C58C1 strain of *A. tumefaciens* harboring the taxadiene synthase gene [107]. The highest taxane production (265% greater than control) was found in the transformed culture in optimal conditions and elicited with methyl jasmonate. 9-cis-epoxycarotenoid dioxygenase gene from *Taxus chinensis* was overexpressed in transgenic *T. chinensis* cells, and about 2.7-fold higher taxol production was recorded [108]. The hairy root culture of *Taxus* x *media* var. Hicksii was established by transformation with C58C1 strain of *A. tumefaciens* harboring the taxadiene synthase gene of *T. baccata* [109]. These transgenic root lines were elicited with different combinations of elicitors (sodium nitroprusside and methyl jasmonate), and 10.78 mg L^{-1} taxol was accumulated upon elicitation with methyl jasmonate and feeding with L-phenylalanine.

4.2 Engineering in Microbes Toward Production of Taxol and Its Precursor

Metabolic engineering within heterologous host such as microbes for the manufacture of plant metabolites includes improvement of regulatory processes that ultimately resulted in the increased yield of a specific compound through rechanneling the carbon flux. In recent year, the execution of recombinant expression systems to

reconstruct natural product pathways has upgraded meaningfully. Among the different microorganism, *Escherichia coli* and *Saccharomyces cerevisiae* have usually been used to bypass complex technical issues associated with the metabolic engineering of plant cell cultures [24, 110, 111]. *E. coli* can be considered as superior host due to its simplicity in the central metabolic pathway and robust regulatory systems. The lacks of posttranslational modification and subcellular compartment and difficulty in the expression of complex protein are the major drawbacks in metabolic engineering of *E. coli*. On the other hand, yeast has ideal characters including larger cell size, better resistance against pH alteration, and unwanted products for final processing of end products. Additionally, mating allows improved cellular engineering that ultimately resulted in robust growth and increased adaptation to the adverse environment. In metabolic engineering, in order to improve product yields, the complete biosynthetic pathways are transferred from native hosts into heterologous organisms. Therefore, for improved synthesis of target product, the optimization of gene expression system, promoter strength precise control of the endogenous regulatory network is utmost required. The isoprenoids are also synthesized in the microbial system [112, 113]. In microbes, they are involved in different cellular activities including light absorption and protein modifications. Among the isoprenoids, biotechnological production of taxol and its precursors through the metabolically engineered microbial system has been enriched over the past few years [114].

The successful synthesis of taxadiene, the intermediate of taxol biosynthesis, was first reported in metabolically engineered *E. coli* in the year 2001 [115]. Overexpression of three genes encoding isopentenyl diphosphate isomerase (IPI), geranylgeranyl diphosphate synthase (GGPS), and taxadiene synthase (TXS) of terpenoid pathway was executed within genetically engineered *E. coli*. In the transgenic *E. coli*, taxadiene was found to be produced at the yield of 1.3 mg L^{-1} of cell culture. This success opened the window for the synthesis of taxoids in non-paclitaxel-producing organisms through metabolic engineering. In a subsequent study, the multivariate-modular approach was implemented to increase the titers of taxadiene up to 1 g L^{-1} [116]. In *E. coli*, methylerythritol-phosphate (MEP) pathway and mevalonic acid (MVA) pathway can produce isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Fig. 5). The taxol can be synthesized from these two building blocks [117]. The researchers have separated the taxadiene biosynthetic pathway into two units: the MEP pathway that generates isopentenyl pyrophosphate (IPP) and heterologous downstream pathway that produces terpenoid. These modules were optimized to exploit the maximum production of taxadiene with reducing the accumulation of indole, the inhibitor of the pathway. In another study, in silico modeling was executed to optimize the biosynthesis of taxadiene in *E. coli* by comparing the maximum theoretical IPP yields and the thermodynamic properties of the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway and MVA pathway using different hosts and carbon sources [118]. They have reported that genetic manipulation of the DXP pathway and chromosomal engineering were powerful tools for heterologous biosynthesis of taxadiene. The pedogenic DPX pathway was modulated by redesigning of codon usage and chromosomal

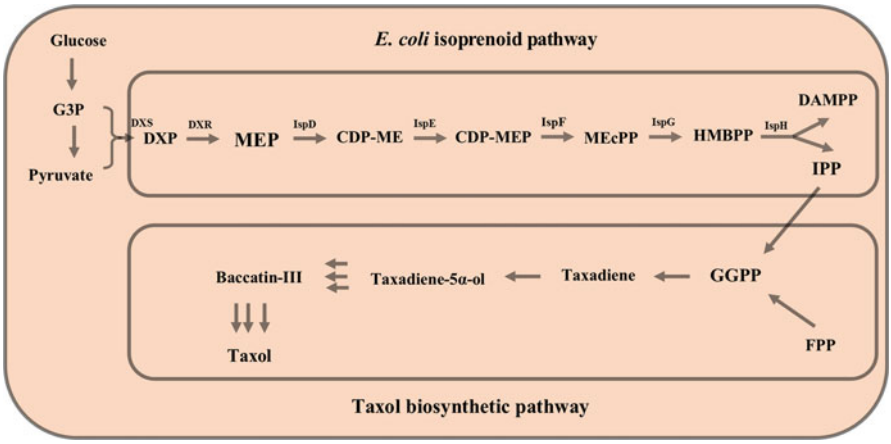

Fig. 5 Biosynthetic scheme for taxol production in *E. coli* (*DXS* DOXP synthase, *CDP-ME* methylerythritol cytidyl diphosphate, *CDP-MEP* 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate, *HMBPP* 4-hydroxy-3-methyl-butenyl-1-diphosphate, *DXP* 1-deoxy-D-xylulose 5-phosphate, *MEP* 2-C-methylerythritol 4-phosphate, *MEcPP* 2-C-methyl-D-erythritol 2,4-cyclodiphosphate, *DAMPP* dimethylallyl pyrophosphate, *DXR DXP* reductoisomerase, *IspD* 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, *IspE* 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, *IspF* 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, *IspG* 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, *IspH* 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase)

engineering that ultimately gave yields of 876 ± 60 mg L^{-1} taxadiene. The K- and B-derived *E. coli* strains were used as a heterologous host for the production of taxadiene [119]. In this study, different inducible promoters (T7, Trc, and T5) and cellular backgrounds were varied during the biosynthesis of taxadiene. The T7 promoter system was examined for upstream MEP pathway, whereas T7, Trc, and T5 promoters were used for downstream MVA pathway. It was reported that K-derivative transgenic *E. coli* synthesized taxadiene about 2.5-fold higher compared to another one. The significant difference in pyruvate metabolism revealed through transcriptomics analysis established the discrepancy in taxadiene biosynthesis between these two strains. Different types of in vivo metabolic chemistries including oxygenation are vital for the production of complex secondary metabolites such as taxol. The expression of cytochrome P450 enzymes was modified to execute oxygenation chemistry in *E. coli*. The highest reported titer of oxygenated taxanes (∼570 ± 45 mg L^{-1}) in *E. coli* was achieved through a series of optimizations including N-terminal modifications [120].

The yeast *Saccharomyces cerevisiae* can also be used for metabolic engineering for the scale-up of taxol biosynthesis. The study was executed to rebuild the initial steps in taxol biosynthetic pathway, and eight genes were expressed in yeast [121]. This initial study was a prerequisite for the construction of engineered yeast for the synthesis of taxol or its precursors. Heterologous genes encoding enzymes of early steps of taxol biosynthetic pathway and the modulation of controlling factor to

prevent deviation toward other undesirable pathways were executed to increase the production of taxadiene within genetically modified *S. cerevisiae* [122]. The impact of these modulations in synthesis taxadiene was monitored. They have observed that only the expression of TXS gene of *T. chinensis* or coexpression of both TXS and GGPS was unable to rise taxadiene content significantly. They have reported that expression of modified 3-hydroxyl-3-methylglutaryl-CoA reductase (HMG-CoA reductase) isoenzyme 1 as well as UPC2-1 (mutant regulatory protein) in transgenic *S. cerevisiae* ultimately induced taxadiene content up to fifty percent. The substitution of *T. chinensis* GGPS gene with its complement part from *Sulfolobus acidocaldarius* and codon optimization resulted in high-level expression in taxadiene (8.7 ± 0.85 mg L^{-1}) that was found to be 40-fold higher compared to control. Later, protein modeling and in silico metabolomic study guided the establishment of transgenic *S. cerevisiae* that can efficiently modulate taxadiene content [123]. In this study, the biosynthesis pathway was reconstructed through transformation with TXS gene along with upregulation of erg20 and thmgr gene. The catalytic efficiency of GGPPS enzymes was anticipated using enzyme-substrate docking strategy. The in silico modeling was correlated with experimental outcomes, and it was found that transgenic yeast with *Taxus cuspidate* GGPPS gene caused the highest synthesis of taxadiene (72.8 mg L^{-1}).

4.3 Engineering in Heterologous Plant

There are a plethora of instances of successful metabolic engineering of taxol biosynthetic genes and enhanced production of taxol in microbial hosts. Nevertheless, complex plant-derived terpenoids including taxol are often problematic to produce within microbial host due to insolubility, membrane associations, as well as different types of posttranslational modifications. Plant cytochrome P450 enzymes are also utmost required for complete biosynthesis of the taxol. There are several obstacles in heterologous expression of terpenoids such as taxol due to the more complex plant transformation techniques for many species, time-consuming recovery of transgenic plants, and slow growth rates compared to microbial hosts. Therefore, plants are more preferable compared to a microbial system for metabolic engineering of natural plant products due to the existence of innate cytochrome P450-related biosynthetic genes. The unavailability of efficient transformation systems for *Taxus* species created a platform to screen alternative plants that can be genetically engineered to produce taxol or its precursors. Metabolic engineering of heterologous host plants for production of taxol has been reported by several research groups. The pioneer steps toward the genetically engineering of taxoids biosynthesis in angiosperms were published in 2004 [124]. The constitutive expression of recombinant *T. baccata* taxadiene synthase (TXS) gene without plastid targeting signature sequence and fusion of C-terminal histidine tagged (*Arabidopsis thaliana*) resulted in the production of taxadiene with associated growth hindrance in transgenic plants. The relatively lower accumulation of carotenoids also indicates relocation of geranylgeranyl diphosphate (GGDP) pool in transgenic plants. The

redirection of GGDP pools toward the production of taxadiene was further supported by the successful establishment of transgenic system expressing a glucocorticoid-mediated system. In another study, the redirection of GGDP toward taxadiene production was reported in transgenic tomato plants. The expression of TXS gene in tomato line missing the capacity to utilize GGDP for carotenoid production ultimately resulted in the production of taxadiene about 160 mg kg^{-1} dried fruit [125]. Metabolic engineering of ginseng (*Panax ginseng* C.A. Meyer) roots with TXS gene of *T. brevifolia* was successfully executed without compromising in growth as well as phenotype [126]. In the transgenic TSS3-2 line accumulation of taxadiene, the precursor of taxol was found to be 9.1 μg g^{-1} dry weight. Elicitation by jasmonate also resulted in about 1.74-fold increment of the taxadiene content. The incorporation of TXS gene within the transcriptional regulation of CaMV 35S promoter in *Nicotiana benthamiana* resulted in the de novo production of taxadiene of 11–27 μg g^{-1} dry weight [127]. Upon elicitation with methyl jasmonate and shunting the terpenoid pathway with suppression of phytoene synthase gene also increased taxadiene content up to 1.9-fold in the transgenic line TSS8. The metabolic engineering of taxol biosynthetic pathway in *Artemisia annua* L plant was also successfully established [128]. The *Agrobacterium tumefaciens*-mediated transformation of TXS gene within pCAMBIA1304 promoter ensued in the accumulation of taxadiene up to 129.7 μg g^{-1} dry mass in the stem without affecting the artemisinin content as well as the growth of the transgenic plant.

5 Conclusions

Cancer is the most devastating disease compared to other life-threatening ailments, and it bears the majority of the burden for the people of low- and middle-income countries. A plethora of research work has been implicated to efficiently detect it in early stage and cost-effective treatment for the people below poverty line. Under such circumstance Taxol (generic name paclitaxel), isolated from the yew tree, was found to be one of the most effective anticancer drugs of plant origin. Unfortunately, due to the relatively lower amount of accumulation in native plant and unavailability of cost-effective chemical synthesis, the demand for the alternative synthesis of taxol is increasing enormously. The application of modern molecular biological techniques to metabolically engineered heterologous plant as well as microbes for the effective synthesis of taxol and its precursor has been successfully implemented. To scale up the biotechnological production of taxol in industrial level, metabolic engineering in a wide range of plant species along with efficient extraction and separation methods of taxol is in utmost priority among the researchers. The expression of taxol biosynthetic genes from the yew tree into diverse plant species through metabolic engineering is appreciated for the taxol biosynthesis, but the direct engineering in the yew species will be more sensible approach. The combinations of genetic modification techniques using *Agrobacterium* and particle bombardment, along with the established cell suspension technology for large-scale production, may lead to the higher productivities of taxol via metabolically

engineered plant cells. More extensive research should be executed to completely unrevealed the regulation of taxol biosynthetic pathway as well as the key genes of rate-limiting steps. Therefore, the future perspective should be the implication of empirical as well as rational approaches of metabolic engineering toward the biotechnological production of taxol.

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