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Enzymes for Synthetic Biology of Ambroxide-Related Diterpenoid Fragrance Compounds

Philipp Zerbe and Jörg Bohlmann

Abstract Ambrox and related ambroxides are highly priced in the fragrance industry, and valued for their delicate odor and fixative properties. Historically, ambrox was obtained from ambergris, a waxy excretion produced by sperm whales, now an endangered species. Synthetic ambroxides have replaced ambergris in perfume manufacture. Plant labdane diterpenoids can serve as starting material for ambroxide synthesis. Among these, the diterpene alcohol sclareol is the major industrial precursor obtained from cultivated clary sage (*Salvia sclarea*). In plants, a large family of diterpene synthase (diTPS) enzymes controls key reactions in diterpenoid biosynthesis. Advanced metabolite profiling and high-throughput sequencing of fragrant and medicinal plants have accelerated discovery of novel diTPS functions, providing a resource for combinatorial synthetic biology and metabolic engineering approaches. This chapter highlights recent progress on the discovery, characterization, and engineering of plant diTPSs with potential uses in ambroxide production. It features biosynthesis of sclareol, *cis*-abienol, and diterpene resin acids, as sources of genes and enzymes for diterpenoid bioproducts.

Keywords Ambrox · Cytochrome P450 · Diterpenoid · Fragrance · Metabolic engineering · Terpene synthase

P. Zerbe (✉) · J. Bohlmann
Michael Smith Laboratories, University of British Columbia, 301-2185 East Mall, Vancouver, BC V6T 1Z4, Canada
e-mail: pzerbe@ucdavis.edu

J. Bohlmann
e-mail: bohlmann@msl.ubc.ca

P. Zerbe
Department of Plant Biology, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

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1 General Introduction

Terpenoids are one of the largest and most diverse classes of natural products, comprising tens of thousands of different metabolites that share a common origin from five-carbon (C_5) building blocks [1, 2]. In plants, the chemical diversity of terpenoids is a reflection of their manifold biological functions [3–5]. Although some terpenoids, such as gibberellins or carotenoids, play important roles in general (primary) plant metabolism, the majority of compounds are specialized (secondary) metabolites mediating ecological interactions of plants with the environment. These specialized terpenoids are often confined to individual plant species, genera, or families, and may be formed or accumulated in specialized anatomical structures. For example, trees of the pine family (Pinaceae) produce large quantities of monoterpenoids and diterpene resin acids (DRAs) as a viscous oleoresin, which accumulates in resin blisters and ducts, and forms part of a chemical defense system against herbivores and pathogens [4, 6]. Similarly, many angiosperms (flowering plants) accumulate specialized terpenoids in glandular trichomes, epidermal protuberances on the surface of leaves and other organs [7]. In addition to their tissue-specific occurrence, these biologically active terpenoids may only be formed upon induction by biotic or abiotic stressors [2, 4, 5].

Owing to their different chemophysical properties that range from highly volatile to semi- and nonvolatile compounds, terpenoids and terpenoid-producing plants have a long history of practical uses as flavors (e.g., menthol), fragrances (e.g., linalool), pharmaceuticals (e.g., Taxol, artemisinin), industrial resins and coatings (e.g., DRAs), and more recently as biofuels (e.g., farnesene, bisabolene) [8–12]. The ancient and modern use of volatile terpenoids as fragrances draws on their wide range of scent qualities. Volatile monoterpenoids with applications in the fragrance industry include pinene, myrcene, and limonene, which are significant by-products of the forest and fruit industries and used as large-volume feedstocks for the production of numerous household products, such as soaps, air fresheners, cosmetics, and perfumes [8, 10]. Semi- or nonvolatile diterpenoids, such as the nor-labdane diterpenoid (-)-ambrox (trademarked by Firmenich) and related ambroxides (Fig. 1)

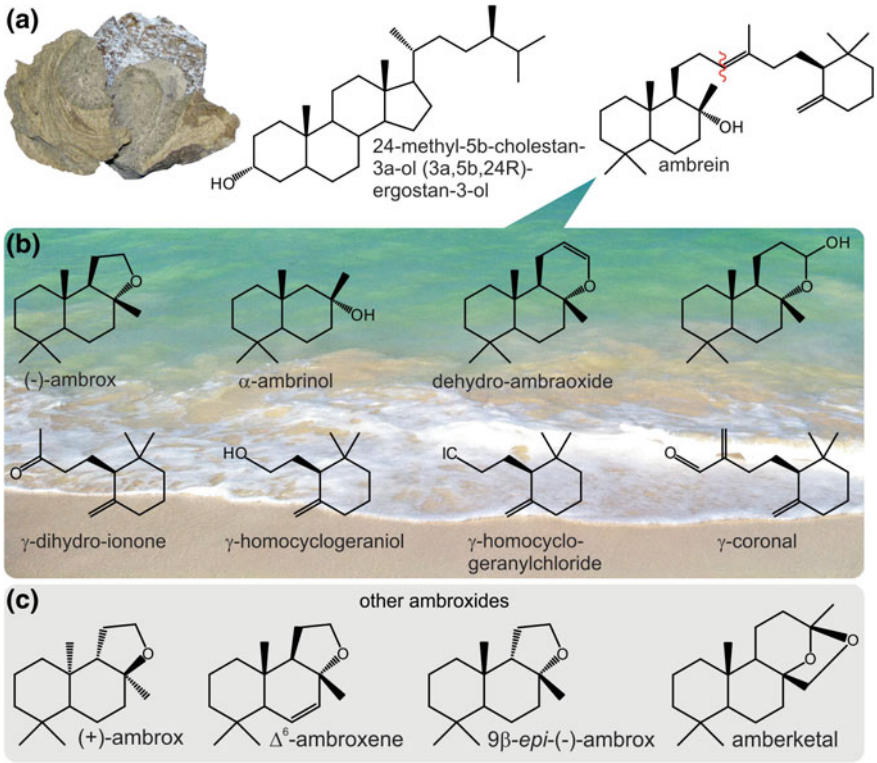

Fig. 1 Ambergris and ambroxide fragrances. Ambergris is an intestinal excretion produced by sperm whales, primarily comprised of cholestanol-type sterols and ambrein (**a**). After regurgitation by the animal, exposure to water and sunlight leads to the oxidation and degradation of ambrein to form (-)-ambrox and related compounds that define the odor characteristics of ambergris (**b**). Today, synthetic ambrox-related compounds produced primarily from plant diterpenoids are used in perfume fragrance production (**c**)

are valued for fragrance manufacture, due to their delicate amber, sweet, and woody odor characteristics and excellent fixative properties to preserve the evanescent top and heart notes of a perfume [13].

Similar to other perfume fixatives, such as musk and civet, (-)-ambrox was historically obtained from an animal product, ambergris or grey amber (Fig. 1). Ambergris is a waxy material formed as a biliary secretion of sperm whales (*Physeter macrocephalus*). Its major constituents are cholestanol-type sterols and the odorless triterpene ambrein. After the material has been regurgitated by the animals, ambrein undergoes oxidative degradation to form (-)-ambrox and numerous other compounds that contribute to the typical ambergris odor [14]. Knowledge of ambergris dates back to its use as ceremonial incense by the ancient Egyptians. Since the tenth century AD, ambergris was harvested on shores or from whale carcasses and traded across the Mediterranean and Europe for its use in

perfumery. (-)-Ambrox remains a rare natural product, found only as a component of ambergris, in the essential oil of gum rockrose (*Cistus ladanifer*, Cistaceae), cypress (*Cupressus sempervirens*, Cupressaceae), and clary sage (*S. sclarea*, Lamiaceae), and the absolute of tobacco (*Nicotiana tabacum*, Solanaceae). It was not before the early 1950s that semi-synthetic ambroxide compounds replaced the scarce natural (-)-ambrox [15]. Today, synthetic (-)-ambrox is available under several trade names, including Ambrox® (Firmenich), Cetalox® (racemic mixture, Firmenich), Amberlyn® (Quest), Ambroxan® (Henkel), and Ambrofix® (Givaudan Roure). It is used as the base note in many popular perfumes, such as *Davidoff Cool Water*®, *Chanel No. 5*®, or *Georgi Armani Si*®. The demand for a reliable and economically viable supply of synthetic ambroxides has motivated the development of numerous total and partial synthetic approaches [16]. In most cases, naturally occurring plant diterpenoids serve as starting material (Fig. 2).

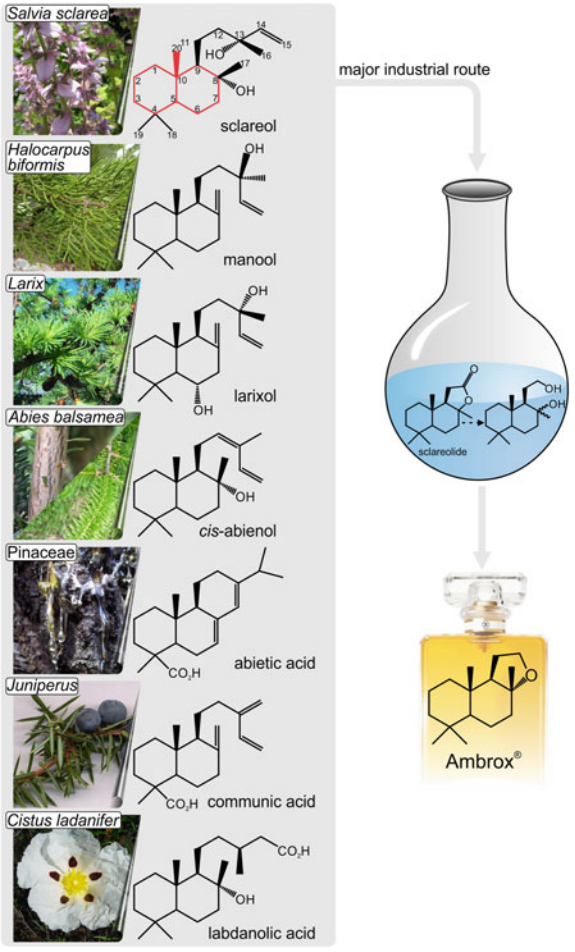

Fig. 2 Ambroxide synthesis from plant diterpenoids. Ambrox® and related ambroxide fragrances can be obtained through synthesis from plant-derived labdane diterpenoids. Sclareol isolated from flowers of clary sage (*S. sclarea*) serves as the major source for (-)-ambrox production in the perfume industry. In addition, a variety of alternative semi-synthetic routes from other labdane diterpene alcohols and acids have been established. For example, *cis*-abienol, labdanolic acid, and communic acid are starting materials for producing (-)-ambrox. Larixol can be converted to Δ^6-ambroxene, and manool is a precursor for the synthesis of amberketal. The *trans*-decalin backbone of labdane diterpenes is highlighted in *red*

Some of these diterpenoids such as DRAs are abundant in nature, however, others can only be obtained in low amounts from wild plant species. Lack of cultivation of many diterpenoid-producing plants and the requirement for protection of some wild species may constrain their use in the fragrance and other industries. Recent advances in transcriptome sequencing and functional genomics, metabolomics, and biochemistry of nonmodel plants have accelerated the discovery of natural product biosynthetic pathways in many previously unexplored species [17–19]. The portfolio of diverse biosynthetic genes and enzymes made accessible through these approaches has, in turn, inspired metabolic engineering and synthetic biology approaches for producing natural plant products, such as terpenoid fragrances, flavors, and pharmaceuticals [11, 17, 18, 20–26]. In this chapter, we highlight recent advances in the discovery, characterization, and metabolic engineering of diterpene biosynthetic pathway genes and enzymes with applications in ambroxide production. On the basis of three examples, sclareol, *cis*-abienol, and DRAs, we discuss new opportunities for combining chemical synthesis with metabolic engineering as alternatives to conventional methods for producing ambroxide fragrances.

2 Chemical Syntheses of Ambroxides

Since the first semi-synthesis of (-)-ambrox from the diterpenoid sclareol in 1950 by Stoll and Hinder [15], several total and partial syntheses toward ambroxide odorants have been established [13, 16]. Kawanobe and coworkers described the total synthesis of (-)-ambrox from β-ionone [27], and several methods for (-)-ambrox production via biomimetic polyene cyclization, for example, from homofarnesol, were described [28]. However, most synthetic routes represent partial syntheses using plant-derived labdane diterpenoids as starting material, because these natural metabolites readily provide the *trans*-decalin scaffold also found in (-)-ambrox [29–34] (Fig. 2). Labdane diterpenoids are widely distributed across the plant kingdom, comprising a large group of several thousand distinct bi-, tri-, or tetracyclic structures, most of which carry extensive functional modifications, including oxygenation, carboxylation, acetylation, methylation, and glycosylation [1, 2, 35].

As the many different synthetic approaches for converting diterpenoids into (-)-ambrox would exceed the scope of this review, only select plant labdane-related diterpenoids with use in ambroxide synthesis are highlighted here. For a detailed review on the underlying chemical procedures, we refer the reader to comprehensive reviews by Frater et al. [13] and Frija et al. [16]. The diterpene-diol sclareol, the primary constituent of clary sage, is currently the major industrial resource for (-)-ambrox production [23] (Fig. 2). Sclareol is isolated from inflorescences either by steam distillation or more efficiently by organic solvent extraction of dried plant material [21, 36]. Several approaches for the conversion of sclareol into (-)-ambrox

have been demonstrated [15, 29, 37]. A critical step is the initial oxidative degradation of the carbon-9 side chain of sclareol to form a mixture of sclareolide diterpene lactones (Fig. 2). Further reduction to the respective diols followed by acid-driven cyclization affords (-)-ambrox. Although so far not industrially competitive, a range of other oxygenated labdane diterpenoids have been employed for the synthesis of (-)-ambrox and its equivalents (Fig. 2). Although the individual synthetic approaches differ, most proceed via reductive degradation of the C-9 side chain and subsequent cyclization as key steps. In particular, conifer trees including species of pine (*Pinus*), spruce (*Picea*), and fir (*Abies*) are a rich source of labdane-related diterpene alcohols and acids, which accumulate in copious amounts in the form of oleoresin [4]. These diterpenoids are obtained as large feedstocks by tapping resin from tree stems or as a by-product of the timber and pulp industry [8, 10]. For example, the tertiary diterpene alcohol, *cis*-abienol, is a feasible starting material obtained from the oleoresin of balsam fir (*Abies balsamea*) and a few other coniferous trees [38]. Barrero et al. [29] demonstrated a three-step conversion of *cis*- abienol into (-)-ambrox in high yield. In addition, synthesis of (-)-ambrox and amberketal (Fig. 1), a structural analogue with similar odor characteristics, from manool has been established [13]. Manool occurs naturally in several plant species and is commercially harvested from yellow pine (*Halocarpus biformis*, Podocarpaceae), a conifer tree endemic to New Zealand. Another diterpene alcohol, larixol, abundant in the hardwood of Dahurian larch (*Larix gmelini*) and other *Larix* species, has also been employed for producing ambroxides. Bolster et al. [32] reported the conversion of larixol into Δ^6-ambroxene, an unsaturated (-)-ambrox analogue, called superambrox for its superb odor qualities. Other diterpene acids with successful use in the synthesis of (-)-ambrox include abietic acid and levopimaric acid as major components of many conifer oleoresins, labdanolic acid from leaf exudates of *C. ladanifer*, and communic acid abundant in the wood and berries of *Juniperus sabina* (Cupressaceae) [30, 31, 33, 34].

In addition to chemical synthesis, bioconversion of natural products using bacteria, fungi, or plant cell cultures have been explored for the manufacture of natural or nature-like diterpenoids [16, 39]. Their advantage lies in the possibility to catalyze regio- and stereospecific reactions under milder conditions, and the modification of functional groups that are difficult to access with conventional chemical methods. For example, fungal cell cultures of *Hyphozyma roseoniger* (Ascomycota) and *Cryptococcus albidus* (Basidiomycota) have been used to convert sclareol into sclareolide and the respective diols as an alternative to the chemical degradation of the side chain of sclareol [40]. Moreover, fungal cultures of *Macrophomina phaseolina* (Ascomycota), as well as plant cell cultures of kiwi fruit (*Actinidia deliciosa*, Actinidiaceae) have been applied for the biotransformation of (-)-ambrox into a range of structural analogues with potentially novel or improved odor qualities [41, 42].

3 Diterpene Biosynthesis and Bioengineering of Ambroxide Precursors

3.1 Introduction

Despite the common utility of plant-derived labdane diterpenoids as starting materials for the semi-synthesis of (-)-ambrox, several factors limit their industrial use. Many natural diterpenoids occur in only minor quantities and often as part of complex mixtures *in planta*, requiring laborious and cost-intensive purification procedures for their application in chemical synthesis. Furthermore, access to wild diterpenoid-producing plant species can be limited, and plant cultivation can be affected by unfavorable environmental conditions. Discovery and engineering of diterpenoid-producing genes and enzymes offers an opportunity to improve and expand the availability of starting materials for ambroxide manufacture. Advanced high-throughput metabolite analysis and transcriptome sequencing are enabling the rapid and cost-efficient investigation of many diterpenoid-forming plant species not previously accessible to comprehensive gene discovery [17, 19]. Recent years have witnessed the identification and definite functional annotation of many diterpene synthases (diTPSs) and cytochrome P450 monooxygenases (P450s), two key enzyme classes in diterpenoid biosynthesis [17, 20, 21, 25, 43–49].

Generally, terpenoids share a common biosynthetic origin from two C_5 intermediates, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Condensation of these building blocks through the activity of prenyl transferases affords a few prenyl diphosphate intermediates of distinct chain length that serve as central precursors for all terpenoids [50]. Diterpenoids derive from the C_{20} intermediate geranylgeranyl diphosphate (GGPP), which is converted by diTPSs through multistep carbocation-driven (cyclo)isomerization reactions to form various linear or cyclic scaffolds [35]. Functional modification of the diTPS products through the activity of P450s and a few other enzyme classes then affords the tremendous chemical diversity of plant diterpenoids [51].

The diversity of diTPS functions is based, in part, on their modular structure that is comprised of variations of three α-helical domains α, β, and γ [52]. The three major diTPS classes are monofunctional class I diTPS, monofunctional class II diTPSs, and bifunctional class I/II diTPSs [2, 35]. These three classes have different domain architectures and differ in the number of active sites and characteristic functional motifs. Class II diTPSs harbor an N-terminal active site in the βγ-domain accompanied by a common DxDD motif that facilitates the protonation-initiated cycloisomerization of GGPP into a variety of bicyclic prenyl diphosphate intermediates of distinct stereochemistry and regiospecific oxygenation. In contrast, class I diTPSs contain a C-terminal active site in the α-domain along with catalytic DDxxD and NSE/DTE motifs for binding the substrate's diphosphate moiety. Class I diTPSs facilitate the transformation of either GGPP or the transformation of products of class II diTPS. Class I diTPSs catalyze ionization-promoted cleavage of the substrate's diphosphate group, and subsequent rearrangement of resulting

carbocations to yield a multitude of diterpene structures. Bifunctional class I/II diTPSs contain both class II and class I active sites and functionalities in one protein. To current knowledge, only mosses, the lycophyte *Selaginella moellendorffii*, and gymnosperms contain bifunctional class I/II diTPSs [53–55]. In angiosperms, the majority of diterpenoids, including all labdane-related compounds, are formed through the sequential activity of pairs of monofunctional class II and class I diTPSs [35]. Repeated gene duplications, followed by sub- and neofunctionalization led to the expansion and functional divergence of the diTPS gene families in the course of evolution. Recent studies in several species suggest that diterpenoid biosynthesis may be organized in the form of modular systems, in which different combinations of class I and class II enzymes can yield different diterpene scaffolds to further increase the chemical space of diterpenoid intermediates and products [17, 25, 44, 45, 56–59].

Information gleaned from the characterization of naturally occurring modular pathways of pairwise acting functionally distinct diTPSs *in planta* can be applied to develop metabolic engineering and synthetic biology strategies based on combinatorial expression of available diTPSs and possibly P450 enzymes [17, 23, 60–62]. Building on the experience gained with established metabolic engineering platforms for high-value terpenoids, such as the anticancer diterpenoid drug Taxol and the sesquiterpenoid artemisinin used in the treatment of malaria [24, 26, 63], new microbial and plant-based platforms for the production of natural and nonnatural diterpene bioproducts are currently being developed in several laboratories [17, 26, 64–66]. In the following sections, we describe three examples of diterpene biosynthesis, sclareol production in clary sage, *cis*-abienol formation in balsam fir, and DRA biosynthesis in the Pinaceae, to highlight recent research on the genetic and enzymatic diversity of diterpenoid metabolism and its application for the fragrance industry.

3.2 Sclareol

Sclareol is presently the most important biologically sourced feedstock for the commercial semi-synthetic production of (-)-ambrox and related ambroxide fragrances for high-end perfume manufacture. In nature, sclareol is found in only a few plant species, including *Cistus creticus* (Cistaceae), *Cleome spinosa* (Brassicaceae), *Nicotiana glutinosa* (Solanaceae), and clary sage (Lamiaceae) [21, 23]. In particular, clary sage accumulates large enough quantities of sclareol that it forms epi-cuticular crystals primarily on the surfaces of flower calyces and bracts [36]. Although sclareol was shown to have antimicrobial properties, its biological function *in planta* is unknown [67]. Owing to its high content of sclareol, clary sage is commercially cultivated in Europe (France, Hungary, Bulgaria), the northern United States, and China. However, annual production levels of sclareol from clary sage can fluctuate as a result of environmental variables, affecting availability and price. Consequently, research on the biosynthesis of sclareol in clary sage and the

development of enzymatic production systems as an alternative to harvesting from plant material have attracted much interest in recent years.

In agreement with the modular architecture of diterpenoid biosynthesis in angiosperms, a pair of monofunctional class I and class II diTPSs is involved in the formation of sclareol [21]. Transcriptome analysis of clary sage calyx tissue led to the identification and functional characterization of the two relevant diTPSs, the class II diTPS labda-13-en-8-ol diphosphate synthase (*Ss*LPS) and the class I enzyme sclareol synthase (*Ss*SCS), which together form sclareol from GGPP [21] (Fig. 3). First, *Ss*LPS forms labda-13-en-8-ol diphosphate (LPP) through protonation-initiated cyclization of GGPP and subsequent regiospecific water capture of the intermediate carbocation at position C-8. *Ss*SCS then converts LPP through ionization of the diphosphate ester, followed by rearrangement of the resulting carbocation and secondary cyclization at C-13 to afford sclareol. In addition to sclareol, the coupled reaction of *Ss*LPS and *Ss*SCS yields small amounts of manool (Fig. 2), which may result from conversion of *ent*-copalyl diphosphate by *Ss*SCS. *In planta*, *ent*-copalyl diphosphate may either be recruited from the gibberellin plant hormone biosynthetic pathway or formed as a minor by-product of *Ss*LPS as observed in vitro. Manool formation through a side reaction of *Ss*LPS and *Ss*SCS is consistent with the low metabolite abundance in clary sage, whereas other *Salvia* species, such as *S. oligophylla* and *S. argentea* accumulate manool rather than sclareol [68].

The complete biosynthesis of sclareol and manool from GGPP is achieved by diTPSs and, unlike many other diterpenoid pathways, it does not require a cytochrome P450-dependent activity to introduce the alcohol functionality. Recent studies suggest that the capacity of diTPSs to facilitate oxygenation of the diterpene backbone is more common than was previously appreciated. Functionally orthologous LPS enzymes include the copal-8-ol diphosphate synthase from *C. creticus* and the recently identified LPSs from tobacco (Solanaceae), *Grindelia robusta* (Asteraceae), and *Coleus forskohlii* (Lamiaceae) [17, 43, 45, 69]. This broad taxonomic distribution indicates that the function of LPS enzymes evolved early in the speciation of angiosperms, but possibly after the split of monocotyledonous and dicotyledonous lineages, inasmuch as no LPSs were found among the large diTPS families in members of the grasses (Poaceae) [57, 70]. The *Ss*SCS-catalyzed transformation of LPP into sclareol via a regiospecific hydroxylation at C-13 is, to current knowledge, unique to clary sage. Other known diTPSs that facilitate oxygenation of the hydrocarbon backbone represent bifunctional class I/II enzymes from the nonseed plants *Physcomitrella patens* and *S. moellendorfii*, and the levopimaradiene/abietadiene synthases (LASs) from gymnosperm species of spruce, pine, and fir [20, 53, 54, 56, 71]. LAS enzymes were previously thought to produce nonoxygenated diterpenes from GGPP but were recently shown to form 13-hydroxy-(8,14)-abietene as an initial unstable product. In contrast to these class I/II bifunctional diTPSs, *Ss*SCS belongs to a group of monofunctional class I diTPSs that adopt an atypical βα-domain architecture through loss of the γ-domain, which is more commonly known from mono- and sesqui-TPSs, as well as casbene synthase-like macrocyclases [52]. Known members of this group are restricted to the

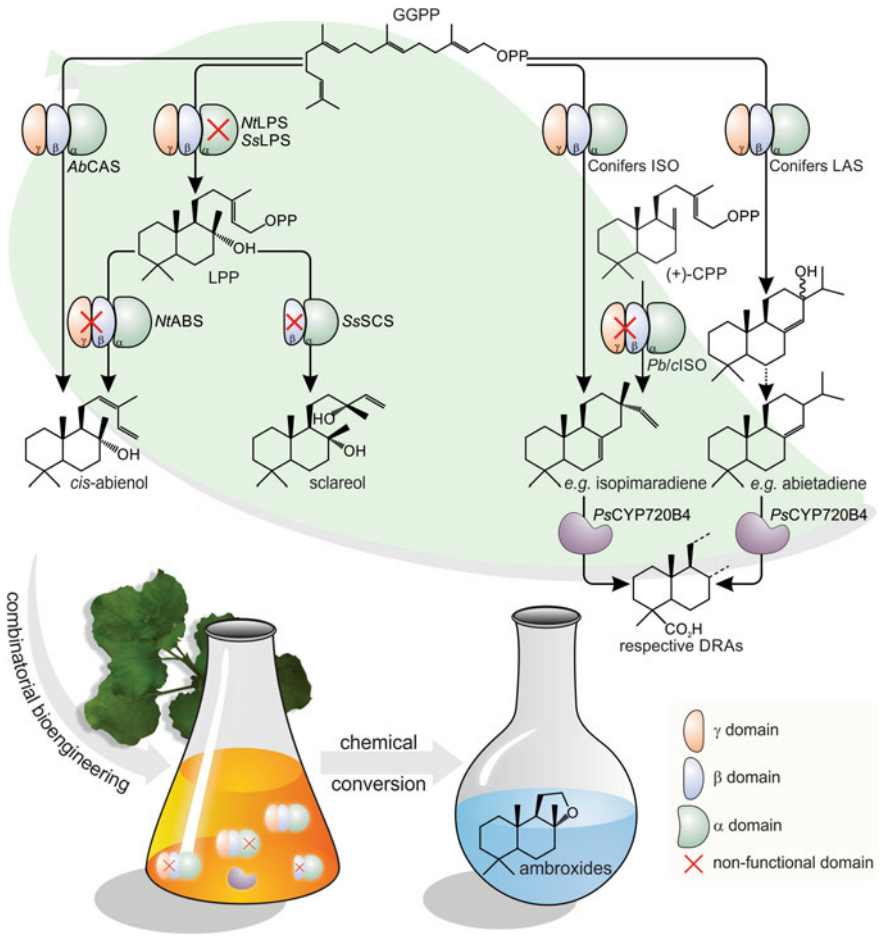

Fig. 3 Modularity of plant diterpenoid biosynthetic pathways and their utility for metabolic engineering of biological systems for ambroxide precursor production. A diverse portfolio of diterpene synthases and cytochrome P450 monooxygenases involved in the formation of plant-derived ambroxide precursors, such as sclareol, *cis*-abienol, and diterpene resin acids, has been discovered in recent years based on transcriptome sequencing and enzyme characterization in nonmodel fragrant plants. Single or combinatorial expression of diterpenoid-biosynthetic enzymes in microbial and plant host systems offers opportunity for metabolic engineering of ambroxide precursors and other diterpenoid bioproducts. These engineered systems enable the production of natural or novel diterpenoids in the form of single target compounds or mixtures of low complexity. Abbreviations: GGPP, geranylgeranyl diphosphate; LPP, labda-13-en-8-ol diphosphate; (+)-CPP, (+)-copalyl diphosphate; *Ab*CAS, *Abies balsamea cis*-abienol synthase; *Nt*LPS, *N. tabacum* LPP synthase; *Nt*ABS, *N. tabacum cis*-abienol synthase; *Ss*LPS, *S. sclarea* LPP synthase; *Ss*SCS, *S. sclarea* sclareol synthase; ISO, isopimaradiene synthase from different conifers; LAS, levopimaradiene/abietadiene synthase from different conifers; *Pb*/*c*ISO, *P. banksiana*/*P. contorta* isopimaradiene; *Ps*CYP720B4, *P. sitchensis* cytochrome P450 720B4; DRA, diterpene resin acid. A *red* X in the schematic depiction of diTPS indicates a nonfunctional active site in monofunctional enzymes

Lamiaceae and wheat (*Triticum aestivum*, Poaceae), with functions in specialized diterpenoid metabolism [21, 25, 45, 72]. Notably, the majority of these bidomain class I diTPSs show substrate promiscuity similar to *Ss*SCS, including manoyl oxide/miltiradiene synthases from *C. forskohlii* and *Rosmarinus officinalis*, and a multifunctional diTPS from *Marrubium vulgare* [21, 25, 44, 45].

As illustrated here for sclareol, the biosynthesis of oxygenated diterpenoids through a modular biosynthetic system of class II and class I diTPSs without requirement for additional P450-catalyzed oxidation steps may have advantages for metabolic engineering of microbial production systems. Recently, Ignea and colleagues demonstrated the successful production of sclareol by combinatorial overexpression of the *C. creticus* LPS with *Ss*SCS in engineered yeast achieving titers of ~400 mg/L [66]. In addition, researchers at Firmenich developed an engineered *E. coli* platform for commercial sclareol production to yield product amounts of up to 1.5 g/L [23]. Here, *Ss*LPS and *Ss*SCS were coexpressed with the bacterial GGPP synthase CrtE and two synthetic operons that encode key enzymes of the mevalonate pathway to improve precursor supply and conversion.

3.3 Cis-*Abienol*

Similar to sclareol, the tertiary diterpene alcohol *cis*-abienol occurs naturally only in a few plant species. For example, *cis*-abienol contributes to the flavor and aroma of cultivars of tobacco, where it accumulates in leaf glandular trichomes [43]. In addition, balsam fir (*A. balsamea*), a coniferous tree native to central and eastern Canada, is a rich source of *cis*-abienol, which accounts for up to 40 % of the aromatic oleoresin accumulating in resin ducts and blisters [20]. Traditionally, balsam fir oleoresin has been traded as "Canada Balsam" for uses as an antifouling agent in naval stores or as glue in the optical industry [38]. As described above, *cis*-abienol is also a suitable starting material for the semi-synthesis of (-)-ambrox [29].

Due to its potential application in the fragrance and flavor industry, details of the biosynthesis of *cis*-abienol *in planta* have been investigated in the last few years. Because biosynthesis of conifer diterpene resin components, such as *cis*-abienol, typically involves bifunctional class I/II diTPSs [55], we had hypothesized that *cis*-abienol formation in balsam fir would likewise be catalyzed by a bifunctional enzyme. To identify the proposed *cis*-abienol synthase (CAS), we generated transcriptome inventories for gene discovery by combining high-throughput transcriptome sequencing with metabolite profiling of balsam fir bark tissue [17, 20]. Querying the transcriptome assemblies against a database of TPS protein sequences resulted in the discovery of three bifunctional class I/II balsam fir diTPSs. Biochemical characterization showed that two candidates, *Ab*LAS and *Ab*ISO, were paralogous to LASs and isopimaradiene synthases (ISO) from other conifers, and the third more divergent enzyme, *Ab*CAS, showed a novel function, directly converting GGPP into *cis*-abienol [20] (Fig. 3). Analogous to angiosperm LPSs, *Ab*CAS catalyzes the cyclohydration of GGPP to form LPP in the enzyme's class II

active site. LPP can then freely diffuse to the class I active site, where *cis*-abienol is formed by ionization of the diphosphate ester without further cyclization (Fig. 3). Formation of a bicyclic diterpenol was a newly discovered feature of gymnosperm bifunctional class I/II diTPSs, whereas all previously known conifer class I/II diTPS enzymes are involved in the formation of tricyclic scaffolds in DRA biosynthesis [17, 56, 73–76]. Notably, a recent structural-functional analysis of grand fir (*Abies grandis*) abietadiene synthase (*Ag*AS) demonstrated that substitution of a single aspartate residue in the enzyme's class II active site altered activity from producing (+)-CPP en route to DRAs to formation of LPP [77]. This aspartate is indeed conserved in *Ab*CAS and may have been critical in the functional evolution of *cis*-abienol biosynthesis, presumably by neofunctionalization of a duplicated diTPS of DRA biosynthesis in a balsam fir ancestor [20].

In parallel with the discovery of *Ab*CAS for *cis*-abienol production in balsam fir, Sallaud and coworkers elucidated an alternative *cis*-abienol biosynthetic pathway in tobacco [43]. In contrast to the bifunctional *Ab*CAS that facilitates class II and class I catalysis, tobacco contains a mechanistically analogous pair of two monofunctional diTPSs that act sequentially to form *cis*-abienol (Fig. 3). Much like sclareol biosynthesis, a class II diTPS (*Nt*LPS) catalyzes the formation of LPP, which is then transformed by a class I diTPS (*Nt*ABS) to afford *cis*-abienol. These two different systems of *cis*-abienol formation suggest that diTPSs involved in *cis*-abienol biosynthesis evolved independently in gymnosperm and angiosperm lineages, highlighting an intriguing example of parallel functional specialization of diTPS enzymes. A similar case of parallel evolution of specialized diterpenoid biosynthesis appears to be the biosynthesis of miltiradiene, which is catalyzed by a class I/II bifunctional diTPS in the primitive land plant *S. moellendorfii* and by pairs of monofunctional diTPSs in several Lamiaceae species [25, 44, 45, 78, 79].

A prototype yeast strain for *cis*-abienol production via coexpression of NtABS with a fusion protein of *C. creticus* LPS and a GGPP-producing variant of the yeast FPP synthase Erg20 has recently been described [66]. It remains to be examined if *Ab*CAS can offer improved *cis*-abienol production, as this bifunctional diTPS requires expression and optimization of only a single gene that provides the naturally evolved scaffold of both active sites, allowing for efficient channeling of reaction intermediates.

3.4 Diterpene Resin Acids

The biosynthesis of conifer DRAs has attracted much attention, due to their important roles in the defense of conifer trees against insects and pathogens, and as a renewable resource for industrial bioproducts [8, 10]. Building on pioneering work by Professors Robert Coates, Rodney Croteau, and their colleagues [80–82], much has been learned over the last two decades about the genomic, molecular, and enzymatic features that define DRA formation in conifers. Numerous bifunctional conifer class I/II diTPSs involved in DRA metabolism have been characterized, all

of which share common catalytic principles in forming pimarane- or abietane-type compounds through conversion of GGPP into (+)-CPP and subsequent rearrangement of distinct secondary and tertiary carbocations (Fig. 3). During the last few years new insight has been gained on the mechanistic, evolutionary, and physiological underpinnings of conifer DRA biosynthesis, which suggest a more complex biosynthetic system than previously known. For example, detailed functional analysis of Norway spruce (*Picea abies*) LAS illustrated that this class I/II diTPS forms the epimeric diterpenol 13-hydroxy-(8,14)-abietene as the initial product, and not a mixture of diterpene olefins as described earlier [71] (Fig. 3). In vitro, and possibly in vivo, these tricyclic diterpene alcohol epimers dehydrate to form the described LAS products, abietadiene, palustradiene, levopimaradiene, and neoabietadiene. In subsequent studies, this reaction was also confirmed for LAS enzymes of balsam fir, jack pine (*Pinus banksiana*), lodgepole pine (*Pinus contorta*), and golden larch (*Pseudolarix amabilis*) and seems to be a specific feature of conifer LASs, whereas the closely related conifer ISO class I/II diTPS enzymes directly form the diterpene olefin product [17, 20, 56]. Recent work also showed that jack pine and lodgepole pine contain both bifunctional class I/II diTPS as well as, surprisingly, a group of monofunctional class I diTPSs for DRA metabolism [56]. The monofunctional diTPSs in pine lack a functional class II active site and convert (+)-CPP, but not GGPP, into pimaradiene and isopimaradiene, respectively. No monofunctional (+)-CPP synthase has been discovered in a gymnosperm species, however, in vitro assays showed that these diTPSs can "hijack" the freely diffusing (+)-CPP intermediate produced by bifunctional conifer diTPSs.

The abietane and pimarane diTPS products serve as substrates for P450s of the gymnosperm-specific CYP720B family of conifer DRA biosynthesis. Members of this family comprise approximately a dozen different genes in a given conifer species suggesting an ancient evolutionary diversification of the gene family [49, 83]. The two functionally characterized members of the CYP720B family, loblolly pine (*Pinus taeda*) CYP720B1 and Sitka spruce (*Picea sitchensis*) CYP720B4, are multifunctional and multisubstrate enzymes, catalyzing the three-step oxidation of all major diterpene olefins via the corresponding C18-alcohols and aldehydes to afford the respective DRAs (Fig. 3). CYP720B4 catalyzes an array of three different oxidation reactions on each of eight different diterpenes accounting for most major and minor DRA components in spruce. A role of CYP720B4 in conifer defense is underpinned by findings of its inducibility upon treatment with methyl jasmonate and high transcript abundance in epithelial cells of cortical resin ducts, the primary site of terpenoid biosynthesis in conifers [6, 49]. The recent publication of draft assemblies of the very large genomes of three conifer species, white spruce (*Picea glauca*), Norway spruce, and loblolly pine, provides a means to better understand the genomic background of DRA diversity in conifers [84–88].

With the large set of characterized diTPSs of DRA biosynthesis and the emerging characterization of diterpene-converting P450s, bioengineering platforms for the stereospecific production of individual DRAs may advance as an alternative to feedstocks from conifer oleoresin, which are complex mixtures of many different terpenoids and, when sourced as by-products of the forest industry, often contain

other lipophilic compounds. Prototype yeast strains have been established that allow the formation of pimarane- and abietane-type DRAs through combinatorial expression of LAS and ISO enzymes with CYP720B4 and the yeast endogenous GGPP synthase [49, 83]. For example, microbially produced abietic acid and levopimaric acid may serve as feasible starting materials for the synthesis of (-)-ambrox [34] (Fig. 2). Other DRA-based biomaterials range from industrial inks and resins to possible biodegradable plastics and rubbers [8, 14, 89].

4 Future Challenges and Opportunities

The above examples illustrate some of the potential of combining genomics-enabled pathway discovery in plants with metabolic engineering and synthetic biology of diterpenoids, specifically for those compounds that may serve as precursors for commercial (-)-ambrox production. It is important to note that both naturally evolved production systems (i.e., plants), and metabolically engineered microbial systems, each have unique advantages and disadvantages. The most obvious advantages of using plants to harvest diterpenoids is the fact that biosynthesis is fueled by sunlight through photosynthesis and operates via optimized metabolic pathways and cell structures, which have evolved over millions of years under natural selection to produce diterpenoids optimized for functions in plant biology.

Some plants that are used for bulk extraction of diterpenoids, such as long-lived conifer trees, require no or only minimal human input for planting or cultivation. A large feedstock of diterpenoid-rich oleoresin is extracted as a by-product from conifers used primarily in the wood or pulp and paper industries. Other diterpenoid-producing plants, such as clary sage, the major source of sclareol, require more intensive crop management, and variation of environmental conditions may substantially affect yield and price of the desired diterpenoid. Finally, some plant systems that produce diterpenoids of interest may not be suitable at the present time for agricultural production, or would be at risk of over-harvesting from their natural environments. Considering all these scenarios, plants appear to remain, for now, the sole major source for all diterpenoids used in commercial ambroxide production.

Alternative production systems, such as engineered microorganisms, in which plant genes for diterpene biosynthesis are heterologously expressed, will have to be competitive in product yield, purity, and production cost. One common limitation inherent to plant production systems is the complexity of their natural product mixtures, especially with regard to mixtures of terpenoids. Plants typically do not produce a single target compound, such as, for example, *cis*-abienol or sclareol, but diverse arrays of metabolites of often similar structures and physicochemical properties, making it difficult to obtain a single compound of interest in pure form. Such arrays of metabolites are likely to have advantages for plants in their natural environments. For example, complex terpenoid defense systems may be harder to breach by pests and pathogens than a defense that is based on a single compound.

For diterpenoids, it is also now known that the suite of metabolites produced in an individual plant, such as the many diterpenoids of conifer oleoresin, are the result of biosynthetic grids with many possible products. Current knowledge suggests that plant diterpenoids are generally not derived from simple linear pathways, but there is growing evidence for dynamic, modular pathway systems, leading to dozens of similar products in a single plant. Considering the genomic (e.g., multigene diTPS and P450 gene families) and biochemical (multiproduct and modular diTPS enzymes, multisubstrate P450 enzymes) multiplicity and promiscuity of plant terpenoid metabolism, it would be difficult to apply traditional selection and plant breeding for the purpose of selectively producing a single target compound. Indeed, this goal may be impossible to achieve unless through use of genetically engineered plants in which existing terpenoid metabolism is redirected or focused to a single major product.

It is here where microbial production systems engineered with individual plant diTPSs or combinations of diTPSs and/or P450s may have the largest advantage. Expression of single enzymes or simple pathways can be optimized for producing one or a few target compounds of interest, thus avoiding or reducing the need for downstream product separation. In addition, engineered microbial systems may offer greater reproducibility, as they are independent of seasonal growth and variable environmental conditions that can affect agricultural or silvicultural production. Engineered microbial systems also have the advantage of (co)expressing optimized diTPS or P450 enzymes, and to explore the vast conceivable space of possible new combinations of diTPS and P450. Following the modular architecture of diterpenoid biosynthesis in nature, combinatorial engineering of diTPSs and P450s of different origin can be utilized for the enantiospecific production of a wide range of natural and nonnatural diterpenoid scaffolds [17, 18, 62].

Issues such as precursor supply, metabolic flux, potential toxicity, accumulation, and transport of diterpenoids in engineered microbial systems can pose limitations in productivity, and will have to be addressed case by case. Host toxicity and often low expression and catalytic efficiency of diTPSs and P450s are some of the major factors constraining diterpenoid production in metabolically engineered microorganisms [26, 90, 91]. However, the succesfull production of sclareol, as well as the sesquiterpenoids farnesene and artemisinin, in *E. coli* and yeast have shown that these types of limitations can be overcome [23, 24, 66, 92]. The scalability of engineered microbial and plant systems continues to improve through optimization of host systems and fermentation processes, as well as enhanced pathway efficiency by establishing a balanced metabolite flow between individual components [23, 26, 47, 61, 65, 90].

As engineered microbial production platforms, and potentially also engineered plant systems, are being advanced for diterpenoids of interest for the fragrance and other industries, plant genomics and plant biochemistry will continue to make important contributions to harness nature's rich resource of genes and enzymes of specialized diterpenoid metabolism [17, 19]. The opportunities and challenges here are inherent to the often high sequence identity and functional divergence of the large diTPS and P450 gene families, which make a priori and ad hoc functional

annotations nearly impossible. In vitro and in vivo biochemical characterization of gene candidates remains an absolute requirement for the discovery of new diTPS and P450 enzymes [2, 17, 51]. Recent systems biology approaches focusing on plant specialized metabolism have already expanded the known catalytic landscape of diTPSs and terpenoid-modifying enzymes, such as P450s [17–19, 93], and the vast majority of plant species still remains to be explored.

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References

1. Davis EM, Croteau R (2000) Cyclization enzymes in the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes. Biosynthesis: Aromatic Polyketides. Isoprenoids, Alkaloids, Springer, pp 53–95
2. Chen F, Tholl D, Bohlmann J, Pichersky E (2011) The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. Plant J 66:212–229
3. Gershenzon J, Dudareva N (2007) The function of terpene natural products in the natural world. Nat Chem Biol 3:408–414
4. Keeling CI, Bohlmann J (2006) Diterpene resin acids in conifers. Phytochemistry 67:2415–2423
5. Schmelz EA, Huffaker A, Sims JW, Christensen SA, Lu X, Okada K, Peters RJ (2014) Biosynthesis, elicitation and roles of monocot terpenoid phytoalexins. Plant J 79:659–678
6. Zulak KG, Bohlmann J (2010) Terpenoid biosynthesis and specialized vascular cells of conifer defense. J Integr Plant Biol 52:86–97
7. Schilmiller AL, Pichersky E, Last RL (2012) Taming the hydra of specialized metabolism: how systems biology and comparative approaches are revolutionizing plant biochemistry. Curr Opin Plant Biol 15:338–344
8. Bohlmann J, Keeling CI (2008) Terpenoid biomaterials. Plant J 54:656–669
9. Peralta-Yahya PP, Zhang F, del Cardayre SB, Keasling JD (2012) Microbial engineering for the production of advanced biofuels. Nature 488:320–328
10. Zerbe P, Bohlmann J (2014) Metabolic engineering of terpene synthases of conifer defense: bioproducts, biofuels and perfumes. Rec Adv Phytochem 44:85–107
11. Philippe RN, De Mey M, Anderson J, Ajikumar PK (2014) Biotechnological production of natural zero-calorie sweeteners. Curr Opin Biotechnol 26:155–161
12. Lange BM, Mahmoud SS, Wildung MR, Turner GW, Davis EM, Lange I, Baker RC, Boydston RA, Croteau RB (2011) Improving peppermint essential oil yield and composition by metabolic engineering. Proc Natl Acad Sci USA 108:16944–16949
13. Frater G, Bajgrowicz JA, Kraft P (1998) Fragrance chemistry. Tetrahedron 54:7633–7703
14. Sell C (1990) The chemistry of ambergris. Chem Ind 20:516–520
15. Stoll M, Hinder M (1950) Odeur et constitution III les substances bicyclohomofarnésiques. Helv Chim Acta 33:1251–1260

16. Frija LMT, Frade RFM, Afonso CAM (2011) Isolation, chemical, and biotransformation routes of labdane-type diterpenes. Chem Rev 111:4418–4452
17. Zerbe P, Hamberger B, Yuen MM, Chiang A, Sandhu HK, Madilao LL, Nguyen A, Hamberger B, Bach SS, Bohlmann J (2013) Gene discovery of modular diterpene metabolism in non-model systems. Plant Physiol 162:1073–1091
18. Facchini PJ, Bohlmann J, Covello PS, De Luca V, Mahadevan R, Page JE, Ro DK, Sensen CW, Storms R, Martin VJ (2012) Synthetic biosystems for the production of high-value plant metabolites. Trends Biotechnol 30:127–131
19. Higashi Y, Saito K (2013) Network analysis for gene discovery in plant-specialized metabolism. Plant Cell Environ 36:1597–1606
20. Zerbe P, Chiang A, Yuen M, Hamberger B, Hamberger B, Draper JA, Britton R, Bohlmann J (2012) Bifunctional *cis*-abienol synthase from *Abies balsamea* discovered by transcriptome sequencing and its implications for diterpenoid fragrance production. J Biol Chem 287:12121–12131
21. Caniard A, Zerbe P, Legrand S, Cohade A, Valot N, Magnard JL, Bohlmann J, Legendre L (2012) Discovery and functional characterization of two diterpene synthases for sclareol biosynthesis in *Salvia sclarea* (L.) and their relevance for perfume manufacture. BMC Plant Biol 12:119
22. Diaz-Chavez ML, Moniodis J, Madilao LL, Jancsik S, Keeling CI, Barbour EL, Ghisalberti EL, Plummer JA, Jones CG, Bohlmann J (2013) Biosynthesis of sandalwood oil: *Santalum album* CYP76F cytochromes P450 produce santalols and bergamotol. PLoS ONE 8:e75053
23. Schalk M, Pastore L, Mirata MA, Khim S, Schouwey M, Deguerry F, Pineda V, Rocci L, Daviet L (2012) Towards a biosynthetic route to sclareol and amber odorants. J Am Chem Soc 134:18900–18903
24. Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A, Eng D, Polichuk DR, Teoh KH, Reed DW, Treynor T, Lenihan J, Fleck M, Bajad S, Dang G, Dengrove D, Diola D, Dorin G, Ellens KW, Fickes S, Galazzo J, Gaucher SP, Geistlinger T, Henry R, Hepp M, Horning T, Iqbal T, Jiang H, Kizer L, Lieu B, Melis D, Moss N, Regentin R, Secrest S, Tsuruta H, Vazquez R, Westblade LF, Xu L, Yu M, Zhang Y, Zhao L, Lievense J, Covello PS, Keasling JD, Reiling KK, Renninger NS, Newman JD (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 496:528–532
25. Zerbe P, Chiang A, Dullat H, O'Neil-Johnson M, Starks C, Hamberger B, Bohlmann J (2014) Diterpene synthases of the biosynthetic system of medicinally active diterpenoids in *Marrubium vulgare*. Plant J 79:914–927
26. Ajikumar PK, Xiao WH, Tyo KE, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G (2010) Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. Science 330:70–74
27. Kawanobe T, Kogami K, Matsui M (1986) New syntheses of (±)-ambrox, (±)-ambra oxide and their stereoisomers. Agric Biol Chem 50:1475–1480
28. Snowden RL (2008) Cetalox® and analogues: synthesis via acid-mediated polyene cyclizations. Chem Biodivers 5:958–969
29. Barrero AF, Alvarez-Manzaneda EJ, Altarejos J, Salido S, Ramos JM (1994) Synthesis of Ambrox® from (-)-sclareol and (+)-*cis*-abienol. Tetrahedron 49:10405–10412
30. Barrero AF, Mar Herrador M, Arteaga P, Arteaga JF, Arteaga AF (2012) Communic acids: occurrence, properties and use as chirons for the synthesis of bioactive compounds. Molecules 17:1448–1467
31. Bolster MG, Jansen BJM, de Groot A (2001) The synthesis of (-)-Ambrox® starting from labdanolic acid. Tetrahedron 57:5657–5662
32. Bolster MG, Jansen BJM, de Groot A (2001) The synthesis of Ambrox®-like compounds starting from (+)-larixol. Tetrahedron 57:5663–5679
33. Castro JM, Salido S, Altarejos J, Nogueras M, Sanchez A (2002) Synthesis of Ambrox® from labdanolic acid. Tetrahedron 58:5941–5949
34. Koyama H, Kaku Y, Ohno M (1987) Synthesis of (-)-Ambrox® from L-abietic acid. Tetrahedron Lett 28:2863–2866

35. Peters RJ (2010) Two rings in them all: the labdane-related diterpenoids. Nat Prod Rep 27:1521–1530
36. Caissard J-C, Olivier T, Delbecque C, Palle S, Garry PP, Audran A, Valot N, Moja S, Nicolé F, Magnard JL, Legrand S, Baudino S, Jullien F (2012) Extracellular localization of the diterpene sclareol in clary sage (*Salvia sclarea* L, Lamiaceae). PLoS ONE 7:e48253
37. Barton D, Parekh S, Taylor D, Tse C (1994) An efficient synthesis of (-)-dodecahydro-3a, 6, 6, 9a-tetramethylnaphthol [2,1-B] furan from (-)-sclareol. Tetrahedron Lett 35:5801–5804
38. Gray PS, Mills JS (1964) The isolation of abienol from Canada Balsam, the oleoresin of *Abies balsamea* (L) Mill. J Chem Soc 1:5822–5825
39. De Carvalho CCCR, da Fonseca MMR (2006) Biotransformation of terpenes. Biotechnol Adv 24:134–142
40. Cheetham PSJ (1993) The use of biotransformations for the production of flavours and fragrances. Trends Biotechnol 11:478–488
41. Musharraf SG, Naz S, Najeeb A, Khan S, Choudhary MI (2012) Biotransformation of perfumery terpenoids, (-)-Ambrox® by a fungal culture *Macrophomina phaseolina* and a plant cell suspension culture of *Peganum harmala*. Chem Cent J 6:82
42. Nasib A, Musharraf SG, Hussain S, Khan S, Anjum S, Ali S, Atta-Ur-Rahman AU, Choudhary MI (2006) Biotransformation of (-)-Ambrox® by cell suspension cultures of *Actinidia deliciosa*. J Nat Prod 69:957–959
43. Sallaud C, Giacalone C, Töpfer R, Goepfert S, Bakaher N, Rösti S, Tissier A (2012) Characterization of two genes for the biosynthesis of the labdane diterpene *Z*-abienol in tobacco (N*icotiana tabacum*) glandular trichomes. Plant J 72:1–17
44. Brückner K, Božić D, Manzano D, Papaefthimiou D, Pateraki I, Scheler U, Ferrer A, de Vos RC, Kanellis AK, Tissier A (2014) Characterization of two genes for the biosynthesis of abietane-type diterpenes in rosemary (*Rosmarinus officinalis*) glandular trichomes. Phytochemistry 101:52–64
45. Pateraki I, Andersen-Ranberg J, Hamberger B, Heskes AM, Martens HJ, Zerbe P, Bach SS, Møller BL, Bohlmann J, Hamberger B (2014) Manoyl oxide (13*R*), the biosynthetic precursor of forskolin, is synthesized in specialized root cork cells in *Coleus forskohlii*. Plant Physiol 164:1222–1236
46. Gao W, Sun HX, Xiao H, Cui G, Hillwig ML, Jackson A, Wang X, Shen Y, Zhao N, Zhang L, Wang XJ, Peters RJ, Huang L (2014) Combining metabolomics and transcriptomics to characterize tanshinone biosynthesis in *Salvia miltiorrhiza*. BMC Genom 15:73
47. Guo J, Zhou YJ, Hillwig ML, Shen Y, Yang L, Wang Y, Zhang X, Liu W, Peters RJ, Chen X, Zhao ZK, Huang L (2013) CYP76AH1 catalyzes turnover of miltiradiene in tanshinones biosynthesis and enables heterologous production of ferruginol in yeasts. Proc Natl Acad Sci USA 110:12108–12113
48. Zi J, Peters RJ (2013) Characterization of CYP76AH4 clarifies phenolic diterpenoid biosynthesis in the Lamiaceae. Org Biomol Chem 11:7650–7652
49. Hamberger B, Ohnishi T, Hamberger B, Séguin A, Bohlmann J (2011) Evolution of diterpene metabolism: Sitka spruce CYP720B4 catalyzes multiple oxidations in resin acid biosynthesis of conifer defense against insects. Plant Physiol 157:1677–1695
50. Beale MH (1990) The biosynthesis of C5-C20 terpenoid compounds. Nat Prod Rep 7:25–39
51. Nelson D, Werck-Reichhart DA (2011) P450-centric view of plant evolution. Plant J 66:194–211
52. Gao Y, Honzatko RB, Peters RJ (2012) Terpenoid synthase structures: a so far incomplete view of complex catalysis. Nat Prod Rep 29:1153–1175
53. Hayashi K-I, Kawaide H, Notomi M, Sakigi Y, Matsuo A, Nozaki H (2006) Identification and functional analysis of bifunctional *ent*-kaurene synthase from the moss *Physcomitrella patens*. FEBS Lett 580:6175–6181
54. Mafu S, Hillwig ML, Peters RJ (2011) A novel labda-7,13*E*-dien-15-ol-producing bifunctional diterpene synthase from *Selaginella moellendorffii*. Chem Bio Chem 12:1984–1987

55. Keeling CI, Bohlmann J (2006) Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. New Phytol 170:657–675
56. Hall DE, Zerbe P, Jancsik S, Quesada AL, Dullat H, Madilao LL, Yuen M, Bohlmann J (2012) Evolution of conifer diterpene synthases: Diterpene resin acid biosynthesis in lodgepole pine and jack pine involves monofunctional and bifunctional diterpene synthases. Plant Physiol 161:600–616
57. Xu M, Wilderman PR, Morrone D, Xu J, Roy A, Margis-Pinheiro M, Upadhyaya NM, Coates RM, Peters RJ (2007) Functional characterization of the rice kaurene synthase-like gene family. Phytochemistry 68:312–326
58. Morrone D, Hillwig ML, Mead ME, Lowry L, Fulton DB, Peters RJ (2011) Evident and latent plasticity across the rice diterpene synthase family with potential implications for the evolution of diterpenoid metabolism in the cereals. Biochem J 435:589–595
59. Zhou K, Xu M, Tiernan M, Xie Q, Toyomasu T, Sugawara C, Oku M, Usui M, Mitsuhashi W, Chono M, Chandler PM, Peters RJ (2012) Functional characterization of wheat ent-kaurene(-like) synthases indicates continuing evolution of labdane-related diterpenoid metabolism in the cereals. Phytochemistry 84:47–55
60. Yadav VG, De Mey M, Lim CG, Ajikumar PK, Stephanopoulos G (2012) The future of metabolic engineering and synthetic biology: towards a systematic practice. Metab Eng 14:233–241
61. Zhou YJ, Gao W, Rong Q, Jin G, Chu H, Liu W, Yang W, Zhu Z, Li G, Zhu G, Huang L, Zhao ZK (2012) Modular pathway engineering of diterpenoid synthases and the mevalonic acid pathway for miltiradiene production. J Am Chem Soc 134:3234–3241
62. Ignea C, Ioannou E, Georgantea P, Loupassaki S, Trikka FA, Kanellis AK, Makris AM, Roussis V, Kampranis SC (2015) Reconstructing the chemical diversity of labdane-type diterpene biosynthesis in yeast. Metab Eng 28:91–103
63. Ro D-K, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA, Eachus RA, Ham TS, Kirby J, Chang MC, Withers ST, Shiba Y, Sarpong R, Keasling JD (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440:940–943
64. Bach SS, King BC, Zhan X, Simonsen HT, Hamberger B (2014) Heterologous stable expression of terpenoid biosynthetic genes using the moss *Physcomitrella patens*. Methods Mol Biol 1153:257–271
65. Brückner K, Tissier A (2013) High-level diterpene production by transient expression in Nicotiana benthamiana. Plant Methods 9:46
66. Ignea C, Trikka FA, Nikolaidis AK, Georgantea P, Ioannou E, Loupassaki S, Kefalas P, Kanellis AK, Roussis V, Makris AM, Kampranis SC (2015) Efficient diterpene pro duction in yeast by engineering Erg20p into a geranylgeranyl diphosphate synthase. Metab Eng 27:65–75
67. Bailey JA, Carter GA, Burden RS, Wain RL (1975) Control of rust diseases by diterpenes from *Nicotiana glutinosa*. Nature 255:328–329
68. Couladis M, Tzakou O, Stojanovic D, Mimica-Dukic N, Jancic R (2001) The essential oil composition of *Salvia argentea* L. Flavour Fragr J 16:227–229
69. Falara V, Pichersky E, Kanellis AK (2010) A copal-8-ol diphosphate synthase from the angiosperm *Cistus creticus* subsp. *creticus* is a putative key enzyme for the formation of pharmacologically active, oxygen-containing labdane-type diterpenes. Plant Physiol 154:301–310
70. Wu Y, Zhou K, Toyomasu T, Sugawara C, Oku M, Abe S, Usui M, Mitsuhashi W, Chono M, Chandler PM, Peters RJ (2012) Functional characterization of wheat copalyl diphosphate synthases sheds light on the early evolution of labdane-related diterpenoid metabolism in the cereals. Phytochemistry 84:40–46
71. Keeling CI, Madilao LL, Zerbe P, Dullat HK, Bohlmann J (2011) The primary diterpene synthase products of *Picea abies* levopimaradiene/abietadiene synthase (PaLAS) are epimers of a thermally unstable diterpenol. J Biol Chem 286:21145–21153

72. Hillwig ML, Xu M, Toyomasu T, Tiernan MS, Wei G, Cui G, Huang L, Peters RJ (2011) Domain loss has independently occurred multiple times in plant terpene synthase evolution. Plant J 68:1051–1060
73. Peters RJ, Flory JE, Jetter R, Ravn MM, Lee HJ, Coates RM, Croteau RB (2000) Abietadiene synthase from grand fir (*Abies grandis*): characterization and mechanism of action of the 'pseudomature' recombinant enzyme. Biochemistry 39:15592–15602
74. Martin DM, Fäldt J, Bohlmann J (2004) Functional characterization of nine Norway Spruce TPS genes and evolution of gymnosperm terpene synthases of the TPS-d subfamily. Plant Physiol 135:1908–1927
75. Keeling CI, Weisshaar S, Ralph SG, Jancsik S, Hamberger B, Dullat HK, Bohlmann J (2011) Transcriptome mining, functional characterization, and phylogeny of a large terpene synthase gene family in spruce (Picea spp.). BMC Plant Biol 11:43
76. Schepmann HG, Pang J, Matsuda SP (2001) Cloning and characterization of *Ginkgo biloba* levopimaradiene synthase which catalyzes the first committed step in ginkgolide biosynthesis. Arch Biochem Biophys 392:263–269
77. Criswell J, Potter K, Shephard F, Beale MH, Peters RJ (2012) A single residue change leads to a hydroxylated product from the class II diterpene cyclization catalyzed by abietadiene synthase. Org Lett 14:5828–5831
78. Gao W, Hillwig ML, Huang L, Cui G, Wang X, Kong J, Yang B, Peters RJ (2009) A functional genomics approach to tanshinone biosynthesis provides stereochemical insights. Org Lett 11:5170–5173
79. Sugai Y, Ueno Y, Hayashi K, Oogami S, Toyomasu T, Matsumoto S, Natsume M, Nozaki H, Kawaide H (2011) Enzymatic (13)C labeling and multidimensional NMR analysis of miltiradiene synthesized by bifunctional diterpene cyclase in Selaginella moellendorffii. J Biol Chem 286:42840–42847
80. Coates RM (1976) Biogenetic-type rearrangements of terpenes. Fortschr Chem Org Naturst 33:73–230
81. Trapp SC, Croteau RB (2001) Genomic organization of plant terpene synthases and molecular evolutionary implications. Genetics 158:811–832
82. Bohlmann J, Croteau R (1999) Diversity and variability of terpenoid defences in conifers: molecular genetics, biochemistry and evolution of the terpene synthase gene family in grand fir (*Abies grandis*). Novartis Found Symp 223:132–145
83. Ro D-K, Bohlmann J (2006) Diterpene resin acid biosynthesis in loblolly pine (*Pinus taeda*): functional characterization of abietadiene/levopimaradiene synthase (PtTPS-LAS) cDNA and subcellular targeting of PtTPS-LAS and abietadienol/abietadienal oxidase (PtAO, CYP720B1). Phytochemistry 67:1572–1578
84. Birol I, Raymond A, Jackman SD, Pleasance S, Coope R, Taylor GA, Yuen MM, Keeling CI, Brand D, Vandervalk BP, Kirk H, Pandoh P, Moore RA, Zhao Y, Mungall AJ, Jaquish B, Yanchuk A, Ritland C, Boyle B, Bousquet J, Ritland K, Mackay J, Bohlmann J, Jones SJ (2013) Assembling the 20 Gb white spruce (*Picea glauca*) genome from whole-genome shotgun sequencing data. Bioinformatics 29:1492–1497
85. Nystedt B, Street NR, Wetterbom A, Zuccolo A, Lin YC, Scofield DG, Vezzi F, Delhomme N, Giacomello S, Alexeyenko A, Vicedomini R, Sahlin K, Sherwood E, Elfstrand M, Gramzow L, Holmberg K, Hällman J, Keech O, Klasson L, Koriabine M, Kucukoglu M, Käller M, Luthman J, Lysholm F, Niittylä T, Olson A, Rilakovic N, Ritland C, Rosselló JA, Sena J, Svensson T, Talavera-López C, Theißen G, Tuominen H, Vanneste K, Wu ZQ, Zhang B, Zerbe P, Arvestad L, Bhalerao R, Bohlmann J, Bousquet J, Garcia Gil R, Hvidsten TR, de Jong P, MacKay J, Morgante M, Ritland K, Sundberg B, Thompson SL, Van de Peer Y, Andersson B, Nilsson O, Ingvarsson PK, Lundeberg J, Jansson S (2013) The Norway spruce genome sequence and conifer genome evolution. Nature 497:579–584
86. Neale DB, Wegrzyn JL, Stevens KA, Zimin AV, Puiu D, Crepeau MW, Cardeno C, Koriabine M, Holtz-Morris AE, Liechty JD, Martínez-García PJ, Vasquez-Gross HA, Lin BY, Zieve JJ, Dougherty WM, Fuentes-Soriano S, Wu LS, Gilbert D, Marçais G, Roberts M, Holt C, Yandell M, Davis JM, Smith KE, Dean JF, Lorenz WW, Whetten RW, Sederoff R, Wheeler

N, McGuire PE, Main D, Loopstra CA, Mockaitis K, Dejong PJ, Yorke JA, Salzberg SL, Langley CH (2014) Decoding the massive genome of loblolly pine using haploid DNA and novel assembly strategies. Genome Biol 15:R59
87. De La Torre AR, Birol I, Bousquet J, Ingvarsson PK, Jansson S, Jones SJ, Keeling CI, MacKay J, Nilsson O, Ritland K, Street N, Yanchuk A, Zerbe P, Bohlmann J (2014) Insights into conifer giga-genomes. Plant Physiol 166:1724–1732
88. Zimin A, Stevens KA, Crepeau MW, Holtz-Morris A, Koriabine M, Marçais G, Puiu D, Roberts M, Wegrzyn JL, de Jong PJ, Neale DB, Salzberg SL, Yorke JA, Langley CH (2014) Sequencing and assembly of the 22-gb loblolly pine genome. Genetics 196:875–890
89. Hillwig ML, Mann FM, Peters RJ (2011) Diterpenoid biopolymers: new directions for renewable materials engineering. Biopolymers 95:71–76
90. Leonard E, Ajikumar PK, Thayer K, Xiao WH, Mo JD, Tidor B, Stephanopoulos G, Prather KL (2010) Combining metabolic and protein engineering of a terpenoid biosynthetic pathway for overproduction and selectivity control. Proc Natl Acad Sci USA 107:13654–13659
91. Keasling JD (2012) Synthetic biology and the development of tools for metabolic engineering. Metab Eng 14:189–195
92. Buijs NA, Siewers V, Nielsen J (2013) Advanced biofuel production by the yeast *Saccharomyces cerevisiae*. Curr Opin Chem Biol 17:480–488
93. Kitaoka N, Lu X, Yang B, Peters RJ (2014) The application of synthetic biology to elucidation of plant mono-, sesqui- and diterpenoid metabolism. Mol Plant. doi:10.1093/mp/ssu104