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•Review•

Biosynthesis and regulation of diterpenoids in medicinal plants

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[ABSTRACT] Plant diterpenoids are widely distributed and abundant natural products with diverse structures and functions in nature, which have been commonly used in pharmaceutical, agricultural and industrial production. In recent years, plant diterpenoids have attracted increasing attention, including their biosynthetic pathways, transcriptional regulatory networks, and biological functions. Herein, the biosynthetic pathways of diterpenoids are summarized in a modular fashion. Further, the regulatory network between diterpene biosynthesis and environmental factors is reviewed. Insights into diterpene metabolism may drive elucidation of complex active diterpene pathways and serve as a knowledge repository for metabolic engineering and cell factory construction.

[KEY WORDS] Diterpenoid; Pharmaceutical value; Biosynthesis; Regulation; Medicinal plant
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Introduction

Terpenoids or isoprenoids derived from 5-carbon isoprene units can be classified into monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids and polyterpenoids based on the number of the 5-carbon unit in a molecule. Among them, plant 20-carbon diterpenoids are a large and structurally diverse class of natural products with important physiological functions for plants and significant pharmaceutical or industrial values for humans. In plants, diterpenoids play a key role in primary metabolism, growth and development, such as gibberellins (GAs) [1], while some of them confer resistance against stress, insects and pathogens, such as diterpenoid resin acids (DRAs) [2] and diterpenoid phytoalexins (DPs) [3, 4]. For humans, some diterpenoids have industrial value as resources of biofuels, food additives and perfumes [5], or exert important pharmacological activities, including antivirus, antibacterial and anticancer effects [6-8].

The biosynthetic pathway of plant diterpenoids greatly contributes to the structural diversity of diterpene products and intermediates, and can be divided into three modules responsible for skeleton formation, oxidation and post-modification. In the first module, various diterpene skeletons are formed under the catalysis of diterpene synthases (diTPSs), followed by oxidation of the skeletons catalyzed by cytochrome P450 monooxygenases (CYPs) or 2-oxoglutarate-dependent dioxygenases (2-ODDs) in the second module, and various post-modification reactions (such as by transferases and isomerases) in the last module [9, 10]. Furthermore, the biosynthesis of plant diterpenoids is regulated at the transcriptional and post-transcriptional levels by biological and abiotic factors, exhibiting spatio-temporal expression patterns. In recent years, a deepen understanding of plant natural products has enhanced interest in investigating the biosynthesis and regulation of diterpenoids in plants. However, there are still limited researches conerning the biosynthetic pathway and metabolism regulation of diterpenoids derived from medicinal plants.

In this review, we first summarized the function and pharmaceutical values of plant diterpenoids, and then generalized the diterpene biosynthetic pathway with modules, followed by introduction of the regulatory factors and regulation modes of diterpenoids in plants, so as to outline a clear flow-based strategy to support pathway identification, gene of interest finding and synthesis of natural medicine.

The Function and Pharmaceutical Values of Plant Diterpenoids

Plants produce thousands of diterpene natural products with diverse physiological and biological functions in growth, development, and stress resistance. The phytohormone GAs,

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as a commonly conserved subset of diterpenoids, are widely involved in the development of plant organs and tissues. It has been extensively studied that the regulatory network between GAs and abscisic acid (ABA) mediates resistance and response to environmental stresses in two of the world's major food crops, maize (*Zea mays*) and rice (*Oryza sativa*) [11]. Moreover, plants also produce myriad specialized diterpenoids with specific species and spatial-temporal distribution that are directly involved in terpenoid-based defense systems. Isopimaric acid, a kind of DRA, is an important metabolite in stress defense with obvious anti-insect and anti-bacterial activities [12, 13]. A diverse array of diterpenoid metabolites in switchgrass were induced in roots and leaves by oxidative stress and ultraviolet irradiation, suggesting their possible role in abiotic stress adaptation [14]. In the dicotyledon plant *Artemisia annua*, isopimaradiene and its derivates were proven to enhance stress resilience [15].

It should be noted that not all diterpene products in plants are required for plant growth or development [16]. Many diterpenoids are of significant industrial value as direct resources or indirect raw materials for pharmaceuticals, fragrances, biofuels and food additives (Table 1). Andrographolide and neoandrographolide, which belong to labdane-related diterpenoids (LRDs) in the Acanthaceae plant *Andrographis paniculata*, have anti-tumor, anti-inflammatory and antiviral effects, and are widely used in the treatment of acute liver and lung injury in China. Its total ester sulfonates can be used to treat COVID-19 pneumonia [17-19]. Clerodane-related diterpenoid, ajugacumbin A, is used as a strongl hemostatic and anti-inflammatory agent for the treatment of acute trauma [20, 21]. Macrocyclic diterpene ginkgolides, especially ginkgolide B, exhibit strong and specific inhibitory effect on platelet activating factor (PAF) receptors [22], and are widely used for the treatment of ischemic stroke [23] and myocardial injury [24]. Taxol, a diterpenoid compound with anti-cancer activity, is a chemotherapy medication for the treatment of various types of cancers, including breast, ovarian, lung and some head and neck cancers [25, 26]. In addition, other diterpenoids such as triptolide [27, 28] and oridonin [29, 30] were proven to have anti-tumor activity. Pimarane can relieve spasm, and is used as a muscle relaxant and antiparasite [31]. Ingenol and its derivatives isolated from *Euphorbia tirucalli* are new promising compounds for HIV reactivation and supporting viral eradication efforts [32, 33]. Diterpenoids act as the valuable resources of fragrances and organic products and are widely used in the biotechnology industry. It is reported that phytol is a diterpene member of the long-chain unsaturated acyclic alcohols and has been widely used as a valuable essential oil for both pharmaceutical and perfume industries [34, 35]. Sclareol is another diterpene with high value for theraputic use (cancer therapy) and perfume manufacturing, and most of commercially-produced sclareol are derived from the extraction of cultivated clary sage (*Salvia sclarea*) [36, 37]. Besides, ambroxides (known as the brand name Ambroxan) and related derivatives are highly priced in the fragrance industry, and valued for their delicate odors and fixative properties. Nowadays, ambroxides have replaced ambergris, a waxy substance produced by sperm whales, in perfume manufacturing for the purpose of wildlife conservation [37]. In another aspect, diterpenoids can be used as biofuels or help bioenergy achieve sustainable production. Phytol and some of its derivatives, as mentioned above, including phytanic acid, exert a wide range of applications in the biotechnological industry [34, 38]. Switchgrass, a perennial herb native to North America, is mainly the next generation of raw materials for lignocellulosic biofuel production, whose diterpenoids produced by secondary metabolism can help them become sustainable biofuels [14]. In addition, some diterpenoids are used as food additives, where the most typical examples are rebaudiosides (*e.g*., Reb A, Reb D and Reb M) [39, 40]. They are commercially important low/no-calorie natural sweeteners from *Stevia rebaudiana* with superior organoleptic properties and have been used by Pepsi-Cola Company for its drink products [41, 42].

The Biosynthetic Pathways of Plant Diterpenoids

Diterpenoids are built up from isopentenyl pyrophosphate (IPP) and dimethyl allyl pyrophosphate (DMAPP) [60], which are supplied from the cytosolic mevalonic acid (MVA) pathway and the plastidic methyl erythritol phosphate (MEP) pathway in plants [61]. One DMAPP molecule is used as the substrate for addition of three IPP molecules to synthesize short-chain geranyl-geranyl pyrophosphate (GGPP) by the condensation reaction. Then, GGPP is used for synthesis of the conserved GAs and the other specialized diterpenoids through three artificially assigned modules, namely the diTPS module, the oxygenase module and the post modification module [9, 10] (Fig. 1). Recent years have seen substantial increases in knowledge and understanding about diterpene biosynthesis in plants, in part based on the development of genome and transcriptome sequencing and synthetic biology.

Formation of diterpene skeletons by diTPSs

In the first module, diTPSs in plants can be divided into three categories (class I, class II, and class I/II) based on the differences in three conserved domains (α, β and γ) within the proteins [62, 63]. Class II diTPS has a conserved DxDD motif in class II active site at the interface of the $\beta\gamma$ domain, while class I diTPS has a conserved DDxxD and a NSE/DTE motif in class I active site buried in the α domain. Due to their catalytic function, diterpene synthases are also known as diterpene cyclases (DTCs). Benefiting from continuous exploration of the real and modeled structures of plant DTCs, as well as some microbial DTCs [64-66], the synthetic mechanism of diterpene diversity has been gradually explained.

In angiosperms, monofunctional class II and class I diTPSs are usually combined in pairs to synthesize a large class of labdane-related diterpedoids (LRDs) from GGPP. In this process, class II diTPS first catalyzes the protonation-initiated cyclization of GGPP to form labda-13-en-8-yl$^+$ diphosphate intermediate and the deprotonation of the methyl group at C-8, finally producing a series of bicyclic skeletons with the diphosphate group, such as labdadienyl/copalyl diphosphate (CPP) with different stereochemical configurations of the methyl and hydride substituents across the C-9,10 bond,

Table 1 The function of diterpenoids in plants

Compouds	Species sources	Functions	Categories	References
GAs	Most plants	Plant growth and development	Plant growth and resistance	[1, 11, 43, 44]
DRAs	Conifers	Plant stress resistance, anti-insect and anti-bacterial activity		[2, 13, 45-47]
DPs	Monocotyledon			[3, 4]
IDMs	*Artemisia annuaPinus contorta*			[12, 13, 15, 48]
Andrographolide Neoandrographolide	*Andrographis paniculata*	Anti-tumor, anti-inflammation, antiviral activity, (treatment of COVID-19), lung and liver injury	Pharmaceutical industry	[17-19]
Ajugacumbin A	*Ajuga decumbens Ajuga forrestii*	Hemostatic and anti-inflammatory activity		[20, 21]
Ginkgolide B	*Ginkgo biloba*	PAF inhibitor, treatment of ischemic stroke and myocardial injury		[22-24]
Paclitaxel/Taxol	*Taxus chinensis Taxus cuspidata*	Anti-cancer activity		[25, 26, 88, 126]
Triptolide	*Tripterygium wilfordii*	Anti-cancer activity		[27, 28]
Oridonin	*Rabdosia rubescens*	Anti-cancer activity		[29, 30]
Pimarane	*Aldama discolor*	Spasmolysis and anti-parasitic activity		[31]
Ingenol	*Euphorbia tirucalli*	Antiviral (anti-HIV) activity		[32, 33]
Salvinorin A	*Salvia divinorum*	Potent and selective agonist of κ-opioid receptor		[49, 50]
Salvidivin A		Antagonist of κ-opioid receptor		
Salvinicin A		Partial agonist of κ-opioid receptor		[50, 51]
Salvinicin B		Antagonist of μ-opioid receptor		
Forskolin	*Coleus forskohlii*	Anti-HIV and anti-cancer activity, treatment of hypertension and heart failure		[52, 53]
Plaunotol	*Croton stellatopilosus*	Anti-bacterial, anti-angiogenic and anti-cancer activity, treatment of gastritis and gastric ulcer		[54, 55]
Tanshinones	*Salvia miltiorrhiza*	Anti-cancer activity, treatment of cardiovascular diseases and prevention of diabetes complications		[56, 57]
Tigilanol tiglate	*Fontainea picrosperma*	Anti-cancer activity		[58]
Diterpene phorbol esters	*Jatropha curcas*	Antibiotics, induction of leukemia cell division		[59]
Phytol	Most plants	Antimicrobial, antioxidant, anxiolytic and anticonvulsant and anti-inflammatory activityPerfume and biotechnology raw material	Pharmaceutical, perfume and biotechnological industry	[6, 34, 38]
Sclareol	*Salvia sclarea*	Anti-cancer activityPerfume raw material		[36, 37]
Ambroxides	*Salvia sclarea*	Perfume raw material	Perfume industry	[37]
Rebaudiosides	*Stevia rebaudiana*	Food additve (sweeter)	Food industry	[39-42]
Steviosides	*Stevia rebaudiana*	Food additve (sweeter)		[40]

*GAs: gibberellins; DRAs: diterpenoid resin acids; DPs: diterpene phytoalexins; IDMs: isopimiradiene derived metabolites; PAF: platelet activating factor.

i.e. ent-CPP, *syn*-CPP or (+)-CPP [67, 68]. The most commonly observed CPP synthases (CPS) in plants is *ent*-CPS, because it is required for gibberellin phytohormone metabolism [9]. For example, it is found that *Aa*CPS1 in *Artemisia annua* [15] and *Sd*CPS1 in *Salvia divinorum* [69] converted GGPP to *ent*-CPP, respectively. Meanwhile, *normal*-CPS is also quite common such as *Cf*TPS1 in *Coleus forskohlii* [52, 70], and *Tw*TPS7v2 and *Tw*TPS9v2 from *Tripterygium wilfordii* [71], and can catalyze GGPP precursor to (+)-CPP. However, *syn*-CPS is not common, with fewer reports like *Os*CPS*syn* in rice [72]. Before deprotonation, the carbocation of the intermediate labda-13-en-8-yl $^+$ may trigger a series of 1,2-hydride and/or methyl shifts or is captured by water, producing other hydrocarbon backbones (*e.g.* clerodienyl diphosphate (CLPP), 8,13-CPP, *endo*-CPP) and hydroxylated variants (*e.g.* 8-OH-CPP, 8β-OH-*ent*-CPP and peregrinol diphosphate (PPP)) (Fig. 1) [73-75]. All these bicyclic skeletons are the origin of LRDs, enhancing the regularity of chemical diversity of the corresponding biosynthetic intermediate.

The majority of class I diTPSs further convert class II diTPS products into a variety of polycyclic (2-5) diterpene scaffolds by removing the diphosphate without catalyzing cyclization [14]. Class I diTPSs can simply remove the diphosphate group of class II products, or continue to catalyze subsequent cyclization, oxidation, and/or rearrangement to generate diverse diterpene backbones [76]. For further catalytic mechanisms, we recommend these two reviews [9, 77]. *ent*-Kaurene, a phytohormone GA's precursor, is synthesized by the cooperation of class II *ent*-CPS and class I kaurene synthase (KS) (Fig. 1). Other class I diTPSs can directly convert GGPP and NNPP (GGPP stereoisomer) into macrocyclic and linear diterpenes, such as the macrocyclic cembratriene-ol synthase from *Nicotiana tabacum* [78] and the linear geranyllinalool synthase from *A. annua*. Some studies also identified several monofunctional class I diTPSs in gymnosperms, such as taxadiene synthases (TS) from yew species [79], and isopimaradiene synthases and pimaradiene synthases from *Pinus contorta* and *Pinus banksiana* [48]. The bifunctional class I/II

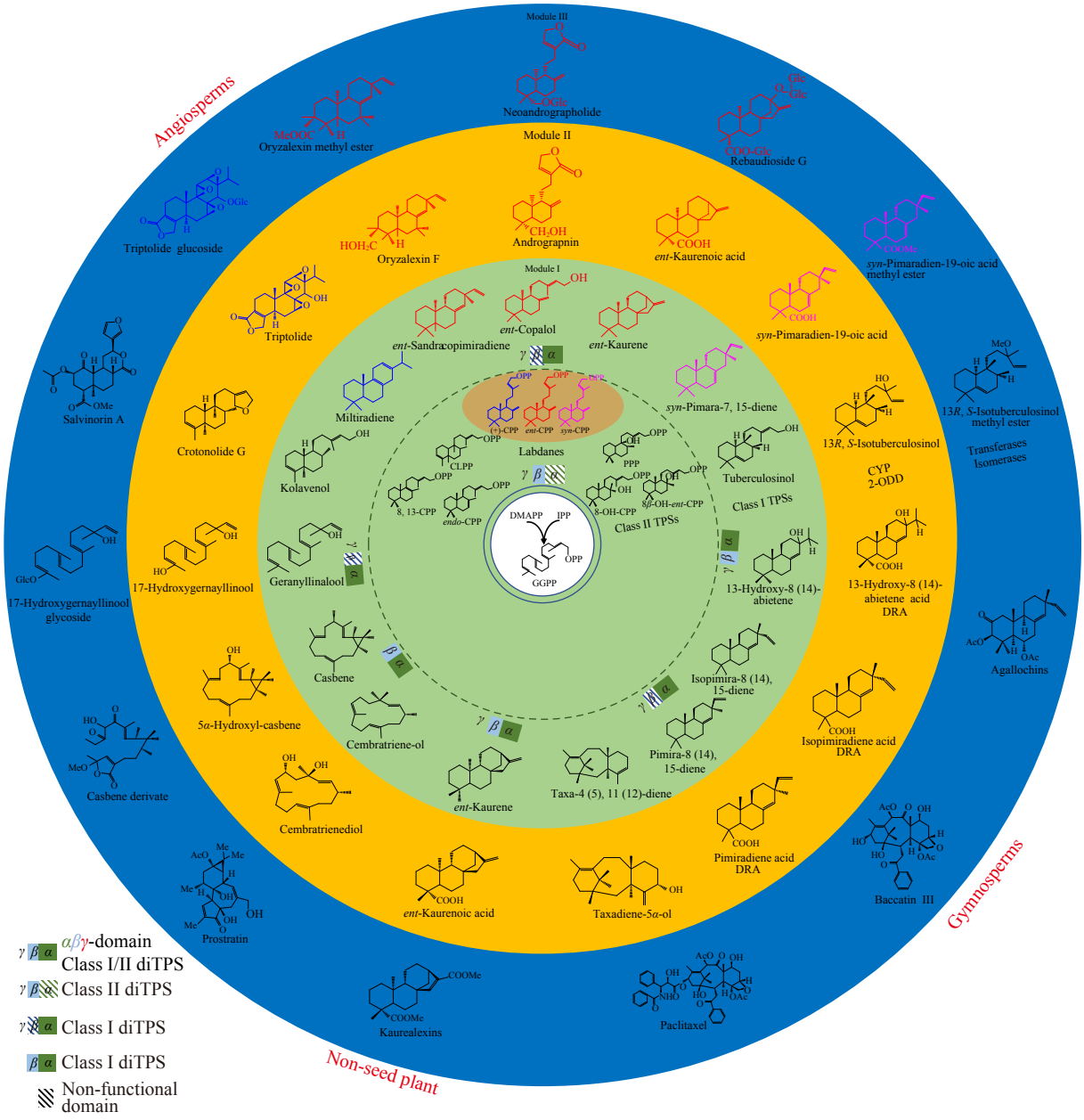

Fig. 1 Diterpenoid biosynthesis in plants. The biosynthetic pathways of diterpenoids are summarized in modular fashion. In module I, geranylgeranyl diphosphate (GGPP), the diterpene precursor, is converted to various diterpene skeletons by diterpene synthases/cyclases (diTPSs/DTCs). Furthermore, the diterpene skeletons are catalyzed by cytochrome P450 monooxygenases (CYPs) and 2-oxoglutarate-dependent dioxygenases (2-ODDs) in module II, and transferases and isomerases in module III for diverse diterpenoids. CPP: labdadienyl/copalyl diphosphate, including different stereoisomers (*ent*-CPP, *syn*-CPP and (+)-CPP); CLPP: clerodienyl diphosphate, including *cis*-*trans*-CLPP (CT-CLPP), kolavenyl diphosphate (KPP) and *ent*-KPP; PPP: peregrinol diphosphate; DRAs: diterpenoid resin acids

diTPS, commonly found in gymnosperms and non-seed plants, combines both class II and class I reactions in a single protein, where the prenyl diphosphate intermediate may freely diffuse from class II to class I active site. The product of first identified class I/II diTPS from *Abies grandis* is 13-hydroxy-8(14)-abietene, which is further degraded into different olefins for producing a large set of diterpene resin acids (DRAs) [46]. In non-seed plants, the diTPS is also the pivotal enzyme for biosynthesis of the diterpenoids. Two diTPSs (*Sm*TPS7 and *Sm*TPS4) in *Selaginella moellendorffii* are bifunctional enzymes for *ent*-kaurene synthesis and share common ancestry with the typical seed plant diTPS [80]. In conclusion, DTCs make a good start for the complex diversity of diterpenoids (Fig. 1).

Oxidation of diterpene skeletons by CYPs/2-ODDs

Over 95% of known diterpene structures carry two or more oxygen atoms to produce active compounds [81], indicating that a few DTCs contribute to the introduction of hydroxyls in the structures. More importantly, extensive participation of CYPs in the second module of the diterpene path-

way broadly increases the complexity of the diterpene structure. *CYP* is the largest gene family involved in plant mentalism with around 1% plant protein-coding genes responsible for the vast majority of diterpene oxidation reactions [82]. In the second module, diverse CYPs/2-ODDs catalyze the oxidation of diterpene skeletons, including hydroxylation, continuous oxidation, ring formation, ring expansion and ring contraction. CYPs are primarily located at the membrane of the endoplasmic reticulum (ER) and their catalytic activity is strictly dependent on the electron supply from membrane-bound proteins NADPH-cytochrome P450 reductase (CPRs). The function characterization of *Croton stellatopilosus Cs*CYP97C27 and *Cs*CPR1 in *Escherichia coli* showed that simultaneous incubation with the substrate geranylgeraniol and cofactor NADPH led to the formation of acyclic diterpene plaunotol, which is used to treat stomach ulcers [54]. Through these CYP-CPR cocatalyzed reactions, the diverse oxygenation patterns of plant natural products by secondary metabolic pathways were realized [83, 84].

Based on the evolutionary view and the type of introns, plant CYPs can be classified into A-type and non-A-type. Among them, the A-type CYPs are considered to be involved in the synthesis of plant specialized natural products, while the non-A-type CYPs are associated with more basic metabolism [85, 86]. Higher plants typically contain in excess of 250 CYPs, which are membrane-bound proteins and can be categorized based on protein sequence identity and phylogenetic analysis. Based on the available sequences, land plant CYPs can be grouped in 11 phylogenetically distinct clans, of which the *CYP71* clan now contains the original A-type CYPs [87]. Therefore, CYPs in the *CYP71* clan are involved in the majority of plant specialized metabolism, including diterpenoid metabolism. Some studies have found that a few CYP members in the *CYP85* clan can also metabolize specialized diterpenoids. The members of the *CYP720B* subfamily from the *CYP85* clan are important for DRA biosynthesis in many conifers, such as spruce and pine [13, 45, 47]. In gymnosperms, *CYP*s involved in specialized diterpene biosynthesis often exist in a cluster with other genes in the same pathway. A recent study has discovered a unique cluster in *Taxus cuspidata* genome containing the *TS*s and several *CYP725A* members from the *CYP85* clan for taxadiene biosynthesis [88]. And another study uncovered a biosynthetic gene cluster on *Ginkgo biloba* chromosome 5 encoding five CYPs and a *Gb*LPS that initiate ginkgolide biosynthesis [89]. More than 50% of known angiosperm *CYPs* involved in specialized diterpene metabolism belong to the *CYP76* and *CYP71* family from the *CYP71* clan.

Benefiting from the rapid development of genome and transcriptome sequencing, many *CYPs* of the *CYP76* family from Lamiaceae have been identified to be involved in the biosynthesis of medicinal LRDs. For example, *SmCYP76AH1*, *SmCYP76AH3* and *SmCYP76AK1* play an essential role in the biosynthesis of tanshinones, the oxygenated diterpenoids from the medicinal plant *Salvia miltiorrhiza* [90]. The genes homologous to *SmCYP76* in other plants are also involved in the biosynthesis of LRDs, such as *CfCYP76AH* members for forskolin synthesis in *C. forskohlii* [91]. Furthermore, it was reported that *Sd*CYP76AH39 (homologous to *Sm*CYP76AH1) in *S. divinorum* catalyzes the biosynthesis of salvinorin A and its analogs, which are the potent selective agonists of κ-opioid receptor [50]. The CYPs from the *CYP71* family in *S. miltiorrhiza*, *SmCYP71D373* and *SmCYP71D375*, catalyzed hydroxylation and cyclization reactions to form the D-ring in the structure of tanshinones [92]. Another study showed that *Pv*CYP71Z25–*Pv*CYP71Z29 from the monocot bioenergy crop switchgrass (*Panicum virgatum*) catalyzed the addition of furan ring to the primary diterpene alcohol intermediates derived from distinct class II diTPS products to form furanoditerpenoids [93]. Interestingly, a special gene *TwCYP728B70* in *T. wilfordii*, which belongs to neither the *CYP71* nor *CYP76* family, is involved in the biosynthesis of labdane-related triptolide, introducing a carboxyl group into the LRD structure, just like *CYP720B* in the DRA synthesis pathway [94], while the special genes in *TwCYP82D* and *TwCYP71BE* subfamilies mediate hydroxylation or ring formation of the LRD structures in the biosynthesis of triptonide [95]. As for the synthesis of some special diterpenoids directly synthesized from GGPP/NNPP by class I diTPSs in dicots, in addition to the *CYP71* and *CYP76* family, other families such as *CYP726* are also involved, and may form gene clusters, for instance the synthesis of casbene-derived diterpenoids in Euphorbiaceae species (*Ricinus communis* and *Euphorbia peplus*) [96, 97] and lycosantalene-related diterpenoids in Solanaceae species (*Solanum lycopersicum*) [98]. In monocotyledons, labdane-related phytoalexin metabolism is catalyzed by diverse diTPSs and CYPs that are organized in distinct gene clusters [4]. It should be noted that in rice, labdane-related diTPSs which code kaurene synthase-like (KSL) enzymes are strictly clustered with the members of the *CYP71* and *CYP76* family on chromosome 2 for phytocassane and oryzalide biosynthesis [99]. In addition to the CYPs, the 2-ODDs have also been identified as the key steps in the diterpenoid biosynthestic pathways, such as the gibberellin pathway [100] and the tashinone pathway. For gibberellin biosynthesis, the final part is catalyzed by multifunctional 2-ODDs, which are encoded by the multigene family. For instance, GA_3-oxidase (GA3ox) catalyzed the last synthetic step of plant hormones GA_1, GA_4 and GA_7 [43], while GA20ox converted the lactone of GA to GA_{24} and GA_9 [44]. It has been reported that 13 *2-ODD*s are involved in tanshinone biosynthesis and the *2-ODD5* plays a crucial role in the downstream biosynthesis of tanshinone by RNAi experiment [101]. Meanwhile, *2-ODD14*, which acts as a dehydrogenase was found to catalyze the aromatization od furan ring in the tanshinone IIA pathway [102].

In summary, the chemical diversity of diterpenoids is achieved first by diTPS-catalyzed formation of a skeleton, followed by the oxidations and rearrangements of the skeleton, which are mostly carried out by oxygenases (CYP and 2-ODD). Although these oxygenases have been characterized in some of the mentioned diterpenoid pathways, there are still a large number of unknown, special and elusive downstream decorative enzymes required to be identified, especially those

in medicinal plants. Unlike those in crops or some monocotyledons, only a few CYPs or 2-ODDs in the active diterpenoid biosynthetic pathways of medicinal plants have been elucidated, such as tanshinone, triptolide, and paclitaxel, etc. (Fig. 2)

Post-modification of diterpene skeletons by transferases

The post-modification of the diterpene skeletons is mainly based on the transfer reactions of the functional group catalyzed by transferases, such as acyl-, methyl- or glycosyltransferases. Glycosyltransferase forms the specific glycosidic bonds between the sugar group and the diterpene skeleton to synthesize diterpene glycoside, which can help to further increase the diversity of diterpenoids, as well as their bioavailability and biological activity when used as drugs. Meanwhile, glycosyltransferases belong to a highly differentiated superfamily, and those involved in the metabolism of diterpenoids are predominantly distributed in several families such as the UGT74 family, *etc*. The *Andrographis paniculata Ap*UGT, a diterpene glycosyltransferase of the UGT74 family, transferred a glucose to the C-19 hydroxyl moiety of andrograpanin to form neoandrographolide, which exhibited anti-herpes simplex virus activities [103]. In *Stevia rebaudiana*, it was reported that a UDP-glycosyltransferase encoded by *SrUGT74G1* catalyzed the conversion of diterpene steviolin to steviol-19-*O*-monoglucoside in leaves. Through the cooperation of four different glycosyltransferases (namely *Sr*UGT74G1, *Sr*UGT76G1, *Sr*UGT85C2 and *Sr*UGT91D2), steviol was further converted into different steviol glycosides, carrying 1-6 glucosyls groups [104].

Various methyltransferases are known to methylate amino group, hydroxyl group, and thiol group, which however are rarely reported in plant diterpene biosynthesis. A classic example is gibberellin deactivation, during which GAMT1 and GAMT2 convert a variety of GAs, including bioactive GAs and their precursors, into the corresponding inactive methyl esters [105]. Acyltransferases catalyze the transfer of acyl groups to form esters and amides, which are usually important rate-limiting steps in biosynthesis of natural products [106, 107]. The donor of acyl groups is mostly acyl-coenzyme A (acyl-CoA). Taxa-4(20),11(12)-dien-5*α*-ol-*O*-acetyl transferase (TAT) is the first reported enzyme catalyzing the acylation step of paclitaxel biosynthesis [108].

In summary, the C20 prenyl substrate geranylgeranyl diphosphate forms more than 18,000 diterpenoids in nature through a complete or partial three-module diterpene pathway [109]. Much of diversity in diterpene natural products arises from complex modification reactions that decorate the diverse backbones of basic diterpene olefin. Although our knowledge of diterpenoids in plants has been substantially expanded in recent years in part based on the rapid development of genome and transcriptome sequencing and annotation, it is still very challenging to identify the CYPs and decoration enzymes with specific functions from the gene superfamily, so as to faciliate the elucidation of the pathways of

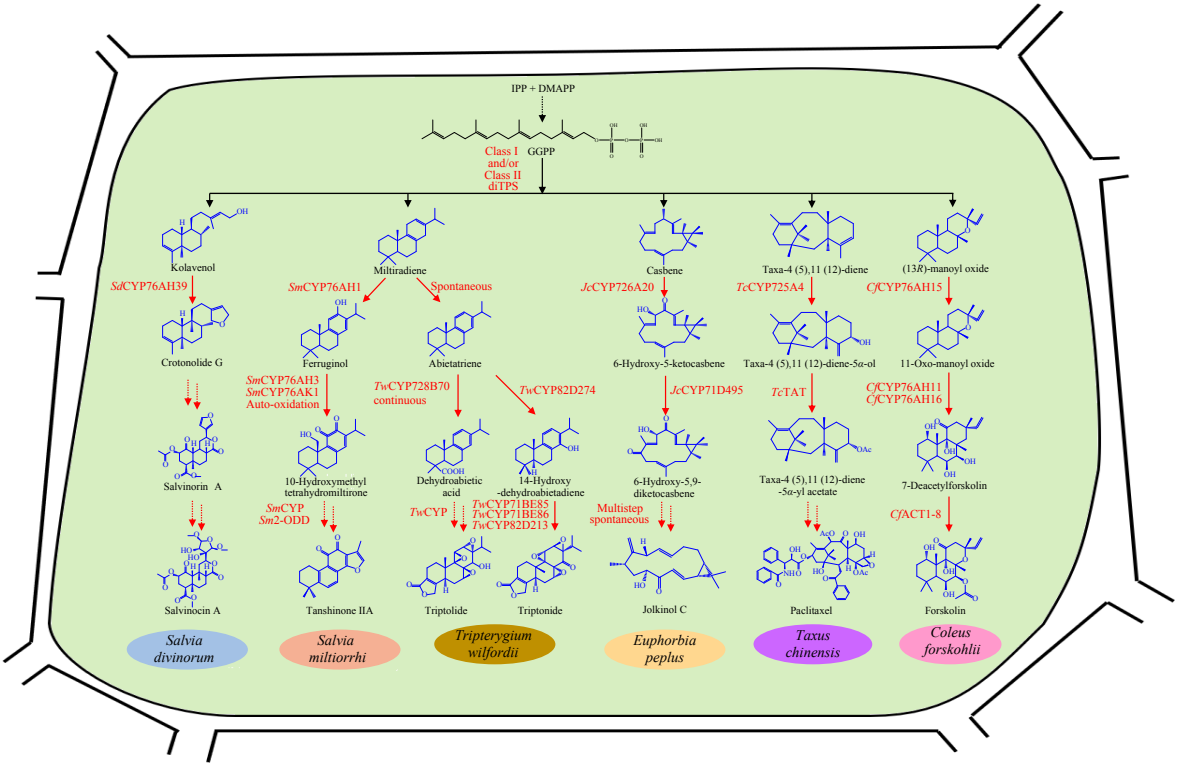

Fig. 2 Oxidation of the diterpene skeletons by cytochrome P450 monooxygenases (CYPs)/ 2-oxoglutarate-dependent dioxygenases (2-ODDs) in medicinal plants. CYPs and 2-ODDs are involved in the oxidation of medicinal diterpenoids in several valuable medicinal plants, such as *Salvia divinorum*, *Salvia miltiorrhi*, *Tripterygium wilfordii*, *Euphorbia peplus*, *Taxus chinensis*, and *Coleus forskohlii*. The solid arrows indicate the catalytic steps that have been identified, while the dotted arrows indicate the catalytic steps that have not been fully clarified

valuable natural products. The complete biosynthetic pathway of most active diterpenoids are still unclear, especially the downstream specific post-modification steps to form specialized metabolites together with CYP related modifications. Therefore, more information is needed to confirm whether these genes are of interest to us.

The Regulation of Diterpene Biosynthesis

The biosynthesis of plant diterpenoids can be affected by many factors, including transcriptional and post-transcriptional regulation, biological and abiotic factors, and spatio-temporal regulation.

Transcriptional and post-transcriptional regulation

The regulation of secondary metabolism, including diterpene biosynthesis, can be achieved at the transcription and post-transcription levels, which are the two most commonly studied aspects. This regulation characterized by changes in compound content is achieved through a complex regulatory network composed of the regulatory factors, such as transcription factors (TFs) and microRNAs. Because of the central role in mediating diterpene biosynthesis, TF seem to be a promising approach to regulate biosynthesis and distribution of plant secondary metabolites [110]. A large number of TFs are involved in the regulation of medicinal diterpenoids in plants, such as the TFs of the MYB, bHLH, AP2/ERF, WRKY, GRAS, bZIP, JAZ and TAR1 families, by binding to different kinds of upstream *cis*-acting elements of target genes. A typical example is *S. miltiorrhiza* TFs from the *bHLH* [111], *MYB* [112] and *AP2/ERF* [113] superfamilies, which can positively or negatively affect tanshinone biosynthesis by regulating the expression of their own target genes. *Sm*bHLH10 up-regulated tanshinone biosynthesis by specifically binding to the G-box in the promotor of several pathway genes [114], while *Sm*ERF6 bound to the GCC-box of *SmKSL1* and *SmCPS1* promotors for up-regulating tanshinone biosynthesis [115]. Three jasmonic acid (JA)-inducible MYC TFs (*Tc*JAMYC1, *Tc*JAMYC2, and *Tc*JAMYC4) of *T. cuspidata* are identified to negatively regulate the biosynthesis of taxol by binding to the E-box (-CANNTG-) in the promoter of pathway genes using a transient expression experiment (Fig. 3) [116]. A transgenic experiment in *T. wilfordii* revealed that RNA interference (RNAi) of *TwMYC2a/b* significantly up-regulated the expression of some genes in the diterpene pathway, including the *TwMS*, *TwCPS*, *TwDXR*, and *TwHMGR1*, and further increased the accumulation of medicinal diterpene triptolide in hairy roots [117]. Besides, the post-transcriptional regulation is gradually being recognized as a key factor regulating diterpene biosynthesis [118]. It is reported that 1-deoxy-D-xylulose-5-phosphate synthase (*DXS*) of the plastidic MEP pathway in *A. thaliana* was post-transcriptionally regulated in the mutants blocked in the MEP pathway [119].

Biological and abiotic regulation of diterpenoids

In general, when plants are threatened by herbivores and insects or infected by pathogens, the biosynthesis of specialized diterpenoids is activated by signal transduction from phytohormones and calcium channels to respond and adapt to these biotic environments. Conifers have evolved complex DRA defenses against herbivores and pathogens [2]. In maize and rice, diterpenoid-based defense systems have been extensively studied [120, 121]. In rice, a set of phytoalexins represented by the momilactone, oryzalexin and phytocassane play an important role in pathogen resistance [122]. Meanwhile, up-regulation of JA biosynthesis and diterpene accumulation upon pathogen infection (e.g. *Pythium arrhenomanes*) revealed the underlying mechanisms in the diterpenoid-related pathogen defense [123]. In maize, kauralexin related diterpenoids confer defense against biotic stresses [124]. The accumulation of these strong diterpene phytoalexins can be induced by biotic stresses and JA. Consistent with the pathogen-inducible accumulation, the transcript abundance of key genes in diterpene pathway increases upon fungal elicitation and JA treatment. In addition to phytoalexins, some volatile diterpenoids may also be released when plants are threatened by herbivores or insects to attract their predators and natural enemies (Fig. 3). Furthermore, the biosynthesis of many medicinal diterpenoids can be induced by different biotic environments and phytohormones, which may be related to plant stress resistance. On the other hand, these responses can also provide important clues for the screening of unknown catalytic enzyme genes. It is reported that the metabolite concentration and gene expression of andrographolide pathway in *A. paniculata* was significantly induced by JA [125]. JA also enhanced the synthesis of the anticancer diterpene triptolide in *T. wilfordii* through a TF *Tw*TGA up-regulating the expression of *TwTPS27a/b* in triptolide pathway [126]. Compared with JA which increases the production of paclitaxel in suspension cells of *Taxus chinensis*, ethylene exhibits an inhibitory effect on paclitaxel synthesis [127]. In summary, plant specialized diterpenoids are usually induced by biotic stresses and phytohormones, where JA in particular has been the most commonly investigated.

Many studies have found that abiotic stresses such as drought, high salt, high humidity and UV exposure can disturb the biosynthesis of diterpenoids in plants. For instance, diterpenoids were accumulatee in response to UV irradiation, drought and oxidative stress in many monocots, such as rice and maize [124, 128]. A recent study has found that the formation of specialized diterpenoids and the expression of corresponding genes are generally induced in response to oxidative stress and UV exposure in switchgrass, suggesting their potential role in conferring abiotic stress resistance [14]. Plants can also produce more diterpenoids in response to the damage caused by high temperature stress [129]. Diterpenoids in medicinal plant *Adhatoda vasica* increase under the responses to UV-B mediated biochemical and metabolic alternation [130]. Moreover, some heavy metal ions also show specific regulatory effects on diterpene biosynthesis and metabolism. For example, the accumulation of tanshinones in *S. miltiorrhiza* and paclitaxel in *T. chinensis*, as well as the expression of corresponding genes, are sensitive to Ag^+ [131].

Spatio-temporal patterns of diterpene biosynthesis

The regulation of diterpene biosynthesis is also influenced by time and space. That is, the synthetic pattern of diterpenoids varies in different tissues and development stages of the plant, as well as in different cellular organ-

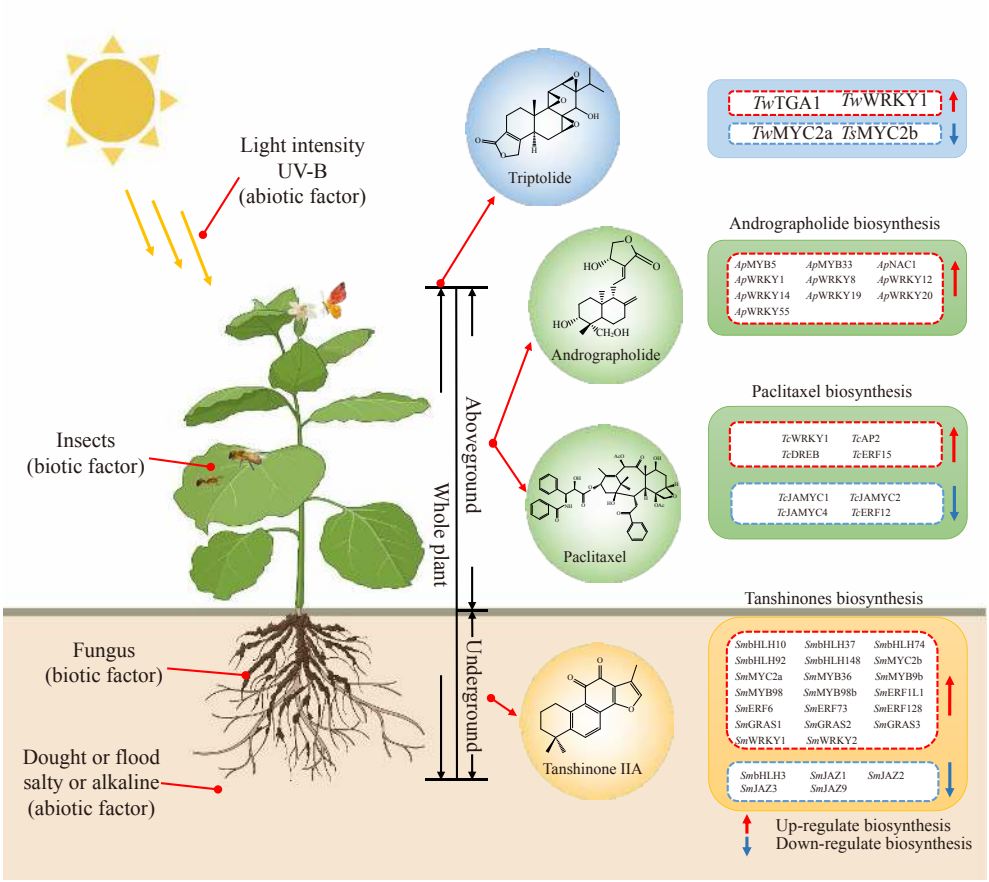

Fig. 3 Regulatory factors are involved in the biosynthesis of valuable diterpenoids in medicinal plants. The biosynthesis of plant diterpenoids can be regulated by biotic and abiotic factors *via* transcription factors. The red and blue arrows represent positively and negatively regulated transcription factors, respectively. Some of the image elements were sourced from https://biorender.com

elles [132]. Thus, the expression of corresponding genes in diterpene pathway performs spatio-temporal specificity. This phenomenon exists in most medicinal plants, so that the specific organs of medicinal plants harvested in the specific season often have the best medicinal value (Fig. 3). Transcriptome analysis showed that the levels of GGPP synthase (GGPPS) and casbene synthase (CS) involved in the biosynthesis of diterpene antibiotics phorbol esters significantly differed at different development stages of *Jatropha curcas* leaves, fruits and seeds [59], indicating its spatio-temporal specificity. In addition, the release of some volatile terpenoids shows circadian rhythm, which is a special temporal expression pattern, and may be related to light intensity [133] and the habits of pollinating and symbiotic insects [134, 135]. It has been reported that only the plastidic MEP pathway plays a role in the formation and release of volatile terpenes, as it operates in a rhythmic manner and is controlled by the circadian clock. In this case, circadian rhythm in volatile diterpenoid biosynthesis based on the MEP pathway is also possible [136].

For medicinal plants with diterpenoids as their active ingredients, the medicinal parts are usually the tissues or organs with high accumulation of diterpene metabolites. For example, tanshinones are highly accumulated in the roots of *S. miltiorrhiza* [36, 137], while ginkgolides are mainly accumulated in the leaves of *Ginkgo biloba*. This tissue-specific distribution is usually consistent with the expression pattern of diterpene pathway genes. According to Garg's study, the concentration of medicinal *ent*-LRDs in *A. paniculata* is found highest in leaves (Fig. 3), compared with germinating seeds, while it is undetected or at very low level in the root. Consistent with the accumulation, and expression of the anticipated *ent*-LRDs pathway, gene *ApCPS2* is significantly different in different organs [138]. Similarly, at the cellular level, plants synthesize various terpenoids and their derivatives in different organelles, due to the different locations of enzymes in the MVA and MEP pathways [139]. Specifically, steroid, sesquiterpene and triterpene skeletons are mainly synthesized in the cytosol, while monoterpene and diterpene skeletons are mainly formed in plastids, and then transferred for further oxidation by CYPs on the endoplasmic reticulum or mitochondria membrane [140], and the post-modification by UGTs in the cytosol [141].

Benefit from the development of sequencing technology and bioinformatics, refined analysis of gene spatio-temporal specific expression is playing an increasingly important role in gene discovery of diterpene pathways. The integrated analysis of *in situ* metabolome and transcriptome is used to construct a complete three-dimensional omics database, so as to quickly narrow the screening range of target genes. Although it has rarely been applied to the identification of diterpene

pathways so far, this strategy has been successfully applied to complete the global omics database of maize endosperm [142] and tomato fruit development [143] for screening genes in interest involved in the development processes. In a word, analysis of spatio-temporal expression patterns can help us formulate corresponding comparative strategies to complete the screening of diterpenoid biosynthetic genes.

Concluding Remarks and Future Perspectives

Diterpenoids in plants play an important role in environmental defenses, and serve as an invaluable source of pharmaceutical use. So far, more than 18,000 diterpenoids have been found from different plant sources. However, due to the complexity and diversity of diterpene biosynthesis, challenges still remain in identifying those synthetic pathways, although rapid development and increasing accessibility of various omics profiling technologies have dramatically accelerated discovery of pathways and related genes. Therefore, it is necessary to clarify diterpene biosynthesis and outline a clearer flow-based strategy to improve research efficiency.

In summary, this article summarizes the general diterpene biosynthetic pathway in modular fashion and the biotic/abiotic factors affecting the synthesis and regulation of diterpenoids. In addition, analysis of the spatio-temporal specific expression pattern can supplement the current data of plant genome and transcriptome to build a more complete three-dimensional omics database, which will provide new research methods and perspectives for plant secondary metabolism research, and may drastically simplify the process of gene screening. With the help of the strategy combining analysis of multiple omics data, phylogenesis, expression pattern and environmental response, gene screening and identification will become more achievable and expand our knowledge of the biosynthesis of valuable diterpenoids.

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