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HARNESSING PLANT BIOMASS FOR BIOFUELS AND BIOMATERIALS

Terpenoid biomaterials

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Summary

Terpenoids (isoprenoids) encompass more than 40 000 structures and form the largest class of all known plant metabolites. Some terpenoids have well-characterized physiological functions that are common to most plant species. In addition, many of the structurally diverse plant terpenoids may function in taxonomically more discrete, specialized interactions with other organisms. Historically, specialized terpenoids, together with alkaloids and many of the phenolics, have been referred to as secondary metabolites. More recently, these compounds have become widely recognized, conceptually and/or empirically, for their essential ecological functions in plant biology. Owing to their diverse biological activities and their diverse physical and chemical properties, terpenoid plant chemicals have been exploited by humans as traditional biomaterials in the form of complex mixtures or in the form of more or less pure compounds since ancient times. Plant terpenoids are widely used as industrially relevant chemicals, including many pharmaceuticals, flavours, fragrances, pesticides and disinfectants, and as large-volume feedstocks for chemical industries. Recently, there has been a renaissance of awareness of plant terpenoids as a valuable biological resource for societies that will have to become less reliant on petrochemicals. Harnessing the powers of plant and microbial systems for production of economically valuable plant terpenoids requires interdisciplinary and often expensive research into their chemistry, biosynthesis and genomics, as well as metabolic and biochemical engineering. This paper provides an overview of the formation of hemi-, mono-, sesqui- and diterpenoids in plants, and highlights some well-established examples for these classes of terpenoids in the context of biomaterials and biofuels.

Keywords: terpenoid synthase, cytochrome P450, conifer diterpene resin acid, short-chain alkanes, biofuel production, poplar.

Introduction

Conservative estimates suggest that at least 40 000 different terpenoids (isoprenoids) exist in nature, many of which are of plant origin (Buckingham, 2004). Many terpenoids are essential for plant growth, development and general metabolism (Croteau *et al.*, 2000). These terpenoids are found in almost all plant species. Their physiological, metabolic and structural roles include, among others, those of light-harvesting pigments in photosynthesis or the regulatory activities of the many terpenoid plant hormones. In addition, a large number of structurally diverse plant terpenoids are known or assumed to have specialized functions associated with interactions of sessile plants with other organisms in the context of reproduction, defence or symbiosis (Gershenzon and Dudareva, 2007). These interactions involve specialized plant terpenoids, for example, in the form of attractants, repellents, anti-feedants, toxins or antibiotics. The terpenoid-mediated interactions of plants with other organisms involve species from all kingdoms and trophic levels. Some specialized terpenoids occur with distinct patterns of taxonomic distribution, whereby individual compounds or groups of related compounds are found only in a few plant species or families.

The chemical diversity of plant terpenoids is probably a reflection of their many biological activities in nature, which have made them a widely used resource for traditional and modern human exploitation, for example, as pharmaceuticals, flavours, fragrances, food supplements in the form of vitamins or sweeteners, or pesticides. Plant terpenoids also

serve as large-volume feedstocks for the production of a suite of industrial materials. Because of their many different structures, plant terpenoids as a group include compounds with many different physical and chemical properties. They may be lipophilic or hydrophilic, volatile or non-volatile, cyclic or acyclic, chiral or achiral. The chemical diversity of plant terpenoids originates from often complex terpenoid biosynthetic pathways.

Much research in the last two decades has concentrated on the molecular biochemistry and genomics of terpenoid biosynthesis, and, to some extent, on their biological functions in nature. There is also long-standing recognition that the diverse pathways for specialized plant terpenoids provide a resource for commercial production of high-value or large-volume chemicals. This resource can be utilized both in their naturally occurring or metabolically engineered forms in crop plants in agriculture, forestry or horticulture, as well as through their biochemical engineering into microbial fermentation systems. A broader awareness of the value of plant terpenoids has created an innovative climate for interdisciplinary research that includes chemistry, biology, chemical engineering and health research, and may lead to new means for the exploitation of terpenoids for human use. Research into plant terpenoid chemicals and terpenoid-producing plants may also provide new leads towards hydrocarbon biofuels, as a complement to the more advanced development of biodiesel or ethanol biofuels.

After a general overview of terpenoid biosynthesis in plants, this paper will focus on examples of a few hemi- (C_5), mono- (C_{10}), sesqui- (C_{15}) and diterpenoids (C_{20}) in the context of terpenoids as a biomaterials resource. Examples are selected to highlight recent research relevant to various aspects of traditional and modern human exploitation of plant terpenoids: (i) menthol (Figure 1), a monoterpenoid that is produced and harvested in large amounts from peppermint (*Mentha* × *piperita*) as an agricultural farm crop; (ii) artemisinin (Figure 1), an anti-malarial sesquiterpenoid pharmaceutical from annual wormwood (*Artemisia annua*) that is being explored for production in metabolically engineered microbial fermentation systems and transgenic plants; (iii) abietic acid and related diterpene resin acids (Figure 1) as a biological feedstock from conifers (Pinaceae) for a large chemical industry that relies to a substantial extent on century-old means of rosin collection; and (iv) Taxol (Figure 1), a high-value diterpenoid-derived anti-cancer drug of limited supply from its initial natural source, the bark of the Pacific yew tree (*Taxus brevifolia*). In addition, in the context of exploring the use of plants such as poplar trees (*Populus* spp.) as a source for cellulose-based biofuels (Doran-Peterson *et al.*, 2008; Li *et al.*, 2008; Pauly and Keegstra, 2008), this paper will briefly address the apparent loss of carbon from plants due to emission of volatile terpenoid hydrocarbons, using the hemiterpene isoprene (Figure 1) as an example. As terpenoids often occur in mixtures with other plant chemicals, the section on conifer

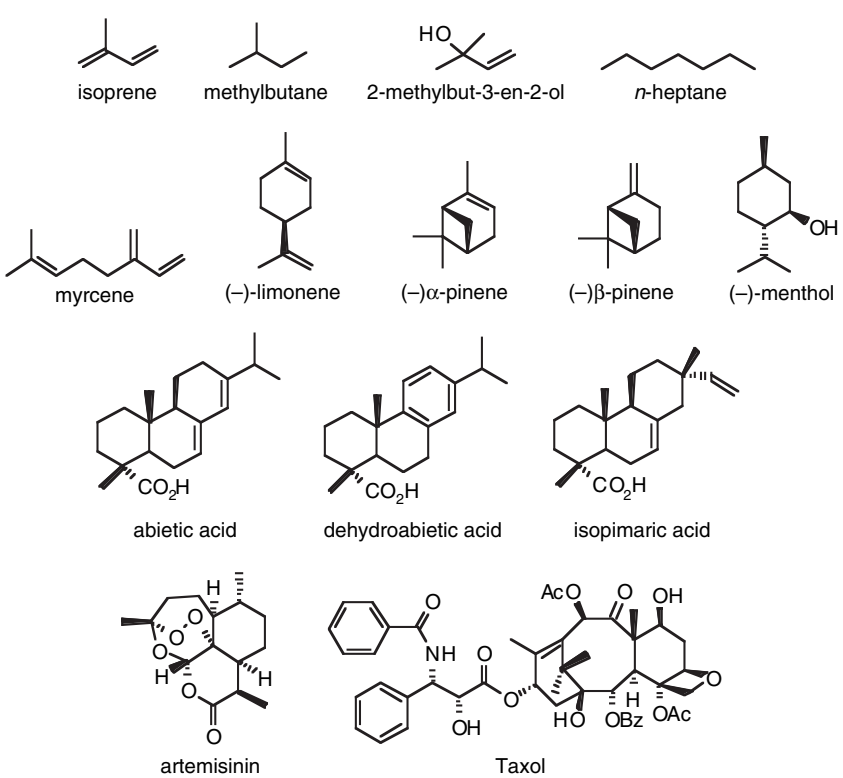

Figure 1. Chemical structures of the hemiterpenoids (C_5) isoprene and methylbutenol; the monoterpenes (C_{10}) myrcene, (–)-limonene, (–)-α-pinene, (–)-β-pinene and (–)-menthol; the sesquiterpenoid (C_{15}) artemisinin; the diterpene resin acids (C_{20}) abietic acid, dehydroabietic acid and isopimaric acid; the diterpenoid (C_{20}) Taxol; and the short-chain alkanes *n*-heptane and methylbutane.

diterpene resin acids also refers to the short-chain alkanes (e.g. *n*-heptane; Figure 1) that are present in some conifer oleoresin secretions. Other plant terpenoids used for plant-derived materials, such as tetraterpenoids (C_{40}) in the form of carotenoids (Tanaka *et al.*, 2008), flavour and aroma compounds derived from mono-, sesqui-, di- and tetraterpenoids (Schwab *et al.*, 2008), as well as the topic of natural rubber, a polyterpene (van Beilen and Poirier, 2008), are covered elsewhere in this issue. The present paper is based, in part, on a recent technical article on plant terpenoids in the *Wiley Encyclopaedia of Chemical Biology* (Keeling and Bohlmann, 2008) and on some excellent recent reviews on plant terpenoids, including reviews on menthol (Croteau *et al.*, 2005) and Taxol (Croteau *et al.*, 2006).

Overview of the biosynthesis of hemi-, mono-, sesqui and diterpenoids in plants

The diverse metabolic pathways of plant terpenoids are all rooted in the formation of only two isomeric five-carbon (C_5) precursors, dimethylallyl diphosphate (DMADP) and isopentenyl diphosphate (IDP) (Cane, 1999). DMADP and IDP are formed in the mevalonic acid (MEV) pathway and in the 2*C*-methyl-D-erythritol-4-phosphate (MEP) pathway (Lange *et al.*, 2000a; Lichtenthaler, 1999; Figure 2). The smallest plant terpenoids, the hemiterpenoids (C_5), can be formed directly from DMADP by terpenoid synthase (TPS) activity (Miller *et al.*, 2001). Alternatively, assembly of two, three or four C_5 units by prenyl transferases (PT) yields geranyl diphosphate (GDP; C_{10}), farnesyl diphosphate (FDP; C_{15}) and geranylgeranyl diphosphate (GGDP; C_{20}) (Takahashi and Koyama, 2006). PT enzymes exist in plants as both homomeric or modular heteromeric enzymes. GDP, FDP and GGDP are the substrates for families of TPS enzymes (Bohlmann *et al.*, 1998; Christianson, 2006; Tholl, 2006; Wise and Croteau, 1999), and serve as the immediate precursors for the diverse groups of all monoterpenoids (C_{10}), sesquiterpenoids (C_{15}) and diterpenoids (C_{20}), respectively. In addition, pairwise condensation of FDP and GGDP gives rise to the classes of triterpenoids (C_{30}) and tetraterpenoids (C_{40}), respectively, and assembly of an undefined number of C_5 precursors yields polyterpenoids. In addition to the regular terpenoids ($C_{n\times5}$), a large number of irregular terpenoids and terpenoid derivatives (e.g. homoterpenes) as well as terpenoid conjugates (e.g. monoterpene indole alkaloids; Facchini and DeLuca, 2008) are formed in plants.

Following formation of the many basic structures of hemi-, mono-, sesqui- and diterpenes in the form of olefins or simple oxygenated terpenoids by TPS, these metabolites can be further functionalized by various cytochrome P450-dependent mono-oxygenases (P450), reductases, dehydrogenases or various classes of transferases. In general, the diversity of thousands of plant terpenoid structures originates from many pathway combinations of TPS and terpenoid-modifying enzymes. TPS and terpenoid-modifying P450 enzymes exist as large and diverse gene families in plants, and the same may be true for other terpenoid-modifying enzymes. As there are only a few biologically relevant isoprenyl diphosphate substrates for TPS, basic characterization of these enzymes is relatively straightforward. In contrast, terpenoid-modifying enzymes, including the P450s (e.g. Kaspera and Croteau, 2006; Mau and Croteau,

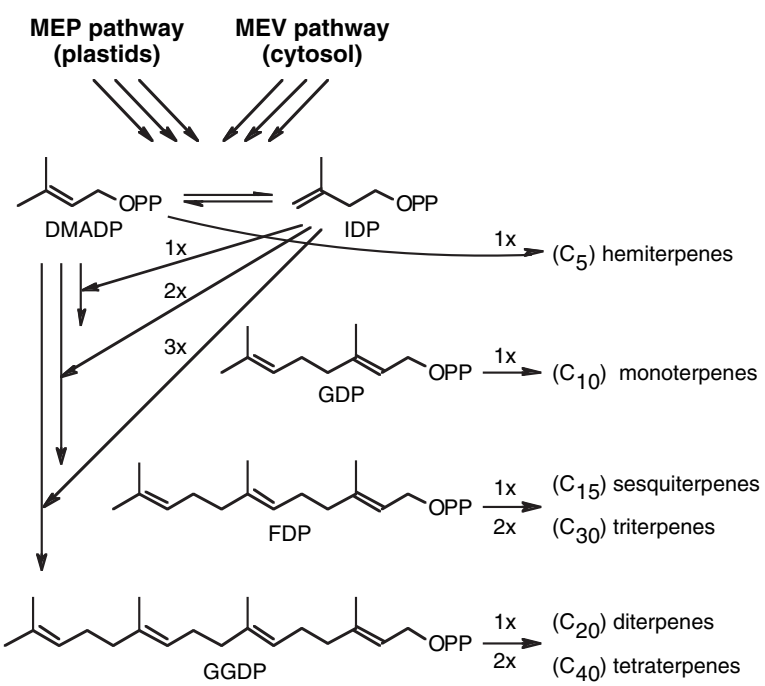

Figure 2. General scheme of plant terpenoid biosynthesis.
DMADP, dimethylallyl diphosphate; FDP, farnesyl diphosphate; GDP, geranyl diphosphate; GGDP, geranylgeranyl diphosphate; IDP, isopentenyl diphosphate; MEP, methylerythritol phosphate; MEV, mevalonate.

2006), are generally more difficult to study, because their substrates cannot be predicted as easily and often are not commercially available.

Mechanistically, TPS ionizes the diphosphate group of the isoprenyl diphosphate substrates, or in some cases protonates GGDP, yielding highly reactive enzyme-bound carbocation intermediates (Christianson, 2006; Starks *et al.*, 1997; Wise and Croteau, 1999). By transient stabilization of these carbocations, TPS allows enzyme-specific isomerizations, various rearrangements, cyclizations, and eventually proton elimination or water termination to yield the many cyclic and acyclic terpenoid carbon skeletons found in plants. TPS and other enzymes in terpenoid biosynthesis also direct stereochemistry, and thus are critical for stereospecific biological activities of terpenoids in other organisms. Many plant TPSs are promiscuous, forming multiple products from a single substrate (e.g. Keeling *et al.*, 2008; Martin *et al.*, 2004; Steele *et al.*, 1998; Tholl *et al.*, 2005). Their general ability to form multiple products, together with identification of specific plasticity residues in the TPS active sites, allows targeted manipulation and the directed evolution of TPS catalysts with new product specificities (Greenhagen *et al.*, 2006; Keeling *et al.*, 2008; Xu *et al.*, 2007; Yoshikuni *et al.*, 2006).

The formation of plant terpenoids involves several subcellular compartments (Croteau *et al.*, 2000), and consequently requires intra- and possibly intercellular transport of intermediates. The early steps of terpenoid biosynthesis of the MEV and MEP pathways occur in the cytosol/endoplasmic reticulum and plastids, respectively. PT and TPS enzymes of the central terpenoid pathway are also found in the cytosol and in plastids. In general, hemi-, mono- and diterpenoids are preferentially formed in plastids using precursors from the MEP pathway, while sesquiterpenoids are preferentially formed in the cytosol using precursors from the MEV pathway. P450 enzymes involved in the modification of mono-, sesqui and diterpenoids are associated with the endoplasmic reticulum. Knowledge of the cellular and subcellular localization of all of the enzymes of a specific terpenoid pathway is important to direct efforts in pathway engineering and to strategically redirect metabolic flux (e.g. Wu *et al.*, 2006).

Many plants accumulate mono-, sesqui- or diterpenoids in quantities that exceed by far the storage capacities of the living cells that produce these compounds. These plants typically have specialized anatomical structures such as oil glands, glandular trichomes, oil or resin cells, resin blisters or resin ducts for terpenoid sequestration (Fahn, 1979). The specialized anatomical structures provide extracellular storage capacities for lipophilic terpenoids and other essential oil compounds that may otherwise interfere with the general metabolism of the terpenoid-producing cells, possibly by disturbing membrane structures or displacement of other essential lipophilic compounds. The high density of specialized anatomical structures for terpenoid accumulation is an important agronomic trait for essential oil production. These structures provide easy access to specialized biochemical cell factories for research into terpenoid metabolism and essential oil biosynthesis in general (Gang *et al.*, 2001; Gershenzon *et al.*, 1992; Nagel *et al.*, 2008; Teoh *et al.*, 2006). Typically, the cells adjacent to terpenoid storage sites are thought, or have been shown (e.g. Turner and Croteau, 2004), to produce terpenoids for secretion into extracellular sites such as the sub-cuticular space of glandular trichomes or the lumen of resin ducts. As terpenoids accumulate with a steep gradient of concentration between the inside and the outside of the cell, some form of active and directional transport across the lipophilic cell membrane is required. However, the mechanisms of terpenoid transport from the biosynthetically active cells into the extracellular storage space are not known. Transport of terpenoids may involve activity of ATP-binding cassette (ABC) transporters (Jasiński *et al.*, 2001), although a role for such a transporter in specialized anatomical structures for terpenoid accumulation remains to be shown.

(–)-Menthol: a model for monoterpenoid essential oil research

Production of monoterpene-rich essential oils from species of *Mentha* and other species in the mint family (Lamiaceae) is an example of both traditional use of plant terpenoids and their modern production from agricultural crops as a biological resource for the pharmaceutical, chemical, food and flavour, and fragrance industries. The monoterpenoid (–)-menthol is the main and characteristic component of the essential oil of peppermint (Croteau *et al.*, 2005), and is valued for its distinct flavour and fragrance properties (Schwab *et al.*, 2008), as well as its anti-microbial properties and mild anaesthetic effects. It is produced commercially on a large scale from peppermint and other mint varieties. The biosynthesis of menthol and related monoterpenes has been studied for more than two decades by Croteau and co-workers at Washington State University (Pullman, WA), and this research serves as a model for biochemical and molecular genetic characterization of monoterpenoid essential oil biosynthesis and its manipulation in plants (Croteau *et al.*, 2005).

The formation of (–)-menthol in peppermint is localized on the surface of leaves in peltate glandular trichomes that allow secretion and accumulation of large amounts of lipophilic terpenoids. Access to the specialized cells of the glandular trichomes, which can be physically separated from other cell types (Gershenzon *et al.*, 1992), has greatly facilitated research on (–)-menthol biosynthesis, and monoterpene biosynthesis in general, at the biochemical, molec-

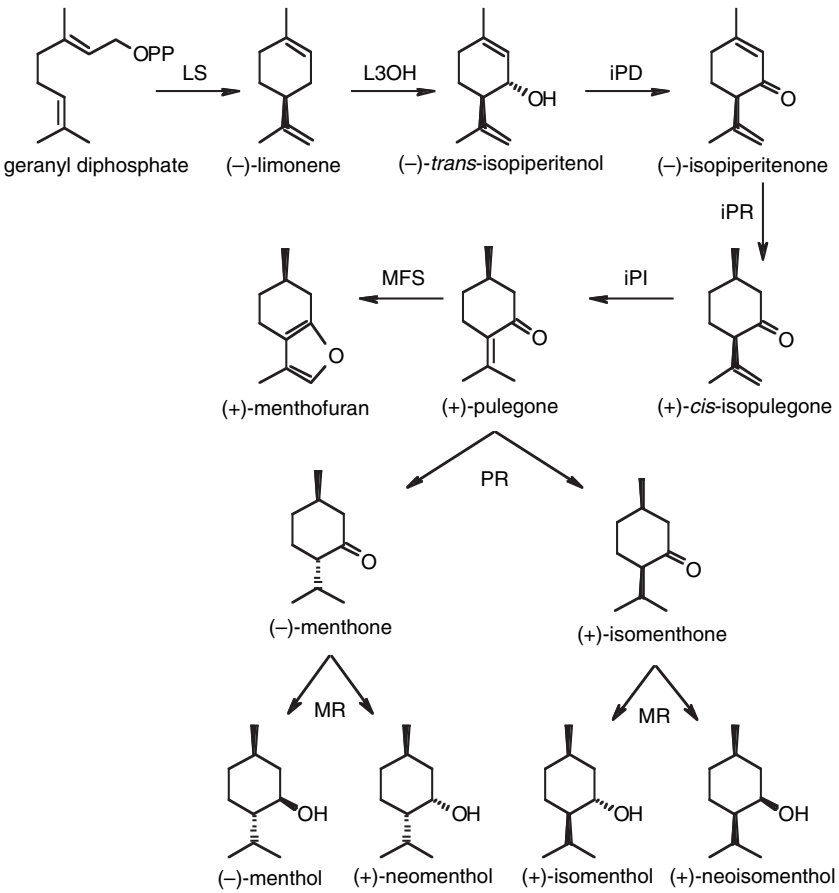

Figure 3. Biosynthesis of (–)-menthol and related monoterpenoids in *Mentha*.
LS, (–)-limonene synthase; L3OH, (–)-limonene-3-hydroxylase; iPD, (–)-*trans*-isopiperitenol dehydrogenase; iPR, (–)-isopiperitenone reductase; iPI, (+)-*cis*-isopulegone isomerase; PR, (+)-pulegone reductase; MR, (–)-menthone reductase; MFS, (+)-menthofuran synthase.

ular, cellular and genomic levels (Croteau *et al.*, 2005). The biochemistry of menthol biosynthesis has been elucidated by *in vivo* substrate feeding using isolated glandular trichomes, cell-free assays using native enzymes, detailed kinetic characterization of cloned and recombinantly expressed enzymes, and enzyme structure–function analyses (Croteau *et al.*, 2005). In brief, the biosynthesis of (–)-menthol (Figure 3) from GDP passes through a series of seven enzymatic reactions starting with formation of the cyclic monoterpene (–)-limonene, followed by a number of redox modifications. Limonene synthase is a typical multi-product plant monoterpene synthase that stereospecifically generates (–)-limonene together with minor amounts of acyclic myrcene and bicyclic (–)-α-pinene and (–)-β-pinene (Colby *et al.*, 1993; Hyatt *et al.*, 2007). Subsequent transformations of (–)-limonene to (–)-menthol involve hydroxylation to (–)-*trans*-isopiperitenol by the P450 limonene-3-hydroxylase, oxidation of (–)-*trans*-isopiperitenol to (–)-isopiperitenone by NAD-dependent isopiperitenol dehydrogenase, formation of (+)-*cis*-isopulegone by NADPH-dependent (–)-isopiperitenone reductase, isomerization of (+)-*cis*-isopulegone to (+)-pulegone by isopulegone isomerise, formation of (–)-menthone by NADPH-dependent (+)-pulegone reductase, and finally formation of (–)-menthol by (–)-menthone reductase. Other metabolites of the same pathway are (+)-menthofuran, (+)-neomenthol, (+)-isomenthol and (+)-neoisomenthol. (+)-Menthofuran is produced from (+)-pulegone by the P450 menthofuran synthase. (+)-Neomenthol is formed from (–)-menthone by an alternative (–)-menthone reductase. (+)-Isomenthone is formed from (+)-pulegone by (+)-pulegone reductase, and converted to (+)-isomenthol and (+)-neoisomenthol by (–)-menthone reductases.

The corresponding genes, in the form of cDNAs, for the complete pathway from GDP to (–)-menthol and its off-products, have been cloned and the corresponding enzymes functionally characterized (Croteau *et al.*, 2005). The current understanding of this pathway provides a starting point for quantitative and kinetic metabolic flux analyses of (–)-menthol biosynthesis (conceptually discussed by Lange, 2006). It has also become possible to strategically alter the monoterpene composition and quality of the essential oil of *Mentha* through metabolic engineering (Mahmoud and Croteau, 2001, 2003; Wildung and Croteau, 2005). By combining metabolic engineering of *Mentha* with existing large-scale agricultural production systems and processing plants, it should also be feasible to utilize the biochemical and agro-industrial production capacities of *Mentha* for future

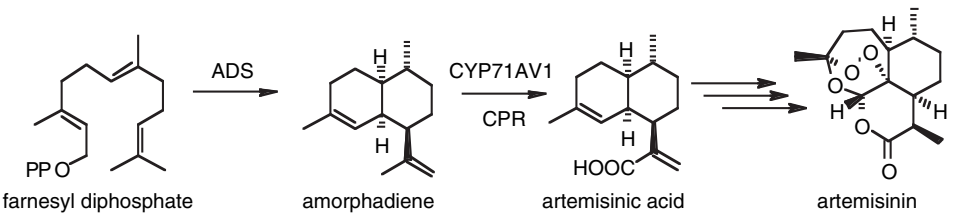

Figure 4. Pathway of artemisinin biosynthesis in *Artemisia annua*. ADS, amorphadiene synthase; CPR, cytochrome P450 reductase.

high-volume production of other valuable mono- or sesquiterpenoid compounds.

Artemisinin: molecular engineering of an anti-malarial sesquiterpenoid in E. coli, yeast and tobacco

The sesquiterpenoid artemisinin (Figure 1) is naturally produced in annual wormwood, which has been used for more than 2000 years in traditional Chinese medicine. Artemisinin, in the form of combination therapies, is today the only effective treatment for multi-drug-resistant strains of the malaria parasite *Plasmodium falciparum*. Thus this compound has retained its place as a terpenoid of wide-ranging pharmaceutical and socio-economic value. Exciting new studies are exploring the biosynthesis and metabolic engineering of artemisinin with the goal of developing cost-effective methods for stable production at large scale and with consistent quality. The biosynthesis of artemisinin (Figure 4) occurs in glandular trichomes on the surface of *Artemisia annua* leaves. It begins with the cyclization of FDP, catalysed by amorpha-4,11-diene synthase, a sesquiterpene synthase (Bertea *et al.*, 2005; Mercke *et al.*, 2000; Wallaart *et al.*, 2001). Subsequent three-step oxidation of amorphadiene to artemisinic acid is catalysed by a multi-functional cytochrome P450 (CYP71AV1; Ro *et al.*, 2006; Teoh *et al.*, 2006). The remaining reactions from artemisinic acid to artemisinin remain to be characterized, but are thought to include non-enzymatic photo-oxidation reactions (Wallaart *et al.*, 2001).

Recent work by Keasling and co-workers at the University of California (Berkeley, CA) on microbial production of artemisinin provides an impressive example of successful synergy between biochemistry, genomics and biochemical engineering of plant terpenoids (Chang *et al.*, 2007; Ro *et al.*, 2006; Shiba *et al.*, 2007). In brief, these authors used amorphadiene synthase, CYP71AV1 and P450 reductase from *Artemisia annua* in combination with introduction of a MEV pathway into *Escherichia coli*, or optimization of flux through the MEV pathway in *Saccharomyces cerevisiae*, for substantial production of artemisinic acid in these microbial hosts. Using a semi-synthetic route from artemisinic acid to artemisinin, their approach resulted in complete synthesis of artemisinin (Chang *et al.*, 2007; Ro *et al.*, 2006; Shiba *et al.*, 2007). In parallel with exploring microbial systems for the production of artemisinin, new and elegant approaches for plant metabolic engineering of amorphadiene and other plant terpenoids have been developed. Wu *et al.* (2006) employed transgenic co-expression of FDP synthase and amorphadiene synthase in plastids for successful, high-level synthesis of amorphadiene in transgenic tobacco (*Nicotiana tabacum*) plants. This approach was designed to avoid competition for a cytosolic pool of FDP by amorphadiene synthase and endogenous FDP-utilizing enzymes. The redirection of additional FDP biosynthesis into plastids, together with targeting of amorphadiene synthase to the same compartment, apparently provided a substantial substrate pool for this engineered pathway without compromising plant growth.

The example of artemisinin highlights recent progress in utilizing plant metabolic engineering as well as microbial biochemical engineering for production of a plant terpenoid at high yield. These studies may result in novel agricultural crops or in efficient microbial fermentations for plant terpenoid production. It is also important to note that the modern socio-economic value of artemisinin as a medicinal compound, and research towards cost-effective and large-scale biotechnological production, are largely founded on traditional knowledge regarding a terpenoid-producing medicinal plant.

Conifer oleoresin: trapping pests and tapping trees for biomaterials and biofuels

Conifer trees of the pine family (Pinaceae) include, among others, the economically important species of spruce (*Picea* spp.), pine (*Pinus* spp.) and true firs (*Abies* spp.). Conifers dominate much of the temperate and boreal forests around the world. Economically, and in the context of biomaterials, conifers are primarily recognized for their worldwide use in the solid wood and pulp-and-paper forest products industries. Wood pellets from forest waste products (e.g. timber destroyed by bark beetles and their associated fungi) can be used as a resource for energy production. Conifers are also a prominent biological system for large-volume production and storage of hydrocarbon chemicals. These hydrocarbons, in the form of oleoresin secretions, provide a

Figure 5. Collection of oleoresin secretions from conifer trees.
Oleoresin is stored under pressure in specialized anatomical structures (e.g. resin ducts) in conifer stems, where it is released to the surface upon wounding. The turpentine (mainly monoterpenes) and rosin (mainly diterpene resin acids) fractions of the oleoresin provide large-volume biological feedstocks for chemical industries. The photographs were taken by Ms Sarah Martz at Tangkubahan Parahu in central Java.

combustible fuel for conifer forest fires, but may also be utilized as a biological feedstock for chemical industries.

Members of the pine family produce large amounts of monoterpene hydrocarbons (Figure 1) together with abundant quantities of diterpene resin acids (Figure 1; Keeling and Bohlmann, 2006a,b; Langenheim, 2003). In addition, several pine species (e.g. Jeffrey pine, *Pinus jeffreyi*) also produce and accumulate substantial amounts of short-chain alkanes such as *n*-heptane (Figure 1; Savage *et al.*, 1996a,b). The monoterpenes, diterpene resin acids and short-chain alkanes are components of the sticky oleoresin secretion (Figure 5) that is formed as a constitutive and inducible defense of conifers against insect pests (e.g. bark beetles) and pathogens (Bohlmann, 2008; Keeling and Bohlmann, 2006a,b; Trapp and Croteau, 2001).

Except for observations from precursor feeding studies using a series of ^{14}C-labelled compounds (Sandermann *et al.*, 1960; Savage *et al.*, 1996a,b), little is known to date about the biosynthesis of short-chain alkanes in conifers, although it probably does not follow the same pathway as terpenoids. The conifer genes and enzymes for short-chain alkanes may provide catalysts for development of short-chain alkane biofuels, which, unlike hygroscopic ethanol, would be compatible with the existing petrochemical infrastructure. *In vivo* tissue feeding studies using $^{14}CO_2$, [^{14}C]sucrose, [^{14}C]pyruvate, [^{14}C]acetate, [^{14}C]mevalonate, [^{14}C]palmitate, [^{14}C]octanal and [^{14}C]1-octanol suggested that different metabolic precursors exist for the formation of short-chain alkanes and monoterpenes in Jeffrey pine (Savage *et al.*, 1996a,b). Together with inhibitor experiments and aldehyde trapping, these feeding studies suggested a pathway for *n*-heptane biosynthesis that involves *de novo* polymerization of acetate via fatty acid synthase-type elongation to produce an octanoyl thioester, followed by two-electron reduction to yield octanal, and subsequent decarbonylation or decarboxylation to *n*-heptane (Savage *et al.*, 1996a,b). More research into the biosynthesis and the genes and enzymes involved in the production of conifer short-chain alkanes is now warranted utilizing existing (e.g. http://www.treenomix.ca) and new conifer genomics resources.

In contrast to the biosynthesis of short-chain alkanes, the genomics, molecular biology and biochemistry of monoterpenes and diterpene resin acids in conifers have been studied in much detail (for recent reviews, see Keeling and Bohlmann, 2006a,b). Unlike the often functionalized monoterpenes in the Lamiaceae, such as (–)-menthol in peppermint (see above), most of the monoterpenes that accumulate in conifer oleoresin are the direct products of TPS and are not typically modified by other enzymes. The many conifer monoterpenes are formed from GDP by families of single- and multiple-product monoterpene synthases (Bohlmann *et al.*, 1997, 1999; Martin *et al.*, 2004; Phillips *et al.*, 2003). Biosynthesis of conifer diterpene resin acids involves two major steps (Keeling and Bohlmann, 2006b) after formation of GGDP: (i) conversion of GGDP to various, mostly tricyclic, diterpene olefin structures, catalysed by diterpene synthases, and (ii) three-step oxidation of the diterpene olefins at C18 to the corresponding diterpene resin acids (Figure 6). Conifer diterpene

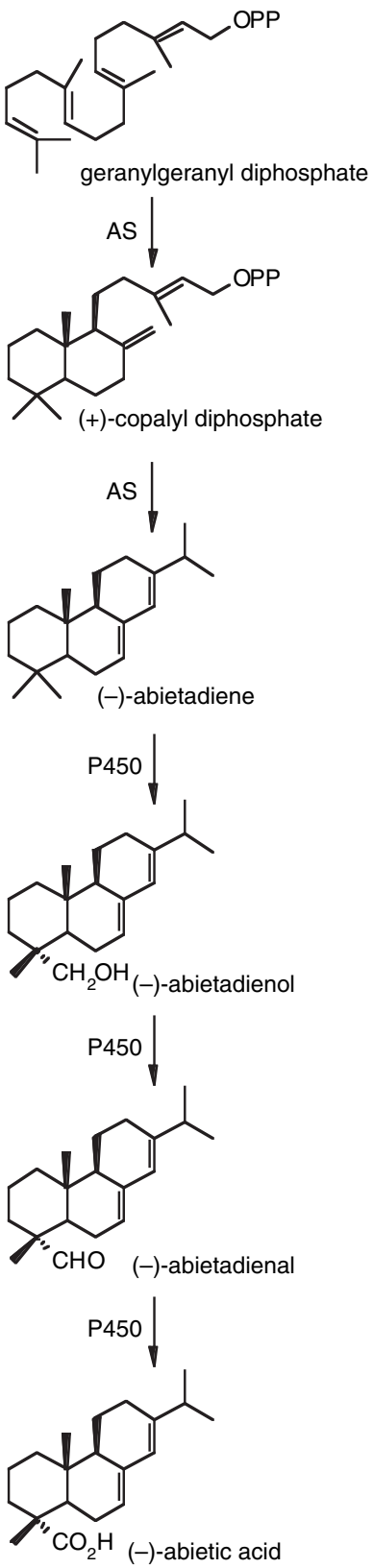

Figure 6. Pathway of diterpene resin acid biosynthesis in conifers.
AS, abietadiene synthase.

synthases are bifunctional enzymes that first cyclize GGDP to (+)-copalyl diphosphate and then cyclize this intermediate at a second active site to the various diterpene olefins (Keeling *et al.*, 2008; Martin *et al.*, 2004; Peters *et al.*, 2003; Stofer Vogel *et al.*, 1996). Diterpene olefins are oxidized to the corresponding resin acids by one or more multifunctional and multi-substrate P450 enzyme(s) (Ro *et al.*, 2005) of the conifer-specific CYP720B gene family (Hamberger and Bohlmann, 2006). Oxidation of diterpene olefins to diterpene resin acids resembles the formation of gibberellic acid by *ent*-kaurene oxidase (Helliwell *et al.*, 1999; Keeling and Bohlmann, 2006b). The conifer diterpene synthases are localized in plastids, and the P450 protein is associated with the endosplamic reticulum (Ro and Bohlmann, 2006).

Oleoresin mixtures of diterpene resin acids and monoterpenes accumulate abundantly in resin cells, resin blisters or reticulate resin duct systems that are part of the bark or wood of conifer stems, roots or needles (Banan, 1936; Fahn, 1979). Oleoresin terpenoids are thought to be produced in specialized epithelial secretory cells that surround the extracellular storage space of resin ducts or resin blisters (Keeling and Bohlmann, 2006a,b). Wounding of conifer stem tissues causes immediate flow of oleoresin to the wound surface where it can trap and kill pests and pathogens. Under natural conditions, the volatile components (turpentine) of oleoresin evaporate, leaving behind a solid structure of diterpene resin acids (rosin) that provides a lasting seal of the wound. Mechanical wounding, insect or pathogen attack, as well as treatment of conifer stems with ethylene or methyl jasmonate, induce *de novo* formation of resin-producing ducts in the stem cambium zone in lieu of differentiation of xylem tracheid cells (Franceschi *et al.*, 2005; Martin *et al.*, 2002), and these stress treatments thus increase the anatomical and biochemical capacities of conifers to produce oleoresin terpenoids (Martin *et al.*, 2002; Miller *et al.*, 2005).

Indigenous people from many parts of the world have developed similar methods for collection of conifer oleoresin secretions by tapping resin from incisions on conifer stems for subsequent separation of turpentine and rosin fractions (Figure 5; Langenheim, 2003). This labour-intensive, traditional process of rosin collection is still practised today in parts of Southeast Asia and China, providing a highly pure diterpene resin acid feedstock for chemical industries. For example, much of the worldwide production of news print ink relies on this traditional method of rosin collection, but this may not be sustainable due to increasing labour costs in the rosin-producing countries. In contrast, diterpene resin acids from more readily available, large-volume by-products (e.g. tall oil) from forest industries are often of insufficient purity for further use as a source for industrial biomaterials. To address this supply challenge,

alternative methods for production and harvesting of diterpene resin acids are needed, which may include the metabolic engineering of diterpene resin acid biosynthesis into faster-growing agricultural crops or improved chemical engineering to better utilize the by-products from wood pulping.

Conifer trees produce oleoresin terpenoids in massive amounts during their entire life, and, unlike agricultural crops, conifers grow in planted forests or plantations for dozens of years without the need for intensive fertilizer or pesticide applications. Thus, conifers provide an enormous biochemical and physical capacity for the sustainable production of terpenoids that has, as of yet, remained untapped for the metabolic engineering of other high-value terpenoids. Using existing conifer transformation platforms (Klimaszewska *et al.*, 2004), and following the lead of terpenoid engineering in other systems (see above), it is conceivable to metabolically engineer key steps for the biosynthesis of other terpenoids (e.g. enzymes specific for Taxol biosynthesis, see below) into the diterpenoid biochemical machinery of plantation conifers. A successful strategy for terpenoid metabolic engineering in conifers may require redirection of existing terpenoid pathways, use of promoters that drive cell-specific expression in secretory cells, and utilization of terpenoid transport mechanisms for extracellular accumulation in resin ducts.

Taxol: meeting demands for a potent diterpenoid anti-cancer drug

Paclitaxel (Figure 1), commonly known under the registered trademark Taxol (Bristol-Myers Squibb, New York), is a powerful diterpenoid anti-cancer drug with an annual market value of several billion dollars. Taxol was first isolated and identified from the bark of Pacific yew (Wani *et al.*, 1971), a slow-growing tree that is adapted to a forest shade environment and a limited area of natural distribution in the Pacific Northwest of North America. The amount of Taxol available from this natural source was not sufficient to provide a stable long-term supply in the face of growing clinical demands for this drug. Thus, the example of Taxol highlights the fact that the term 'natural product' is not necessarily synonymous with a 'sustainable' resource, unless detailed knowledge of biosynthesis can be harnessed for engineering of biotechnological or semi-synthetic production systems. Limited supply from the original source, together with a lack of cost-effective total synthesis (Xiao *et al.*, 2003), prompted much of the research of the last 15 years into the biochemistry and molecular biology of Taxol (reviewed by Croteau *et al.*, 2006). Knowledge of Taxol biosynthesis supported the successful development of alternative biological or semi-synthetic production systems that can use more readily available pathway intermediates from regenerating foliage

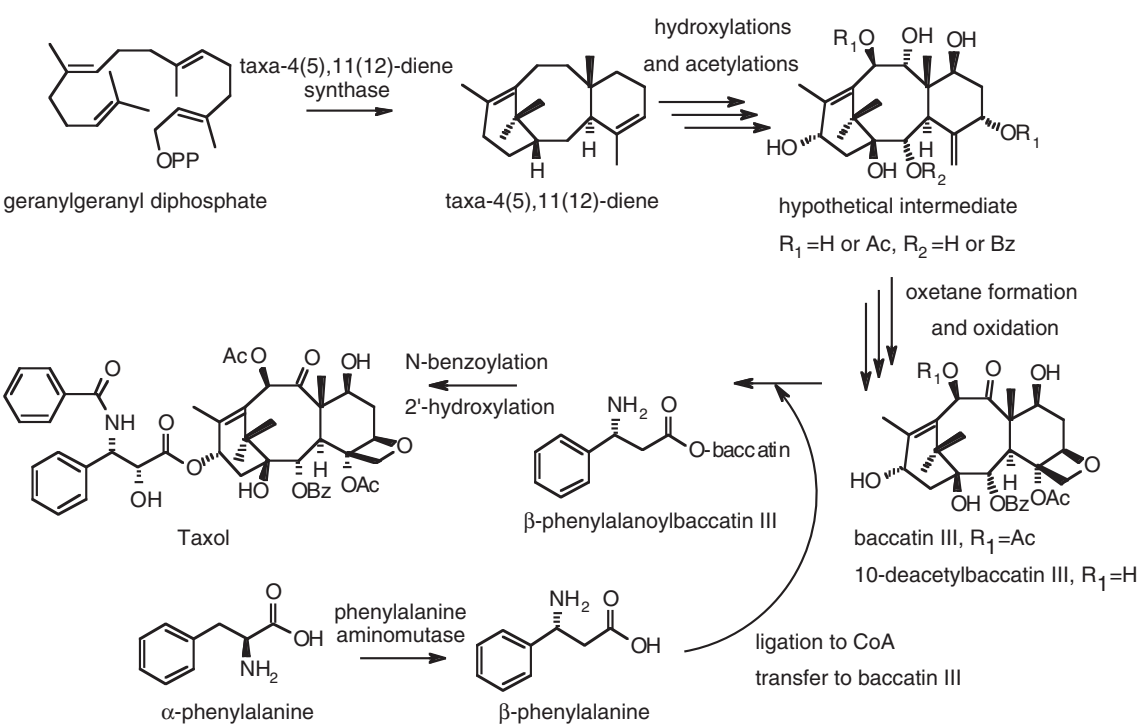

Figure 7. Pathway of Taxol biosynthesis in *Taxus*.

of faster-growing *Taxus* species or that can build on the methyl jasmonate-inducible biosynthetic machinery of *Taxus* cell cultures. These biological and semi-synthetic production systems can also overcome the challenges of chemical synthesis of Taxol associated with its specific stereochemistry at 11 chirality centres.

Much of the biosynthesis of Taxol (Figure 7) has been elegantly deciphered by Croteau and co-workers (reviewed in detail by Walker and Croteau, 2001; Jennewein and Croteau, 2001; Croteau *et al.*, 2006; Kaspera and Croteau, 2006). Their work serves as a fascinating example for rationalizing and empirically exploring a specific and complicated terpenoid biosynthetic pathway within a complex metabolic grid of more than 400 taxoid metabolites using chemical, biochemical, molecular and functional genomics approaches. Their approach included the synthesis of various putative precursors and intermediates and testing of these using cell-free assay systems and recombinant proteins, the cloning of genes in the form of cDNAs using homology-based approaches as well as random cDNA sequencing from inducible Taxol-producing cell culture systems, and detailed kinetic characterization of recombinant enzymes. The ability to induce the formation of Taxol and other taxoids in *Taxus* cell cultures has substantially aided the discovery of genes and enzymes involved in Taxol biosynthesis (Jennewein *et al.*, 2004; Ketchum *et al.*, 1999). In brief, the biosynthesis of Taxol involves formation of GGDP (Hefner *et al.*, 1998), followed by a series of 19 predicted pathway-specific enzymatic steps, many of which have been characterized with the corresponding cDNAs and recombinant proteins (Croteau *et al.*, 2006; Kaspera and Croteau, 2006; Walker and Croteau, 2001). Formation of the first pathway-specific intermediate is catalysed by a diterpene synthase yielding taxa-4(5),11(12)-diene (Wildung and Croteau, 1996). The tricyclic taxadiene hydrocarbon is hydroxylated by several P450 enzymes and further functionalized by a group of acyl and aroyl transferases to yield a putative intermediate with seven alcohol or ester groups, followed by oxidation to introduce a ketone function and formation of the characteristic oxetane ring of baccatin III (Croteau *et al.*, 2006; Kaspera and Croteau, 2006; Walker and Croteau, 2001). Phenylalanine aminomutase, a putative CoA ligase, baccatin III C13-phenylpropanoyl CoA transferase and an *N*-benzoyl transferase are involved in formation of the aromatic side chain of Taxol (Croteau *et al.*, 2006; Walker and Croteau, 2001).

A substantial challenge in discovery of the biosynthesis of Taxol and the development of efficient production systems was the fact that Taxol is only one of hundreds of closely related metabolites all derived from the same complex metabolic system active in *Taxus* trees. Knowledge of the pathways leading not only to Taxol but also to other Taxol-like compounds can now be used to explore redirection of metabolic flux towards Taxol and away from less desirable metabolites using the emerging genetic engineering of *Taxus* cell cultures (Ketchum *et al.*, 2007).

Isoprene: terpenoid hydrocarbons blown into the wind

In the Northern hemisphere, plantation forests of fast-growing poplars, cottonwoods or aspens are one of several possible sources for cellulose for the production of bio-ethanol. Poplars are also an established renewable resource of cellulose fibre for the pulp-and-paper industry. When considering poplars as a source for ethanol production, or for high-yield plantation forestry in general, it is important to note that poplars emit large amounts of volatile hydrocarbons into the atmosphere in the form of the hemiterpene isoprene (2-methyl-1,3-butadiene; Figure 1). The formation of isoprene in poplars (and other plants) is relevant in the context of research into biomaterials and biofuels for two reasons: (i) isoprene is a versatile starter molecule for chemical syntheses, and (ii) emission of isoprene creates a substantial loss of carbon from biomass-producing plants.

Isoprene is the simplest terpenoid found in plants. Its formation only requires DMADP and isoprene synthase, which catalyses ionization of DMADP followed by deprotonation (Miller *et al.*, 2001), a reaction mechanism reminiscent of the TPS-catalysed formation of other acyclic terpenes such as the monoterpene myrcene or the sesquiterpene farnesene. Genes encoding plant isoprene synthase have been cloned as cDNAs and characterized in poplar (Miller *et al.*, 2001) and kudzu (*Pueraria montana*; Sharkey *et al.*, 2005). Using the plant isoprene synthase gene, and building on recent advances in metabolic engineering as demonstrated by mono-, sesqui- and diterpenoid formation in *E. coli* and yeast (Chang *et al.*, 2007; Martin *et al.*, 2003; Reiling *et al.*, 2004; Shiba *et al.*, 2007), biochemical engineering of hemiterpenoids should also be possible. In addition to isoprene, the hemiterpenol 2-methyl-3-buten-2-ol (Figure 1), which is naturally produced and emitted by some conifers (Rosenstiel *et al.*, 2002), and the reduced forms of these hemiterpenoids (e.g. 2-methylbutane and 2-methylbutan-2-ol, respectively) could provide targets for biochemical engineering of a biofuel resource.

Isoprene as a major emission from poplars also poses a very different challenge for biologists in the context of utilization of poplars for plantation forestry. Isoprene is the most abundant biogenic volatile organic compound emitted from plants, with annual rates of emission of more than 1×10^{12} kg (Guenther *et al.*, 2006), and it is highly reactive. Despite the importance of isoprene in the context of global carbon cycles, the physiological roles of isoprene in plants are not entirely clear (Sharkey and Yeh, 2001). It is therefore uncertain whether emission of isoprene from poplars can be reduced without affecting essential physiological processes, and whether carbon flux into isoprene can be redirected for

increased carbon sequestration and biomass production. Two of the main functions of isoprene are thought to be protection of plant tissues from thermal and oxidative stress (Behnke *et al.*, 2007; Loivamäki *et al.*, 2007; Sharkey *et al.*, 2005). Although a function of isoprene in thermotolerance has been established through the use transgenic poplar plants with down-regulated transcript levels of isoprene synthase (Behnke *et al.*, 2007), the long-term effect of down-regulation of isoprene synthase and reduced emission of isoprene on perennial growth and yield remains to be tested on field plantations under various abiotic conditions.

Similar to the situation with poplars, other forest tree species such as *Eucalyptus* spp. and conifers, which are widely used for production of biomass, traditional forest products and industrially valuable terpenoids, also emit large amounts of terpenoid volatiles, mostly in the form of monoterpenes, together with sesquiterpenes and hemiterpenes. Due to their large quantities, these emissions are often visible as a blue haze over large forest areas (hence the name Blue Mountains). More research is needed to understand the biological role(s) of these emissions in poplar, eucalypts and conifers, and to address whether reducing emission of these volatile hydrocarbons could drive an increase in carbon fixation and biomass production in plantation forests.

Conclusions: an emerging genomics perspective on plant terpenoids

The great chemical diversity of plant terpenoids has been utilized by humans since ancient times, and they remain one of the most important classes of plant-derived, biologically active chemicals and industrial materials, and could potentially become a source for the development of new biofuels. The chemical diversity of plant terpenoids, illustrated here with only a very few examples, reflects the complexity and diversity of the pathways that biosynthesize them. Current knowledge of plant terpenoids is fundamentally based on research in specialized plant chemistry and biochemistry. Powerful genomic approaches have also advanced the discovery of genes and enzymes for biosynthesis of plant terpenoids. The recent sequencing of the genomes of a few plant species, together with large collections of expressed sequence tags and full-length cDNAs from many other plants, are continuously yielding new candidate genes for terpenoid biosynthesis (e.g. Aubourg *et al.*, 2002; Jennewein *et al.*, 2004; Lange and Ghassemian, 2003; Lange *et al.*, 2000b; Peters, 2006). Given the rapid evolution of TPS and P450-encoding genes and possibly other genes involved in specialized terpenoid biosynthesis, straightforward gene discovery approaches in hitherto unsequenced plant species are guaranteed to yield new catalysts for terpenoid biosynthesis, but almost all of these genes will require biochemical characterization for functional annotation. The number of terpenoid-forming genes in the few plant species for which complete genome sequences are now available also suggests a much wider range of chemical diversity and distribution of terpenoids than previously anticipated. For example, there are at least 32 putatively functional TPS genes in *Arabidopsis thaliana* (Aubourg *et al.*, 2002), at least 15 in rice (*Oryza sativa*; Goff *et al.*, 2002; Peters, 2006), at least 47 in poplar (*Populus trichocarpa*; Tuskan *et al.*, 2006), and at least 89 in a highly inbred grapevine (*Vitis vinifera* Pinot Noir; Jaillon *et al.*, 2007) and other grapevine varieties (Lücker *et al.*, 2004; Martin and Bohlmann, 2004). The large majority of these genes have not yet been characterized for their biochemical functions. Given that most TPS form multiple products from a single substrate, and given that these products are often modified by the action of additional enzymes such as P450 mono-oxygenases, the number of terpenoids found in any given plant species is likely to exceed the number of TPS genes present. Comparative and functional genomics studies, in particular of the large gene family of TPS, which is key in generating the structural diversity of plant terpenoids, have also provided new insights into evolutionary events of repeated gene duplication and subsequent neo-functionalization, as well as the role of allelic variations for new terpenoid biosyntheses (e.g. Keeling *et al.*, 2008; Köllner *et al.*, 2004; Martin *et al.*, 2004; Xu *et al.*, 2007).

In conclusion, the combination of chemistry, biochemistry (specifically of metabolic pathway enzymes) and genomics provides a very powerful approach for discovery of complete sets of genes and enzymes of terpenoid biosynthetic pathways. In addition, understanding the biosynthesis of specialized plant terpenoids is critically important to fully capture their economic value via plant metabolic engineering and biochemical engineering of microbial systems. A significant benefit of exploring plant terpenoids as a renewable resource is that societies could become less reliant on petrochemicals for the production of specialized chemicals, chemical feedstocks and possibly transportation fuels than the present and previous generations. The selected studies on terpenoid products highlighted in this paper are valuable examples of a much-needed new funding environment that permits the often expensive but innovative multidisciplinary research required to harness the powers of plant and microbial systems for production of economically valuable plant terpenoid compounds.

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