Review

CellPress

Plant diterpene synthases: exploring modularity and metabolic diversity for bioengineering

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Plants produce thousands of diterpenoid natural products; some of which are of significant industrial value as biobased pharmaceuticals (taxol), fragrances (sclareol), food additives (steviosides), and commodity chemicals (diterpene resin acids). In nature, diterpene synthase (diTPS) enzymes are essential for generating diverse diterpene hydrocarbon scaffolds. While some diTPSs also form oxygenated compounds, more commonly, oxygenation is achieved by cytochrome P450-dependent mono-oxygenases. Recent genome-, transcriptome-, and metabolome-guided gene discovery and enzyme characterization identified novel diTPS functions that form the core of complex modular pathway systems. Insights into diterpene metabolism may translate into the development of new bioengineered microbial and plant-based production systems.

Multipurpose metabolites: ecological and economic relevance of plant diterpenoids

Plants produce many different, predominantly polycyclic, 20-carbon (C_{20}) isoprenoids (see Glossary), collectively termed diterpenoids. Some diterpenoids occur broadly across the plant kingdom and play key roles in general (i.e., primary) metabolism, growth, and development of plants, such as gibberellin (GA) phytohormones and the phytol side chain of chlorophyll. However, most diterpenoids are specialized (i.e., secondary) metabolites that are of limited taxonomic distribution and often represent signature molecules of certain plant species or families [1,2]. Biosynthesis or accumulation of specialized diterpenoids can further be limited to specific cell types, tissues, or organs, and may be regulated in response to environmental perturbations. In an ecological context, some specialized diterpenoids have been shown to confer resistance against pests or pathogens: for example, diterpene phytoalexins in rice (*Oryza sativa*) and maize (*Zea mays*) [3], diterpene resin acids (DRAs) as major components of the oleoresin defense system of conifer trees (Pinaceae) [4,5], 17-hydroxygeranyllinalool glycosides mediating anti-herbivore defense of wild tobacco [6,7], and the below-ground defense metabolite rhizathalene in *Arabidopsis thaliana* [8] (Figure 1).

Evidence of human appreciation of plant diterpenoids dates back to the Neolithic age, where amber (i.e., fossilized oleoresin) was used as jewelry. Throughout history, diterpenoid-producing plants and their extracts served as ceremonial incense, traditional medicines, fragrances, flavors and biomaterials, leading up to today's wide range of industrial applications worth several billion dollars annually. Major commercial diterpenoids include the anti-cancer drug taxol (paclitaxel) from pacific yew (*Taxus brevifolia*) [9], sclareol from clary sage (*Salvia sclarea*) used as a precursor for ambroxide fragrance and fixatives in perfume manufacture [10,11], the natural sweetener steviol from *Stevia rebaudiana* [12], and conifer DRAs as large-scale feedstock for industrial coatings and inks [13,14] (Figure 1).

Glossary

CPP: copalyl diphosphate; is the product of class II diTPS via conversion of GGPP and serves as key intermediate in diterpenoid biosynthesis.
diTPS: diterpene synthase; form various diterpene scaffolds as the first committed reaction in diterpenoid biosynthesis.
DMAPP: dimethylallyl diphosphate; together with IPP forms the central intermediate in isoprenoid biosynthesis.
DRA: diterpene resin acid; formed as an oleoresin in conifers that function as defense metabolites against insect pests and pathogens.
DXS: 1-deoxy-D-xylulose 5-phosphate synthase; is a key enzyme of the MEP pathway.
GA: gibberellic acid; serves as phytohormone with essential functions in plant growth and development.
GGPP: geranylgeranyl diphosphate; formed through sequential condensation of IPP and DMAPP molecules and represents the common precursor for diterpenoids.
IPP: isopentenyl diphosphate; together with DMAPP forms the central intermediate in isoprenoid biosynthesis.
Isoprenoid: isoprenoids comprise a group of organic compounds characteristically composed of two or more five-carbon isoprene (C_5H_8) units that are derived from the MVA or MEP pathways.
LPP: labda-13-en-8-ol diphosphate (also termed copal-8-ol diphosphate); is an isomer of CPP that contains a hydroxy group at carbon 8.
NNPP: nerylneryl diphosphate; represents the *cisoid* isomer of GGPP.
P450: cytochrome P450-dependent mono-oxygenase; catalyzes functional modifications of diterpene scaffolds, such as oxygenation and carboxylation reactions.
Plasticity: defined as the capability of an enzyme to undergo functional change with only a few amino acid substitutions.
TXS: taxadiene synthase; is the diterpene synthase forming the central intermediate in the biosynthesis of the anti-cancer agent taxol (paclitaxel).

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Keywords: diterpene synthase; plant chemical diversity; synthetic biology; metabolic engineering; natural product biosynthesis.

0167-7799/
 http://dx.doi.org/10.1016/j.tibtech.2015.04.006

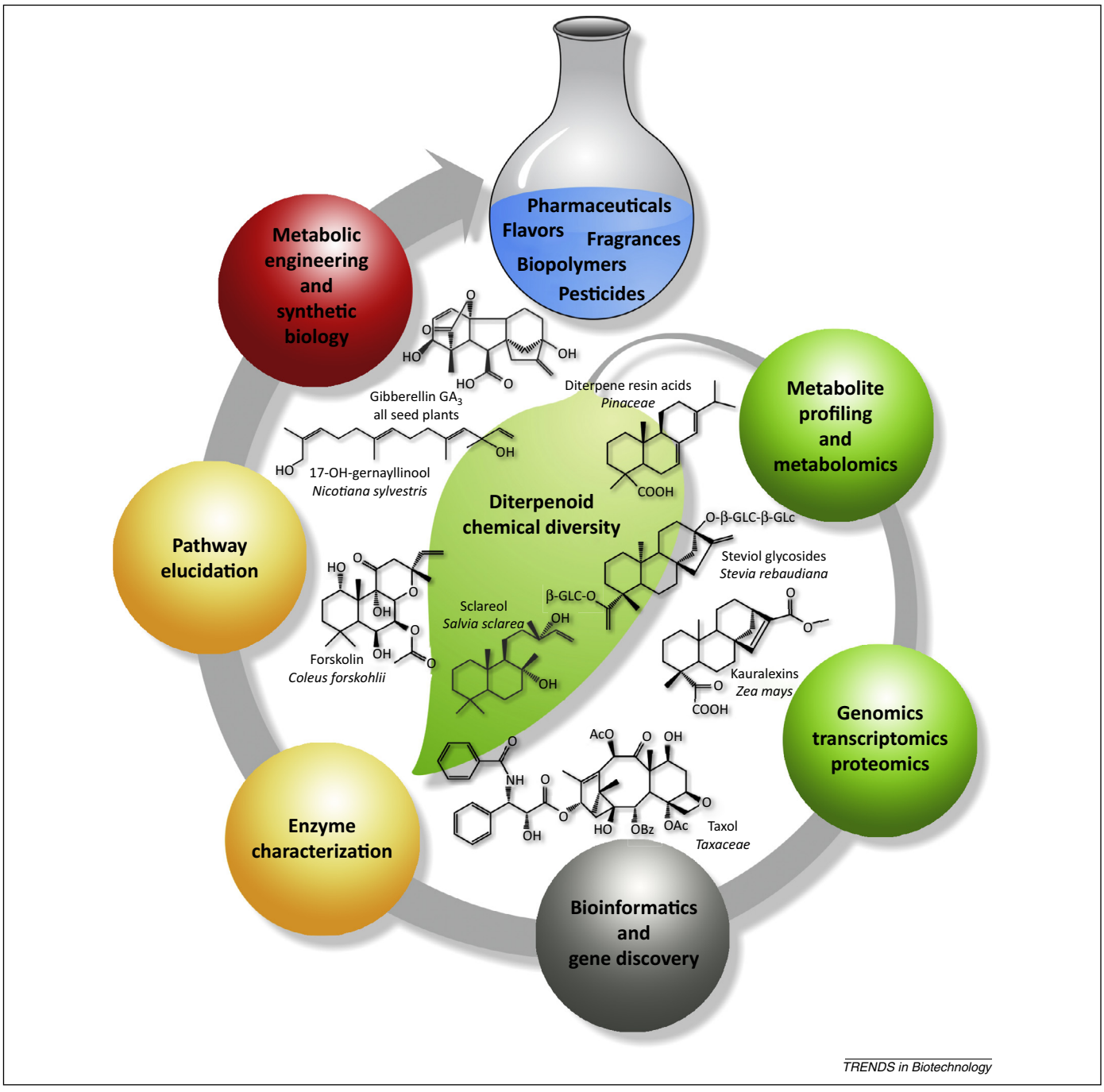

Figure 1. Discovery and application of genes and enzymes of plant diterpenoid metabolism for high-value bioproducts. Plants produce several thousand different diterpenoid metabolites; several of which are used by humans as high-value pharmaceuticals, fragrances, food additives, and precursors for various chemicals and bioproducts. Multidisciplinary systems biology strategies offer new avenues to explore and harness this natural resource. Tissue-specific identification of known and novel compounds through metabolite profiling or metabolomics inform genomics-based discovery and elucidation of diterpenoid-biosynthetic genes, enzymes and pathways. Drawing on the expanding portfolio of modular enzymes and pathways, combinatorial plant and microbial metabolic engineering and synthetic biology approaches can be developed for plant-based bioproducts.

With our increasing need for food, pharmaceuticals, and renewable fuels, plant natural products and biotechnological means for their production, including synthetic biology approaches, have come back to the forefront of a growing attention. A few diterpenoids, such as conifer oleoresin diterpenoids, can be obtained in large quantities directly from plant sources. However, access and availability of some high-value diterpenoids (e.g., taxol) may be limited by low abundance in nature, lack of cultivation or geographic access, and risk of overharvesting of undomesticated plant species. Also, plants typically produce complex diterpenoid mixtures, which can create challenges for isolation of individual target compounds.

Alternative production systems, such as total or semi-synthesis (e.g., (+)-ingenol, the core precursor of the anti-cancer drug ingenol mebutate [15]) and plant cell cultures (e.g., taxol [16]), are being used for commercial diterpenoid production. However, these approaches may not be applicable for many diterpenoid natural products. Beyond plants serving as a rich resource for biologically active

diterpenoid metabolites, research in plant sciences has advanced the discovery of biosynthetic genes, enzymes, and pathways, which can be utilized to advance the biotechnological or semisynthetic production of high-value diterpenoids. Rapid progress in plant systems biology, which combines metabolite profiling, genome or transcriptome sequencing, bioinformatics of gene annotation, and biochemistry have accelerated gene discovery across a diversity of non-model plant species [17–20] (Figure 1). Drawing on the results of such research into diterpenoid biosynthesis, metabolic engineering of plant and microbial systems has become a forward-looking strategy for diterpenoid production [11,21,22].

Building on a number of comprehensive reviews on diterpenoid biochemistry and molecular biology [2,23–27], this article focuses on recent research on diTPSs and their critical role in generating diterpenoid diversity. The evolutionary diversification of the diTPS gene family and the expansive catalytic space of diTPS enzymes are providing a tool kit 'made in nature' for the metabolic bioengineering and synthetic biology of diterpenoid production.

Modularity of diterpenoid biosynthesis fuels chemical diversity

The many thousands of plant diterpenoids derive from only two five-carbon (C_5) isoprenoids, isopentenyl diphosphate (IPP), and dimethylallyl diphosphate (DMAPP), produced via the cytosolic mevalonate (MVA) and the plastidial 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathways [28,29]. Sequential condensation of these units by prenyl transferases yields a handful of central prenyl diphosphate intermediates in terpenoid biosynthesis. Diterpenoids originate predominantly from the MEP pathway. Rooted in geranylgeranyl diphosphate (GGPP) as the general precursor, diterpenoid biosynthesis in plants can be perceived as a 'LEGO*-like' modular system [20,30], where combining a few enzyme modules along a common blueprint, facilitates the formation of thousands of different metabolites (Figure 2). DiTPSs and cytochrome P450-dependent mono-oxygenases (P450s) are the major enzyme modules to generate diterpenoid diversity. DiTPSs facilitate the committed carbocation-driven cyclization and/or rearrangement of GGPP into various diterpene scaffolds [2,20,24], which are then modified through P450-catalyzed oxygenations and further functional decorations [31–37]. Diterpenoid pathways may encompass a single diTPS as in *cis*-abienol biosynthesis in balsam fir (*Abies balsamea* [38]) or combinations of multiple diTPS and P450 modules as for diterpenoid biosynthesis in cereal crops [3,39,40].

In addition to this modular pathway architecture, the diTPS enzymes themselves are also organized in a modular fashion. In general, diTPSs share a conserved modular structure comprised of two or three α-helical domains α, β, and γ (Figure 3) [41–43]. Variations in this modular domain structure, along with variations in the associated active sites and signature motifs, define three major diTPS classes: monofunctional class I and class II diTPSs, and bifunctional class I/II diTPSs [2,24,26] (Figure 3). Class II diTPSs contain an N-terminal active site formed by the γ and β domains with a conserved DxDD motif that is critical for facilitating the protonation-initiated cyclization of GGPP into several bicyclic prenyl diphosphates. The bicyclic prenyl diphosphate products of different class II diTPSs include copalyl diphosphate (CPP) of *ent* (9*R*,10*R*) [20,39,44–46], normal (i.e., (+) or 9*S*,10*S*) [47–50] or *syn* (9*S*,10*R*) [39] stereochemistry, as well as the oxygenated labda-13-en-8-ol diphosphate (LPP) [10,20,48,51,52] and peregrinol diphosphate (PPP) [49] (Figure 2B). Conversely, class I diTPSs possess a functional α domain that harbours two catalytic motifs, DDxxD and NSE/DTE, which coordinate the Mg^{2+}-mediated substrate binding [26]. The majority of class I diTPSs convert class II diTPS products via ionization of the diphosphate group and subsequent cyclization, oxygenation and/or rearrangement of the resulting carbocations into a variety of scaffolds [24] (Figure 2A). Bifunctional class I/II diTPSs combine both reactions in a single protein, where the prenyl diphosphate intermediate may freely diffuse from the class II to the class I active site [53–57]. Some class I diTPSs act directly on GGPP or its stereoisomer nerylneryl diphosphate (NNPP), without requiring prior class II diTPS cyclization, to produce linear [6,20,58] or macrocyclic diterpenes [8,9,36]. Beyond these variations in the modular structure of different diTPS, the functional space of the many diTPS enzymes is further expanded by mechanistic plasticity in the steric and electrostatic control of the intermediate carbocations formed through substrate protonation (class II) or ionization (class I) [26,59,60], allowing for minor changes in the two active sites to dramatically alter enzymatic activity. As little as a single amino acid substitution can lead to redirection of diTPS product specificity, highlighting the functional plasticity of diTPSs and their capacity to evolve new functions [61–68].

Plants employ both diTPS structural modularity and diterpene pathway modularity. This combined modular system maximizes the diversity of possible diterpenoid products. Considering diTPSs as LEGO modules of a diterpene pathway, diTPSs can act either in form of single bifunctional or monofunctional proteins or as combinations of monofunctional proteins with plant lineage- and pathway-specific combinatorial patterns (Figure 2A). For example, biosynthesis of GAs in seed plants (spermatophytes) and specialized labdane-type diterpenoids in flowering plants (angiosperms or magnoliophyta) invariably requires pairs of monofunctional class I and II diTPSs. While mechanistically analogous, formation of specialized labdane-related metabolites in mosses [55], the lycopode *Selaginella moellendorffii* [56,57], and several gymnosperm species [20,38,53,54,69] involves bifunctional class I/II diTPSs capable of catalyzing both reactions. The formation of non-labdane scaffolds is catalyzed through direct conversion of GGPP or NNPP by monofunctional class I diTPS enzymes, with members of this group widely dispersed across the plant kingdom, including the *Taxus*-specific taxadiene synthases [9], casbene-type macrocyclases abundant in Euphorbiaceae [36,70], and geranyllinalool synthases in various plant lineages [6,20,58].

Discovery of diterpene synthase genes and functions

Recent genomics, metabolomics, and biochemical approaches have advanced the discovery of genes and enzymes of plant specialized metabolism far beyond established model

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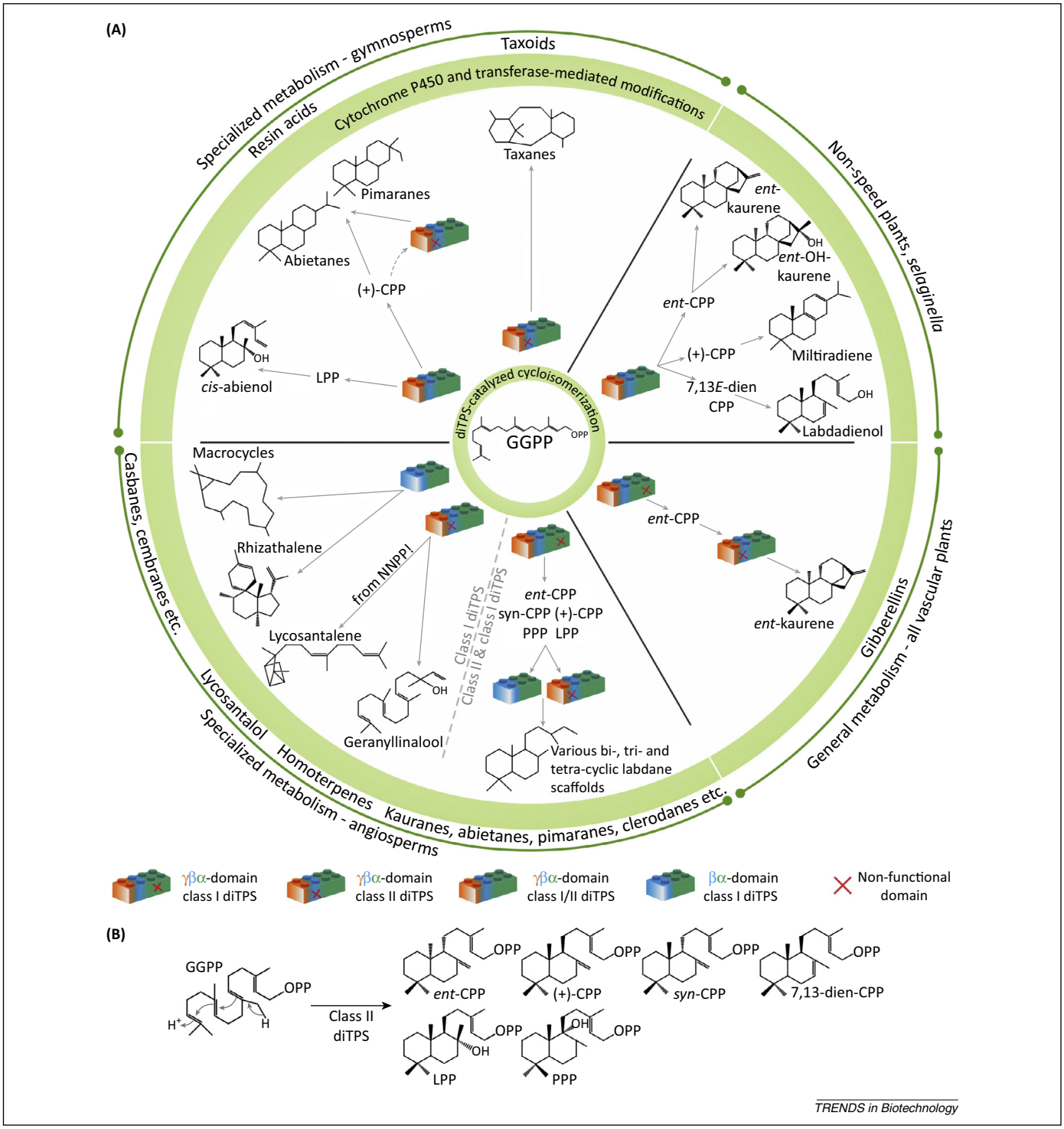

Figure 2. DiTPSs are central to modular diterpenoid pathways. DiTPSs convert GGPP, the general isoprenoid precursor of diterpenoids. **(A)** Illustrated as LEGO blocks (i.e., modules) are different types of monofunctional class II and class I and bifunctional class I/II diTPSs of modular diterpene pathway systems. Following a generic blueprint, these enzyme modules can function in combination or in parallel with each other to generate a large variety of different acyclic, polycyclic or macrocyclic diterpene scaffolds. DiTPSs themselves are modular enzymes that are organized in form of a common three-domain (α, green; β, blue; γ, orange) structure with variations across the diTPS family (see also Figure 3). Downstream of the diTPSs, cytochrome P450 dependent monooxygenases and various transferases are common modules of diterpene pathways that further enhance chemical diversity. **(B)** Schematic overview of class II diTPS conversion of GGPP and known products. Abbreviations: CPP, copalyl diphosphate; DiTPS, diterpene synthase; GGPP, geranylgeranyl diphosphate; LPP, labdadienol diphosphate; NNPP, nerylneryl diphosphate (i.e. *cis*-GGPP); PPP, peregrinol diphosphate. LEGO is trademarked by The Lego Group (Denmark).

species. Specifically for gene discovery of diterpenoid biosynthesis in non-model plants, strategies that efficiently explore plant transcriptome and genome sequence inventories in the context of spatial and temporal patterns of diterpenoid metabolite profiles have proven successful [18,20] (Figure 1).

An important toolset that has advanced the gene and enzyme discovery of diterpenoid biosynthesis are the

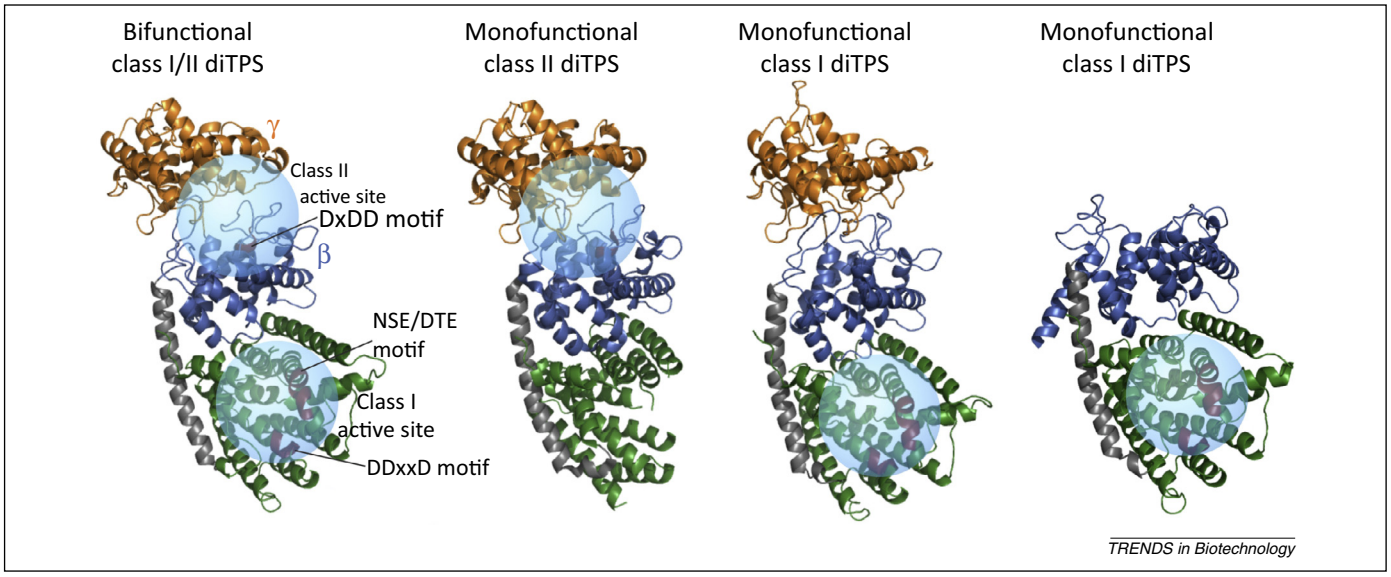

Figure 3. Modular domain architecture of DiTPSs. All DiTPSs share a conserved structure of three α-helical domains γ, β, and α. Variations in the γβα domain architecture and composition of the class II or class I active sites largely contribute to the enzymes substrate and product specificities. Representative diTPS structures from left to right: *Abies grandis* abietadiene synthase (PDB code 3S9V [41]), *Arabidopsis ent*-CPP synthase (PDB code 4LIX [43]), *T. brevifolia* taxadiene synthase (PDB code 3P5R [42]), homology model of *Marrubium vulgare* 9,13-epoxy-labd-14-ene synthase [49]. DiTPS, diterpene synthase.

databases and phylogenies of diTPSs and P450s, which were used to interrogate large transcriptome or genome sequence resources [20,36,40]. Although many diterpenoid pathways remain to be explored, these approaches identified novel diTPS functions and almost doubled the number of functionally characterized diTPSs over the past 5 years (Figures 2 and 4).

Newly discovered diTPSs that form novel diterpene scaffolds include the tomato (*Solanum lycopersicum*) TPS21 as the first diTPS to convert the *cisoid* isomer of GGPP (NNPP) into lycosantalene [71,72] and the class I rhizathalene synthase in *Arabidopsis* [8]. The discovery of several diTPSs that produce oxygenated scaffolds was another novelty [10,38,48,49,51,57,73]. Following on the discovery of *Physcomitrella patens ent*-kaurene/*ent*-hydroxy-kaurene synthase [55], Mafu *et al*. described a bifunctional diTPS from *S. moellendorffii* that forms labdadien-15-ol by replacing the diphosphate ester with a hydroxy group [57]. Keeling *et al*. demonstrated that the Norway spruce (*Picea abies*) levopimaradiene-abietadiene synthase (LAS) forms epimers of the diterpenol 13-hydroxy-8(14)-abietene as initial product which dehydrates to a set of olefin products [73]; a function later confirmed for other conifer LASs [20,38,69]. In angiosperms, class II diTPSs that enable water quenching of the labda-13-en-8-yl carbocation were found in six different plant families [10,20,48,49,51,52]. These class II diTPSs can pair with class I enzymes, converting oxygenated prenyl diphosphates through ionization with or without secondary cyclization or additional hydroxylation to afford diterpene alcohols, including manoyl oxide, sclareol and *cis*-abienol, that are of interest in the fragrance industry [10,48,49,51]. These recent discoveries illustrate that the diTPS functional space is not limited to forming hydrocarbon scaffolds, but extends to regiospecific oxygenation reactions both in the class II and class I reactions. Thus, initial oxygenation of the diterpene scaffold is not solely a function of P450 activity. Rapid development of genome and transcriptome resources for a large range of plant species is certain to reveal more diTPS functions, providing a deeper knowledge of the diTPS catalytic space and new targets for diterpenoid engineering. In addition, on the genome level a significant number of diterpenoid biosynthetic gene clusters have been discovered in several plant species, suggesting a role of genomic structural organization in the evolution of diterpenoid pathways with important implications for the discovery of diterpene metabolic pathways [3,36,71,74]. Biochemical characterization of new diTPS candidates remains important for functional identification and accurate annotation.

Diterpene synthases function as modules in combinatorial metabolic systems

As more diTPSs are characterized, a growing number of studies have illustrated that depiction of diterpenoid biosynthesis as one-dimensional (i.e., linear) pathways appears to oversimplify the multibranched diterpenoid pathway systems or metabolic grids as they exist in plants. In a given plant species, individual class I and class II members of multigene diTPS families can interact in different combinations to increase metabolic diversity (Figure 2). Indeed, while class II catalysis appears to be regio- and stereoselective, comprehensive analysis of the large class I diTPS families in rice and wheat (*Triticum aestivum*) revealed substrate promiscuity for several members that can utilize *ent*-CPP, *syn*-CPP and (+)-CPP produced by the respective class II diTPSs [40,75]. Similarly, multifunctional class I diTPSs have been described in several Lamiaceae species that act in combination with different class II enzymes to form manoyl oxide, miltiradiene, and other diterpene scaffolds [48–50]. Variable combinations of class II and class I diTPS modules are not only found in angiosperms, where

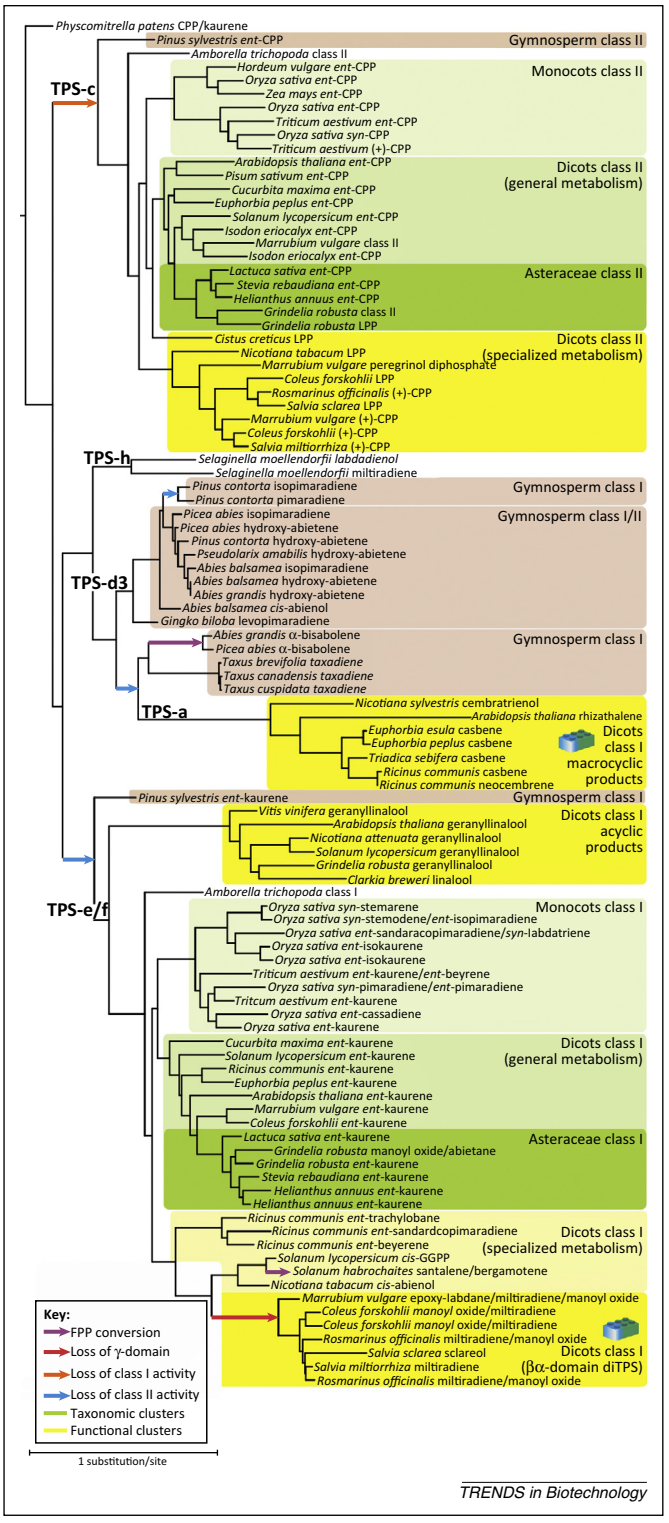

Figure 4. Divergent evolution of the plant diterpene synthase family produced the many different enzyme functions for forming diterpenoid metabolic diversity. Illustrated is the phylogeny of functionally characterized plant diTPSs, highlighting the evolutionary diversification of the enzyme family through repeated events of gene duplications, domain fusion and/or loss, as well as sub- and neofunctionalization. Abbreviations: DiTPS, diterpene synthase; CPP, copalyl diphosphate; GGPP, geranylgeranyl diphosphate; LPP, labdadienol diphosphate.

sequentially acting monofunctional diTPSs are prevalent. In species of pine, several monofunctional diTPSs were recently identified that lack class II activity and convert (+)-CPP but not GGPP into abietane and pimarane diterpenes [69]. While no monofunctional (+)-CPP synthase has yet been reported in any gymnosperm, these diTPSs may bridge pathways by utilizing (+)-CPP produced as a freely diffusing intermediate by bifunctional class I/II LAS and isopimaradiene synthases [54].

DiTPS phylogenies and annotation of diTPS gene functions

Computational prediction of specific diTPS functions appears to be nearly impossible. This is due to the following facts: (i) within a given plant species or taxonomic group of closely related species diTPSs of high sequence identity often have different functions; (ii) diTPSs of more distantly related species may have independently evolved similar or identical functions, but typically lack high sequence identity [2,24]. The events of evolutionary diversification of plant diTPSs involved gene duplications, domain losses, and sub- or neofunctionalizations. These events resulted in phylogenetic topographies of the TPS gene family with clusters dominated by taxonomic rather than functional relatedness. Examples of such taxonomic diTPS clusters are the gymnosperm-specific TPS-d3 clade, and diTPSs groups of the Poaceae (monocot) and Asteraceae families in the TPS-c (class II diTPSs) and TPS-e/f (class I diTPSs) clades (Figure 4). A few emerging patterns of diTPS branches related by function, such as LPP synthase-type diTPSs and specialized class I diTPSs with an unusual βα-bi-domain architecture (Figure 4), may improve phylogeny-guided gene annotation. However, accurate annotation of new diTPS candidate genes continues to require functional characterization using biochemical approaches, including expression of genes in heterologous hosts [2,20,76]. Genetic tools, specifically mutant lines, to assess diTPS functions in plants are largely limited to a few established reference systems. Such tools are not currently available for most non-model species, which produce medicinally active or otherwise biotechnologically interesting diterpenoids. Following the advances in genome and transcriptome sequencing of non-model plant species to discover genes of natural product biosynthesis, genome editing, virus-induced gene silencing and other forward or reverse genetic tools should be further developed to support the annotation of diTPS gene functions.

Metabolic engineering of diterpenoid pathways

Metabolic engineering of diterpenoid production may serve as an alternative or complementary approach to chemical synthesis or plant biomass extraction. This includes the traditional concept of reconstruction of naturally occurring plant diterpenoid pathways in heterologous host systems, as well as new combinatorial approaches using 'new-to-nature' combinations of diTPSs from different plant species. While combinations of monofunctional diTPSs of different species have already been successfully used to identify new diTPS gene functions [20,40,49,50], the same approach can be extended to test the larger catalytic space of diTPS combinations for producing new nature-like diterpenoids. In this context, the increasing knowledge and number of characterized diTPSs and their diverse functions in modular biosynthetic pathways provide as of yet underexplored opportunities for metabolic bioengineering of diterpenoids (Figure 2).

Building, in part, on experience with biotechnological production systems for sesquiterpenoids [77–79], a number of diterpenoid engineering platforms have been described that use plant diTPSs (and in some cases P450s) to produce, for example, the fragrance precursors sclareol and *cis*-abienol [11,38,80], DRAs [32,81], and precursors of pharmaceuticals such as taxadiene [9,21], miltiradiene [22,82], 13*R*-manoyl oxide [83], levopimaradiene [64] and casbene [70]. *Escherichia coli* and yeast (*Saccharomyces cerevisiae*) are currently the preferred microbial hosts for terpenoid production, due to their robustness, scalability and the broad repertoire of tools for DNA assembly and host optimization (reviewed in [84,85]). However, most diterpenoid-producing platforms are less advanced than established sesquiterpenoid production systems. Insufficient precursor supply, flux of precursors into competing pathways, low enzyme expression and activity, and toxic or inhibitory effects of intermediates are some of the barriers limiting diterpenoid pathway productivity. Overexpression of rate-limiting MVA or MEP pathway genes [21,77,78,82,86,87] and, in the case of yeast, downregulation of the endogenous squalene synthase *ERG9* [88] have been successfully used to increase formation of isoprenyl diphosphate intermediates in terpenoid production. However, enhancing GGPP formation remains challenging. Ignea *et al*. recently improved GGPP supply in yeast by redirecting the product specificity of farnesyl diphosphate synthase towards formation of GGPP [80]. Balanced expression of enzymes along the pathway of interest, for example, by control of gene copy number and promoter strength, has proven critical to enhance carbon flux by minimizing toxicity and enzyme inhibition caused by accumulating intermediates [21,22,86]. Additionally, fusion proteins of consecutively acting enzymes can be employed to optimize metabolite channeling [11,21,22,80]. Future bioengineering efforts can be expected to combine these tools with systems biology and computational modeling approaches to predictively optimize pathway regulation and performance as demonstrated for production of the sesquiterpenoid artemisinin precursor amorphadiene [89]. Beyond pathway engineering, fermentation optimization is critical for improving toxicity tolerance and host performance [11,21,82]. For example, Schalk *et al*. used fed-batch fermentation along with an organic solvent overlay for *in situ* product removal to develop an industrial-scale platform for sclareol production [11]. Furthermore, structure-guided protein engineering has proven feasible to alter diTPS product specificity and improve the commonly low catalytic rates of diTPSs [61,64,65]. In this vein, structural and mechanistic insights into catalysis of functionally distinct diTPSs may be explored to design and synthesize diTPS enzymes with improved or novel activities. To better utilize the potential of protein engineering in support of biotechnological diterpenoid production, it is important to develop robust high-throughput diTPS assay systems to facilitate screening of large protein variant collections.

Plants continue to be important biological systems for diterpenoid engineering [90,91]. One key advantage of plants is that they naturally produce diterpenoids, and that they accomplish diterpenoid biosynthesis with precursorformation powered by solar energy through photosynthesis, eliminating the need for supplying carbon precursors. *Nicotiana benthamiana* is already widely used for *in vivo* functional characterization and combinatorial expression of diTPSs [20,48,49,92]. Endogenous biosynthesis of GGPP, ease of combinatorial gene expression, and availability of cell-type-specific promoters are some of the advantages of using cultivated tobacco species as a production system. Possible obstacles, including interference with endogenous diterpenoid pathways, and potentially undesired product glycosylation, should be possible to be overcome. For example, Brückner *et al*. improved diterpenoid engineering in *N. benthamiana* through coexpression with a tomato 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and a tobacco GGPP synthase [93]. The moss *P. patens* has also attracted some recent attention as a possible diterpenoid production system, as it can be grown in tissue culture and contains only a single endogenous and non-essential diTPS gene that can be silenced and replaced with heterologous diTPSs [94,95]. Successful metabolic engineering of essential oil compositions in peppermint (*Mentha x piperita*) [96] and redirection of carotenoid metabolism towards taxadiene production in tomato fruits [97] are encouraging examples for plant terpenoid metabolic engineering. Future research may also focus on as-of-yet underexplored species, such as conifers, that produce large quantities of diterpenoids without intensive cultivation. For example, conifers already provide a commercial source for high-volume terpenoid supply as byproducts in the forest industry. The metabolic capacity of conifer trees for producing DRAs may be redirected through metabolic engineering towards other valuable diterpenoids. Metabolic engineering of diterpenoids in intact plants will benefit from a comprehensive understanding of the complex terpenoid metabolism such as to circumvent pathway diversion into undesired byproducts as was observed with the overexpression of a prenyl diphosphate synthase in spruce [98]. Specialized plant cell and tissue types, such as trichomes of many angiosperm species or as resin duct epithelia of conifers, have been optimized through millions of years of natural evolution to produce and accumulate various terpenoids. Cell- or tissue-specific gene expression in metabolically engineered plants may allow for biosynthesis of diterpenoids in these specialized cells and tissues [91]. In select cases, cell cultures have proven successful for producing tanshinones, rosmarinic acid and taxol, the latter representing the world's largest commercial cell culture application [16,99]. Metabolic engineering of other photoautotrophic organisms, such as algae and cyanobacteria, may offer alternative systems for high-volume terpenoid synthesis [100].

Ultimately, for both microbial or plant-based systems, scalability, ease of multigene combinatorial gene expression (including the more difficult expression of P450s), and cost-effective extraction procedures will be key factors for the commercially viable biotechnological production of diterpenoid natural products.

Concluding remarks

Integrating metabolome, genome, and transcriptome analyses and biochemical enzyme characterization has delivered many new genes and enzymes of modular pathways of

diterpenoid metabolism in model and non-model plant species. To take full advantage of these genes and enzymes for synthetic biology and metabolic bioengineering, it will now be critical to better understand the regulation of complex diterpene biosynthetic systems beyond the characterization of individual enzymes.

Acknowledgments

The authors' work has been supported with funds from the University of British Columbia (JB), the Natural Sciences and Engineering Research Council of Canada (JB), Genome Canada (JB), Genome British Columbia (JB) and the University of California at Davis (PZ). Some of the work discussed in this paper resulted from the Tria Project (www.thetriaproject.ca), the Treenomix Project (www.treenomix.ca), the SMarTForests Project (www.smartforests.ca) and the PhytoMetaSyn Project (www.phytometasyn.ca). JB is a UBC Distinguished University Scholar.

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