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Volatile science? Metabolic engineering of terpenoids in plants

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Terpenoids are important for plant survival and also possess biological properties that are beneficial to humans. Here, we describe the state of the art in terpenoid metabolic engineering, showing that significant progress has been made over the past few years. Subcellular targeting of enzymes has demonstrated that terpenoid precursors in subcellular compartments are not as strictly separated as previously thought and that multistep pathway engineering is feasible, even across cell compartments. These engineered plants show that insect behavior is influenced by terpenoids. In the future, we expect rapid progress in the engineering of terpenoid production in plants. In addition to commercial applications, such transgenic plants should increase our understanding of the biological relevance of these volatile secondary metabolites.

Importance of terpenoids

Isoprenoids, also known as terpenoids, are the largest family of natural compounds, consisting of >40 000 different molecules. The isoprenoid biosynthetic pathway generates both primary and secondary metabolites that are of great importance to plant growth and survival. Among the primary metabolites produced by this pathway are: the phytohormones gibberellic acid (GAs), abscisic acid (ABA) and cytokinins; the carotenoids, chlorophylls and plastoquinones involved in photosynthesis; the ubiquinones required for respiration; and the sterols that influence membrane structure. Monoterpenoids (C_{10}), sesquiterpenoids (C_{15}), diterpenoids (C_{20}) and triterpenoids (C_{30}) are considered to be secondary metabolites (Figure 1). Many of the terpenoids are commercially interesting because of their use as flavors and fragrances in foods and cosmetics (e.g. menthol, nootkatone and sclareol) or because they are important for the quality of agricultural products, such as the flavor of fruits and the fragrance of flowers (e.g. linalool) (Figure 1) [1,2]. In addition, terpenoids can have medicinal properties such as anti-carcinogenic (e.g. Taxol and perilla alcohol), antimalarial (e.g. artemisinin), anti-ulcer, hepaticidal, antimicrobial or diuretic (e.g. glycyrrhizin) activity (Figure 1) [3–7]. The terpenoids have also been shown to be of ecological significance [8,9]. Compounds such as the bitter triterpenoid cucurbitacins and the pungent diterpenoid polygodial have been shown to be involved in insect resistance (Figure 1) [10,11]. Other terpenoid compounds are involved in interactions between plants, between plants and microorganisms, and between plants and arthropod herbivores [e.g. (*E*,*E*)-α-farnesene, which is induced in cucumber by spider mite feeding] (Figure 1) [12–14].

The commercial and ecological importance of terpenoids makes their metabolic engineering an attractive subject for investigation [15]. On the one hand, engineering could lead to the improvement of many input and output traits in crops. These include disease and pest resistance, weed control (e.g. by producing allelopathic compounds), improved fragrance of ornamentals and pollination of seed crops (both by altering floral scent), enhanced aroma of fruits and vegetables, and the production of pharmaceuticals in plants. On the other hand, transgenic plants with modified terpenoid production could make an important contribution to fundamental studies of the biosynthesis and regulation of these compounds and their importance in ecological relationships. In this article, we describe the latest exciting developments in the engineering of terpenoids in plants. We limit ourselves to the secondary metabolite class of terpenoids as defined above and depicted in Figure 1.

Biosynthesis of terpenoids in plants

Terpenoids are derived from the mevalonate pathway, which is active in the cytosol, or from the plastidial 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway (Figure 2) [16,17]. Both pathways lead to the formation of the C_5 units isopentenyl diphosphate (IDP) and its allylic isomer dimethylallyl diphosphate (DMADP), the basic terpenoid biosynthesis building blocks. In both compartments, IDP and DMADP are used by prenyl transferases in condensation reactions to produce larger prenyl diphosphates, such as the monoterpene precursor geranyl diphosphate (GDP), the sesquiterpene precursor farnesyl diphosphate (FDP) and the diterpene and C_{40} carotenoid precursor geranylgeranyl diphosphate (GGDP) (Figure 2). Condensation of two units of FDP produces squalene, the precursor of triterpenes and sterols. Although there is increasing evidence that there is exchange of intermediates between these compartments [6,18–24], the cytoplasmic mevalonate pathway is generally considered to supply the precursors for the production of sesquiterpenes and triterpenes (including sterols)

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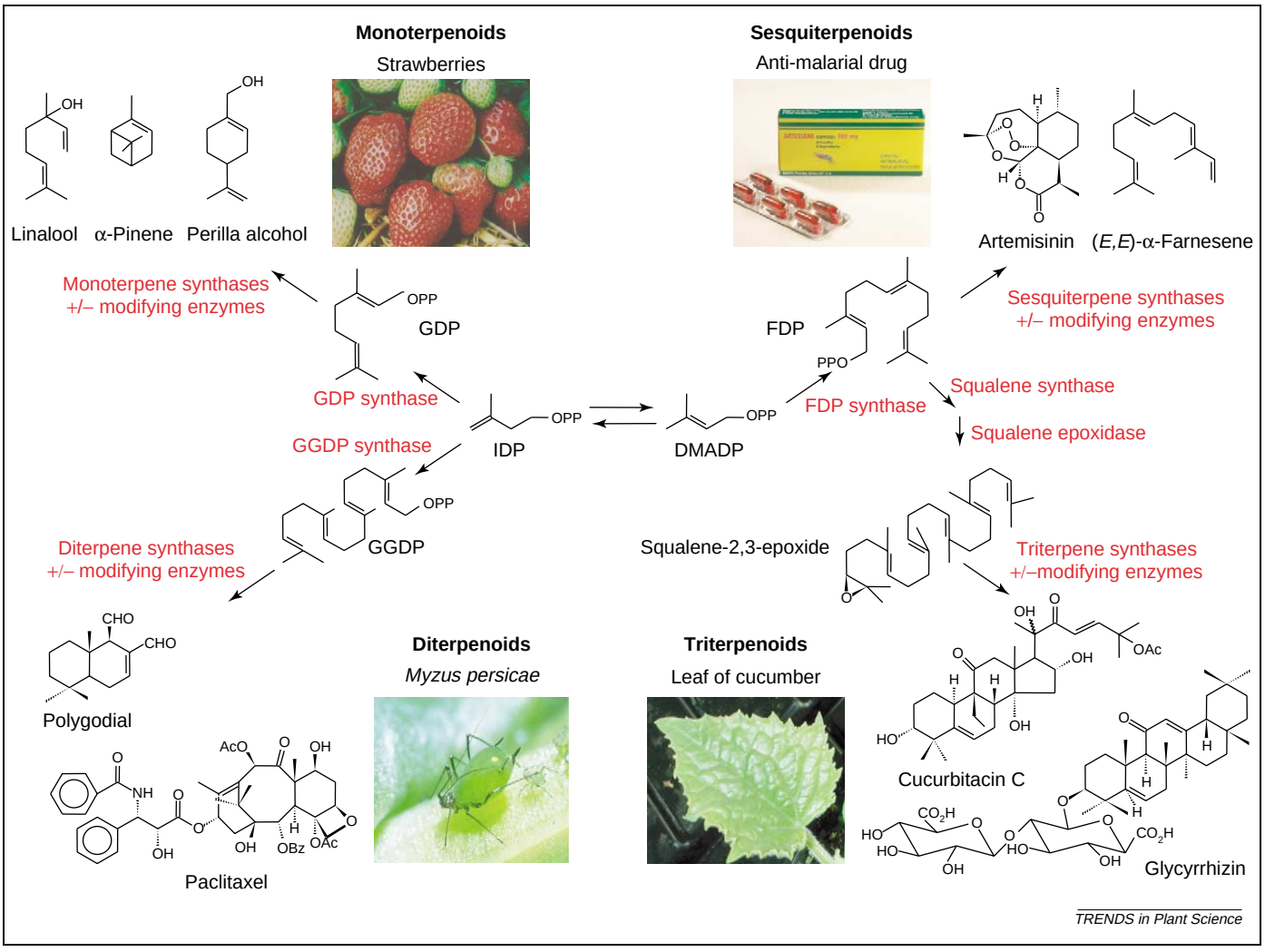

Figure 1. Schematic overview of the biosynthesis of the monoterpenoids, sesquiterpenoids, diterpenoids and triterpenoids. For some representatives of these classes, structures and pictures related to their biological importance are shown. Enzymatic steps are indicated in red. Abbreviations: DMADP, dimethylallyl diphosphate; GDP, geranyl diphosphate; GGDP, geranylgeranyl diphosphate; FDP, farnesyl diphosphate; IDP, isopentenyl diphosphate.

and to provide precursors for protein prenylation and for ubiquinone and heme-A production in mitochondria. In the plastids, the MEP pathway supplies the precursors for the production of isoprene, monoterpenes, diterpenes (e.g. GAs) and tetraterpenes (e.g. carotenoids) (Figure 2).

Following the formation of the acyclic precursors GDP, FDP and GGDP, terpenoid scaffolds are generated through the action of terpene synthases (TPSs). Primary terpene skeletons formed by TPSs can be further modified by the action of various other enzyme classes, such as the cytochrome P450 hydroxylases (EC 1.14.14.1), dehydrogenases (EC 1.1; alcohol and aldehyde oxidoreductases), reductases, glycosyl transferases (EC 2.4) and methyl transferases (EC 2.1.1) [25].

Metabolic engineering of terpenoids

Engineering precursor availability

The availability of precursors is an important issue in metabolic engineering. Whether the concentration of a certain isoprenoid precursor is limiting for the production of terpenoids probably depends on the plant species, the tissue and the physiological state of the plant. The initial step towards the biosynthesis of IDP through the MEP pathway is catalyzed by 1-deoxy-D-xylulose-5-phosphate (DXP) synthase (DXS). Overexpression and downregulation of *DXS* in *Arabidopsis* affected the levels of various isoprenoids including chlorophylls, tocopherols, carotenoids, ABA and GAs [26]. Upregulation of DXP reductoisomerase (DXR), which converts DXP to methylerythritol phosphate (Figure 2), in transgenic peppermint (*Mentha* × *piperita*) plants resulted in a 50% increase in essential oil yield [27]. As well as DXS and DXR, hydroxymethylbutenyl diphosphate reductase (HDR), the enzyme conducting the last step in the pathway generating IDP and DMADP, has also been suggested to be a rate-limiting step in the MEP pathway in both tomato (*Lycopersicon esculentum*) and *Arabidopsis* (Figure 2) [28]. When plants expressing taxadiene synthase (TXS) (see below under 'Engineering diterpenoids and triterpenoids') under the cauliflower mosaic virus (CaMV) 35S promoter [29] were crossed with plants overexpressing *DXS* or *HDR*, increases of 6.5 times and

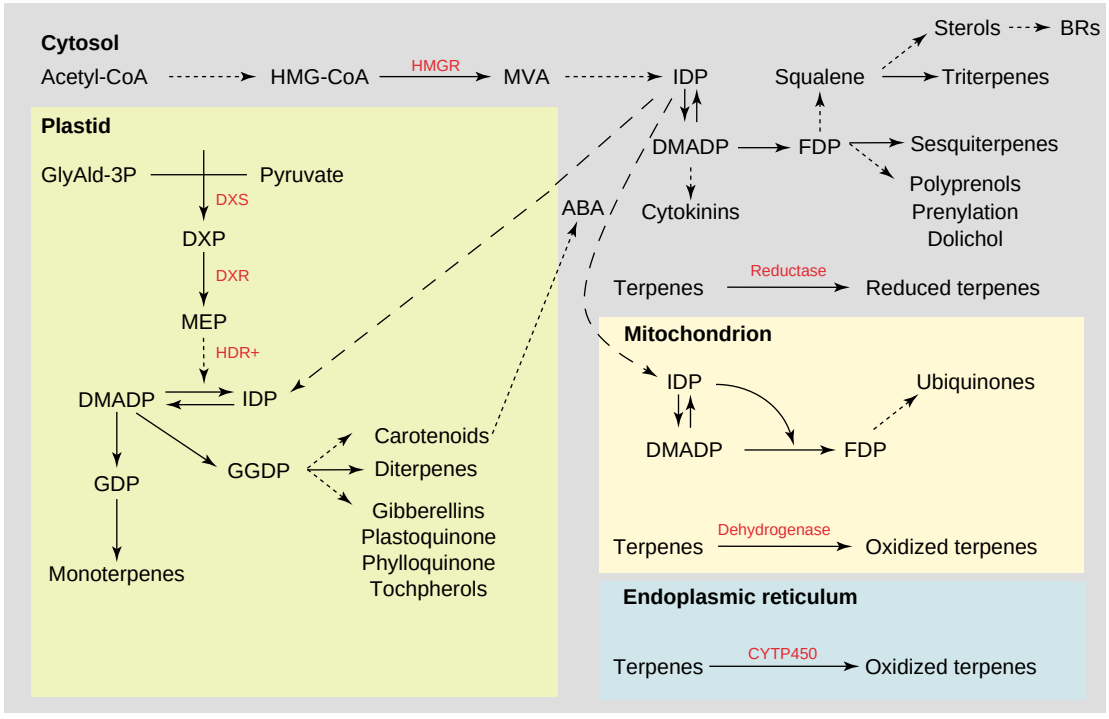

Figure 2. The isoprenoid pathway in plants. Solid arrows, broken arrows with short dashes and broken arrows with long dashes represent single and multiple enzymatic steps, and transport, respectively. Selected enzymes are depicted in red. Abbreviations: ABA, abscisic acid; BRs, brassinosteroids; CYTP450, cytochrome P450 hydroxylases; DMADP, dimethylallyl diphosphate; DXP, deoxyxylulose-5-phosphate; DXR, DXP reductoisomerase; DXS, DXP synthase; FDP, farnesyl diphosphate; GDP, geranyl diphosphate; GGDP, geranylgeranyl diphosphate; GlyAld-3P, glyceraldehyde 3-phosphate; HDR+, hydroxymethylbutenyl diphosphate reductase plus several other enzymes that are required to generate DMADP and IDP from MEP); HMG-CoA, hydroxymethylglutaryl CoA; HMGR, HMG-CoA reductase; IDP, isopentenyl diphosphate; MEP, methylerythritol 4-phosphate; MVA, mevalonic acid. 'Terpenes' include terpenes from all classes and originating from the various organelles.

13 times, respectively, were detected in the accumulation of taxadiene compared with plants overexpressing only *TXS* [28].

The results of many of the studies reported to date suggest that, in general, the direct precursor for monoterpene biosynthesis (i.e. GDP) is largely available to introduced monoterpene synthases and, in some cases, does not seem to be limiting. For example, Joost Lücker *et al.* [30] reported that, in the flowers of transgenic tobacco (*Nicotiana tabacum*) plants expressing three different monoterpene synthases, the levels of products corresponding to the three enzymes were high but did not affect the level of the endogenous linalool production.

Enhancing the precursor supply for the biosynthesis of isoprenoids in the mevalonate pathway has been attempted mainly by altering expression levels of the gene encoding 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), which is considered to be a rate-limiting step in the pathway (Figure 2) [31–33]. Constitutive overexpression of *HMGR* in tobacco resulted in a three- to tenfold increase in total sterol levels [31]. Levels of other isoprenoids, including sesquiterpenes, were not altered, possibly because of compartmentation, channeling or the presence of other rate-limiting steps. *Arabidopsis* plants overproducing FDP synthase did not have enhanced levels of sterols [34]. Levels of sesquiterpenes were not examined in these transgenic plants. In an earlier study, overproduction of FDP synthase in *Artemisia annua* resulted in two- to threefold increases in levels of the sesquiterpene artemisinin in transgenic plants compared with those in wild-type plants [35].

Engineering monoterpenoids

The first reports of the feasibility of altering the monoterpene profile of plants focused on mint species (*Mentha* spp.) and petunia (*Petunia hybrida*) (Table 1). Early work with the aim of overexpressing the gene from spearmint (*Mentha spicata*) encoding limonene synthase (*MsLS*) in peppermint (*Mentha* × *piperita*) reported stable integration of the gene, but the terpene profile of the plants was not clearly altered [36]. Later, transgenic peppermint and cornmint (*Mentha arvensis*) plants overexpressing *MsLS* were reported to have quantitative changes in levels of monoterpenes [37]. Levels of several monoterpene pathway components were altered, including 1,8-cineole and β-ocimene (one-step enzymatic products from GDP), and monoterpene end products such as pulegone or piperitone (formed from limonene via isopiperitenone). The effects were caused not only by overexpression but probably also by co-suppression resulting from the introduction of a homologous gene.

In petunia, Joost Lücker *et al.* [38] showed that overexpression of the gene encoding the *Clarkia breweri S*-linalool synthase (*Lis*) [39], a heterologous monoterpene synthase, indeed resulted in monoterpene production. However, they also showed that the newly formed linalool is efficiently converted to the non-volatile

Table 1. Reports of metabolic engineering of monoterpenoids, sesquiterpenoids and diterpenoids, and their derivatives and precursors

Terpene class	Engineered species	Target	Subcellular location	Regulation	Altered terpene profile[a]	Refs
Precursors	Mint	DXR	Plastid	Constitutive	Total essential oil ↑↓[b]	[27]
	Tobacco	HMGR	Cytosol	Constitutive	No change	[31]
	Arabidopsis	DXS	Plastid	Constitutive	Taxadiene ↑	[28]
	Arabidopsis	HDR	Plastid	Constitutive	Taxadiene ↑	[28]
	Artemisia	FDP synthase	Cytosol	Constitutive	Artemisinin ↑	[35]
Monoterpenes	Mint	Limonene synthase	Plastid	Constitutive	No change	[36]
	Mint	Limonene synthase	Plastid	Constitutive	Several products ↑↓	[37]
	Mint	Limonene synthase	Plastid	Constitutive	No change	[50]
	Petunia	Linalool synthase	Plastid	Constitutive	Linalool glycoside ↑	[38]
	Tomato	Linalool synthase	Plastid	Fruit specific	Linalool ↑, hydroxylated linalool ↑	[40]
	Carnation	Linalool synthase	Plastid	Constitutive	Linalool ↑, linalool oxides ↑	[41]
	Arabidopsis	Linalool/nerolidol synthase	Plastid	Constitutive	Linalool ↑, hydroxylated and glycosylated linalool ↑	[42]
	Potato	Linalool/nerolidol synthase	Plastid	Constitutive	Linalool ↑, hydroxylated and glycosylated linalool ↑	[42]
	Tobacco	γ-Terpinene synthase, β-pinene synthase and limonene synthase	Plastid	Constitutive	γ-Terpinene ↑, limonene ↑, β-pinene ↑ and side products ↑	[30]
	Tobacco	Limonene synthase	Cytosol	Constitutive	Limonene ↑	[53]
	Tobacco	Limonene synthase	Plastid	Constitutive	Limonene ↑	[53]
	Tobacco	Limonene synthase	ER	Constitutive	No change	[53]
Sesquiterpenes	Tobacco	Trichodiene synthase	Cytosol	Constitutive	Trichodiene ↑, oxygenated trichodiene[c] ↑	[44]
	Tobacco	Amorpha-4,11–diene synthase	Cytosol	Constitutive	Amorpha-4,11–diene ↑	[45]
	Arabidopsis	Linalool/nerolidol synthase	Plastid	Constitutive	Nerolidol ↑	[42]
	Arabidopsis	Germacrene A synthase	Cytosol	Constitutive	Germacrene A ↑	[42]
	Arabidopsis	Linalool/nerolidol synthase	Mitochondria	Constitutive	Nerolidol ↑, 4,8-dimethyl-1,3(*E*),7-nonatriene ↑	[47]
Diterpenes	*Arabidopsis*	Taxadiene synthase	Plastid	Constitutive, induced	Taxadiene ↑	[29]
Modified terpenes	Mint	Menthofuran synthase	ER	Constitutive	Menthofuran ↓, pulegone ↓, menthol ↑	[27]
	Tobacco	Cembratrien-ol hydroxylase	ER	Constitutive	Cembratriene-diol ↓, cembratrien-ol ↑	[51]
	Mint	Limonene hydroxylase	ER	Constitutive	Limonene ↑, menthofuran ↓, isomenthone ↓, menthol ↓, menthone ↓	[50]
	Tobacco	Limonene hydroxylase	ER	Constitutive	Isopiperitenol and derivatives ↑	[52]

Abbreviations: DXR, deoxyxylulose 5-phosphate reductoisomerase; DXS, deoxyxylulose 5-phosphate synthase; FDP, farnesyl diphosphate; HDR, hydroxymethylbutenyl diphosphate reductase; HMGR, hydroxymethylglutaryl CoA reductase.
[a]Terpenoids that have been reduced (↓) or increased (↑) in amount.
[b]Overexpression sometimes resulted in co-suppression. Therefore, in different plants, levels of certain compounds were up- or downregulated.
[c]Formation of oxygenated trichodiene was detected after induction by an elicitor.

S-linalyl-β-D-glucopyranoside, probably by the action of an endogenous glucosyl transferase. Expression of *Lis* in tomato under the control of a late fruit-ripening promoter (E8 promoter) resulted in the accumulation of *S*-linalool and 8-hydroxy-linalool in ripening fruit [40]. The same gene was also constitutively expressed in carnation (*Dianthus caryophyllus*), and both free linalool and its *cis* and *trans* oxides could be detected by headspace analysis of leaves and flowers of transgenic plants [41]. In tobacco, Joost Lücker *et al.* [30] achieved substantial production of three new monoterpene products [γ-terpinene, (+)-limonene and (−)-β-pinene] by introducing the three corresponding lemon (*Citrus limon*) monoterpene synthases into a single plant. The products were emitted by the leaves as well as the flowers of the transgenic plants.

A model plant such as *Arabidopsis* would be extremely helpful for evaluating metabolic engineering strategies.

Flowers of *Arabidopsis* produce many mono- and sesquiterpenes, whereas its leaves produce only trace amounts of limonene and β-myrcene [13,42,43]. To evaluate the potential of *Arabidopsis* to produce monoterpenes and sesquiterpenes, we used the strawberry (*Fragaria* × *ananassa*) gene *FaNES1* (*Nerolidol synthase 1*) [1]. This gene encodes a dual-function monoterpene and sesquiterpene synthase that catalyzes the formation of both linalool from GDP and nerolidol from FDP with equal efficiency, and hence is called a linalool/nerolidol synthase. Leaves of transgenic *Arabidopsis* plants constitutively overexpressing *FaNES1* produced free, hydroxylated and glycosylated linalool derivatives [42]. The newly formed monoterpene alcohol was converted to various derivatives by endogenous enzymes, probably hydroxylases and glycosyl transferases. In chrysanthemum (*Chrysanthemum* × *grandiflorum*), transformation with the same construct resulted in the emission of large amounts of unmodified linalool (M.A. Jongsma *et al.*, unpublished). The different profiles of linalool derivatives identified in all these transgenic plants illustrate the dramatic influence of the plant genetic makeup on the outcome of metabolic engineering [42].

Engineering sesquiterpenoids

Production of sesquiterpenes in transgenic plants is a more challenging task than generating monoterpenes. Tobacco plants have been transformed with a fungal trichodiene synthase [44] and with the *A. annua* amorpha-4,11-diene synthase [45] (Table 1). In both cases, only low levels of the expected sesquiterpenoids could be detected. The levels of trichodiene in cell suspension cultures of the transgenic tobacco expressing trichodiene synthase could be increased tenfold by elicitation with cellulase [46]. The elicited transgenic suspension culture also accumulated an oxygenated trichodiene derivative. Expressing strawberry *FaNES1* in *Arabidopsis* using plastidic targeting resulted in the production of small amounts of the sesquiterpene nerolidol [42]. This was unexpected because it is generally assumed that sesquiterpenes are only produced in the cytosol (Figure 2) but this shows that FDP is also available in the plastids. Because mitochondria are also involved in isoprenoid biosynthesis (they are the site of ubiquinone biosynthesis) (Figure 2) and *Arabidopsis* has an FPP synthase with a mitochondrial-targeting signal, we have also targeted FaNES1 to the mitochondria [47]. The transgenic plants indeed produced 3(*S*)-*E*-nerolidol (and no linalool), confirming that FDP is also present (and available) in the mitochondria. The nerolidol produced was partly converted, by endogenous *Arabidopsis* enzymes, to the C_{11} homoterpene 4,8-dimethyl-1,3(*E*),7-nonatriene [(*E*)-DMNT]. Transgenic *Arabidopsis* expressing a *bona fide* cytosolically targeted sesquiterpene synthase – the chicory (*Cichorium intybus*) germacrene A synthase gene (*CiGASlo*) – only produced low levels of germacrene A [42].

Engineering diterpenoids and triterpenoids

The diterpenes are again a product of plastidic isoprenoid biosynthesis (Figure 2) and, to date, there is only one example of diterpene engineering. The enzyme catalyzing the first step in Taxol biosynthesis, TXS [48], has been produced in *Arabidopsis* (Table 1) [29]. When an inducible promoter was used, taxadiene levels in *Arabidopsis* leaves reached $\sim 0.6\ \mu g\ g^{-1}$ (dry weight) [29], which is 10–60 times higher than the levels obtained with sesquiterpene synthases in tobacco [45] but 1000 times lower than obtained with monoterpenes in *Arabidopsis* [42]. With regard to triterpenes, there are no examples of metabolic engineering yet, with the exception of the above-described enhancement of sterol formation caused by increasing precursor availability. It is unclear whether no attempts were made to modify triterpene metabolism or whether these attempts were unsuccessful. Indeed, the fact that triterpenes are also cytosolic products and derived from FDP suggests that – just as for sesquiterpenoids – engineering is not straightforward.

Engineering modifying steps

In many cases, the primary terpene scaffolds produced by the terpene synthases are further modified by one or more modifying enzymes, hence creating the myriad of different terpenoid molecules (Figure 1). As described above, endogenous plant enzymes will sometimes accept the terpenoids introduced through genetic engineering as substrates. Examples include glycosylation, hydroxylation and reduction or oxidation [38,40–42,47]. However, modifying enzymes can also be introduced or altered in activity by genetic engineering. To reduce the levels of +-menthofuran, an undesirable monoterpenoid in peppermint, Soheil Mahmoud and Rodney Croteau [27] blocked the expression of the gene encoding the cytochrome P450 menthofuran synthase (*MFS*), the enzyme that catalyzes the hydroxylation of +-pulegone to +-menthofuran [49]. This reduction in *MFS* expression resulted in reduced levels of menthofuran without changing the yield of essential oil [27]. Surprisingly, the substrate [+-pulegone] did not accumulate and even decreased compared with the wild-type plants. The decrease in +-pulegone is an additional positive compositional change because, above a certain level, this compound is undesirable in peppermint essential oil. It also illustrates the possibility of unexpected outcomes in metabolic engineering approaches.

In the same biosynthetic pathway leading to menthol in peppermint, the primary monoterpene skeleton −-limonene is converted to −-*trans*-isopiperitenol by the activity of limonene-3-hydroxylase (LH) [50]. Overexpression of the gene encoding this enzyme under the control of the strong CaMV 35S promoter in transgenic peppermint plants resulted in several lines with reduced *LH* expression because of a co-suppression effect [50]. These plants accumulated limonene up to 80% of the total essential oil, compared with only 2% in wild-type plants, and were depleted of the downstream metabolites in the pathway. The increase in the precursor compound limonene did not affect the activity of limonene synthase and other enzymes of monoterpene biosynthesis in peppermint. Sense, co-suppression and antisense experiments using a cytochrome P450-encoding gene have been conducted in tobacco [51]. The authors suppressed the activity of cembratriene-ol hydroxylase, which catalyzes the biosynthesis of cembratriene-diol, a

diterpene constituting 60% of trichome exudate weight in the tobacco cultivar investigated. As a result, the concentration of cembratriene-diols decreased, whereas that of its precursor (cembratriene-ol) increased.

Although the production of the two mint cytochrome P450 enzymes described above was reduced in the homologous plant species, a recent study examined the possibility of generating a hydroxylated monoterpene in a heterologous system [52]. Transgenic tobacco plants already expressing three lemon monoterpene synthases, including the *C. limon* limonene synthase (ClLS), were retransformed with a construct designed for overexpression of an *M. spicata* limonene-3-hydroxylase encoding cDNA [52]. Plants combining the expression of the four genes emitted +-*trans*-isopiperitenol, the product of limonene C-3 hydroxylation, and some of its derivatives. Because ClLS activity in the transgenic tobacco was targeted to the plastids and the cytochrome P450 protein was targeted to the endoplasmic reticulum (ER) by its natural targeting signal (Figure 2), this result demonstrates that the 'normal' transport mechanisms operating in terpene-producing plants and cells (also see below under 'Subcellular compartmentation') also operate in transgenic plants and, hence, that multistep metabolic engineering of monoterpene biosynthesis involving two (or more) different cellular compartments is feasible [52].

Subcellular compartmentation

As described above, the isoprenoid precursors are present in several different cell compartments and so it should be possible to generate terpenoids by metabolic engineering in more than one cell compartment. Transgenic *Arabidopsis* plants expressing *FaNES1* fused to a plastid targeting signal produced high levels of the monoterpene alcohol linalool but also low levels of the sesquiterpene alcohol nerolidol [42]. This might be explained by transport of FDP from the cytosol to the plastids or by release of FDP by other prenyl transferases active in the plastids, such as GGDP synthase. By targeting the same enzyme to the mitochondria, the presence of FDP in these organelles was also established [47]. In another study, three different expression constructs designed for plastidic, cytosolic and ER localization of the *Perilla frutescens* limonene synthase (PfLS) protein were introduced into tobacco plants [53]. Limonene formation was detected in plastid- as well as cytosol-localized PfLS transgenic plants. PfLS activity and *in vivo* limonene production could not be demonstrated in the case of ER targeting, possibly because of incorrect folding or instability of the protein.

Changes in the precursor pool in one compartment might also affect isoprenoid formation in another compartment. For example, the pool of IDP in the plastids might affect the formation of sesquiterpenes in the cytosol given that transport of isoprenoid precursors is known to occur from the plastids to the cytosol [18,23,54]. The level of the cytosolically produced sesquiterpene β-caryophyllene emitted by flowers of transgenic tobacco plants overexpressing the three plastid-targeted lemon monoterpene synthases described above was lower than the level in wild-type flowers, probably because of a decrease in the amount of IDP transported from the plastids to the cytosol [30]. Recently, Natalia Dudareva *et al.* [20] showed, by feeding stable isotope-labeled isoprenoid precursors to snapdragon (*Antirrhinum majus*) flowers, that IDP used for the production of the sesquiterpene nerolidol in the cytosol of snapdragon petals indeed originates from the plastidic MEP pathway. In strawberry fruit, the biosynthesis of linalool was suggested to occur in the cytosol rather than in the plastids, as would be expected for monoterpenes [1]. This was based, for example, on the absence of a plastid-targeting sequence in *FaNES1*. These results were corroborated by the finding that feeding of labeled mevalonic acid to strawberries resulted in incorporation of the label into linalool, whereas feeding of the plastidic precursor 1-deoxy-D-xylulose did not result in labeled linalool (Mathias Wüst, personal communication).

The complexity of the intracellular compartmentation of terpene biosynthetic enzymes in the formation of menthol has recently been demonstrated [55]. The primary product limonene is produced in the plastids of secretory cells of peltate glandular trichomes. Subsequent hydroxylation by LH occurs in the ER, oxidation by −-*trans*-isopiperitenol dehydrogenase in the mitochondria and reduction by (+)-pulegone reductase to menthone in the cytosol. The mechanisms involved in the transport of intermediates between organelles are still unclear but might involve a combination of active (e.g. carrier proteins and membrane pumps) and passive (e.g. membrane contacts and diffusion) mechanisms, possibly depending on their relative solubilities in water. There is evidence that ABC transporters are involved in diterpene secretion from tobacco epidermal cells [56]. Modifying aspects of these complex interconversions and subcellular translocations in host plants should provide a fascinating toolbox to achieve the formation of a desired derivative.

Costs of terpenoid engineering

Metabolic engineering of volatile terpenoids can impose a cost on plant growth and fitness [42]. This might be caused not only by the reduced supply of precursors to branching (primary metabolite) pathways but also by the toxicity of the resulting compounds to plant cells. For example, transgenic peppermint plants in which DXR was co-suppressed exhibited chlorophyll deficiency, grew more slowly and had lower essential oil yield than did wild-type plants [27]. Transgenic *Arabidopsis* plants overproducing FaNES1 in their plastids also showed delayed growth and development, and this phenotype was inherited and correlated with the levels of linalool and its derivatives produced in the transgenic plants [42]. The cause for this phenotype could not be determined, although depletion of several plastid-derived compounds (chlorophylls, lutein and β-carotene) was ruled out because no change in their levels could be detected in transgenic compared with wild-type plants. Also the expression of *TXS* in *Arabidopsis* retarded growth and induced a pale-green phenotype [29]. In other cases, transgenic plants with altered terpenoid profiles were obtained without any other detectable effect on the plant phenotype [38,53].

Until now, genes have been expressed in plants using constitutive promoters such as CaMV 35S. Our group has recently started using the Rubisco small subunit promoter from chrysanthemum, which is light inducible and imparts much higher expression levels [about eight times higher (http://www.impactvector.com/)] than the CaMV 35S promoter [57]. As expected, the use of this strong promoter resulted in a more extreme range of phenotypes, such as reduced growth and bleaching of leaves. The negative effects of terpenoid engineering on plant growth could be overcome by better spatial and temporal control of transgene expression. Organ-specific promoters such as the E8 late fruit-ripening-specific promoter, used for the expression of *lis* in tomato, could be used [40]. Although not yet demonstrated, the use of gland-specific promoters in plants with glandular trichomes (such as mint) might provide a secure method of producing large quantities of volatile terpenoids without affecting plant growth and development [58]. Secretory tissues can contain a much higher concentration of terpenoids without any toxic effect to the plant; for example, the wild tomato *Lycopersicon hirsutum f. typicum* has concentrations of zingiberene in type VI trichomes of up to 2.8% of leaf fresh weight [59]. Alternatively, inducible gene expression methods such as the glucocorticoid-mediated system could be used, as demonstrated recently for the overexpression of *TXS* in *Arabidopsis* [29].

Biological effects and practical implications

Although the past few years have been fruitful in terms of generating plants producing various mono- and sesquiterpene derivatives, the biological effects and possible practical applications of these compositional changes in the terpenoid profiles have hardly been tested. The first indications of the possibility of altering insect behavior through terpenoid metabolic engineering were provided by transgenic tobacco plants producing higher levels of the diterpene cembratriene-ol [51]. Not only did exudates from transgenic plants have higher aphidicidal activity but also, in *in vivo* assays, these plants showed greatly reduced aphid colonization compared with that shown by wild-type plants. Aphid behavior was also altered in transgenic *Arabidopsis* plants producing linalool [42]. In dual-choice assays with *Myzus persicae*, transgenic lines producing linalool significantly repelled the aphids [42]. These observations were recently extended with even more convincing results: transgenic chrysanthemum producing linalool repelled western flower thrips (*Frankliniella occidentalis*) [M.A. Jongsma (2004) Novel genes for control and deterrence of sucking insect pests, http://www.isb.vt.edu/news/2004/Nov04.pdf]. The *Arabidopsis* plants expressing *FaNES1* with mitochondrial targeting signal and emitting (3*S*)-*E*-nerolidol and (*E*)-DMNT became attractive to carnivorous predatory mites (*Phytoseiulus persimilis*), the natural enemies of spider mites [47].

A first attempt at sensory analysis of transgenic plants engineered for terpenoid production by a human panel was described by Mazen El-Tamer *et al.* [60]. Transgenic tobacco plants producing three new monoterpenes [γ-terpinene, (+)-limonene and (−)-β-pinene] [30] were used. The human panel detected a significant difference between the transgenic and the control plants, although this difference could not be associated with sensory attributes, probably as a result of insufficient panel training [60].

Conclusions and future prospects

Several research groups have made significant progress in the metabolic engineering of terpenoids during the past few years, particularly the monoterpenes. Several studies of a range of plant species have shown that a high level of production of monoterpenes, including modified products, can be obtained using metabolic engineering. The engineering of sesquiterpenes has been much less successful even though the precursor for the sesquiterpenes, FDP, is a ubiquitous molecule that supposedly is present in relatively large quantities, if only because it is the precursor for sterol biosynthesis. In 1995, Joseph Chappell discussed the possibility that metabolic channeling might occur in isoprenoid biosynthesis at a subcellular level [31]. In metabolic channeling, enzymes from the same pathway (typically those catalyzing successive steps in a metabolic pathway) will interact and form a protein complex, allowing more efficient reaction and regulation of the pathway [61]. Metabolic channeling has since been reported to occur in primary metabolism, but has recently also been described in secondary metabolism, such as phenylpropanoid and dhurrin biosynthesis [61,62]. This might also occur in isoprenoid biosynthesis, which could explain why FDP (which is produced in relatively large amounts for sterol biosynthesis) is not or hardly available to introduced sesquiterpene synthases. Perhaps this obstacle to successful sesquiterpene metabolic engineering can be solved by artificial channeling, using fusion constructs of two or more enzymes from a pathway. Work with a fusion construct of FDP synthase and the sesquiterpene synthase *epi*-aristolochene synthase produced in *Escherichia coli* shows that such fusion constructs can be functional [63]. During the coming years, experiments with plants will hopefully demonstrate this *in planta* and work could then also be extended to larger fusion constructs containing genes that encode several enzymes from one pathway. We also anticipate that experiments will be carried out to demonstrate whether channeling in isoprenoid biosynthesis occurs in unmodified systems. Finally, with the exception of excellent work on the WRKY transcription factor, which regulates sesquiterpene biosynthesis in cotton [64], there are virtually no data available on the involvement of transcription factors in isoprenoid biosynthesis. This contrasts with the situation in, for example, flavonoid and indole alkaloid biosynthesis [65,66]. Also, with regard to transcription factors, we expect great progress in the field of terpenoid metabolic engineering. The positive results with monoterpene engineering using native plastidic targeting promise more successes for the future, in which the apparent ample supply of isoprenoid precursors in this compartment will also be used for the production of terpenoids other than monoterpenoids.

Apart from progress in the engineering of terpenoids, we expect to witness a dramatic increase in the use of transgenic plants with altered terpenoid profiles for experiments aimed at elucidating the biological significance of terpenoids. Experiments examining tri-trophic interactions, fungal and bacterial resistance, allelopathy, and the importance of root exudates are only a few examples of future activities with such transgenic plants. From the applied point of view, the first field trials with commercial varieties with engineered terpenoid profiles are expected to be carried out soon and, one day, the terpenoid-containing agricultural products that we use daily will almost certainly originate from engineered rather than conventional plants.

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References

1 Aharoni, A. *et al*. (2004) Gain and loss of fruit flavor compounds produced by wild and cultivated strawberry species. *Plant Cell* 16, 3110–3131
2 Pichersky, E. *et al*. (1994) Floral scent production in *Clarkia* (Onagraceae): I. Localization and developmental modulation of monoterpene emission and linalool synthase activity. *Plant Physiol.* 106, 1533–1540
3 Bertea, C.M. *et al*. (2005) Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua*. *Planta Med.* 71, 40–47
4 Haudenschild, C. and Croteau, R. (1998) Molecular engineering of monoterpene production. *Genet. Eng.* 20, 267–280
5 Lin, Z.J. *et al*. (2005) Simultaneous determination of glycyrrhizin, a marker component in *Radix glycyrrhizae*, and its major metabolite glycyrrhetic acid in human plasma by LC-MS/MS. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 814, 201–207
6 McCaskill, D. and Croteau, R. (1998) Some caveats for bioengineering terpenoid metabolism in plants. *Trends Biotechnol.* 16, 349–355
7 Rodriguez-Concepcion, M. (2004) The MEP pathway: a new target for the development of herbicides, antibiotics and antimalarial drugs. *Curr. Pharm. Des.* 10, 2391–2400
8 Degenhardt, J. *et al*. (2003) Attracting friends to feast on foes: engineering terpene emission to make crop plants more attractive to herbivore enemies. *Curr. Opin. Biotechnol.* 14, 169–176
9 Pichersky, E. and Gershenzon, J. (2002) The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Curr. Opin. Plant Biol.* 5, 237–243
10 Balkema-Boomstra, A.G. *et al*. (2003) Role of cucurbitacin C in resistance to spider mite (*Tetranychus urticae*) in cucumber (*Cucumis sativus* L.). *J. Chem. Ecol.* 29, 225–235
11 Powell, G. *et al*. (1997) Laboratory evaluation of antifeedant compounds for inhibiting settling by cereal aphids. *Entomol. Exp. Appl.* 84, 189–193
12 Arimura, G. *et al*. (2000) Herbivory-induced volatiles elicit defence genes in lima bean leaves. *Nature* 406, 512–515
13 Chen, F. *et al*. (2004) Characterization of a root-specific *Arabidopsis* terpene synthase responsible for the formation of the volatile monoterpene 1,8-cineole. *Plant Physiol.* 135, 1956–1966
14 Mercke, P. *et al*. (2004) Combined transcript and metabolite analysis reveals genes involved in spider mite induced volatile formation in cucumber plants. *Plant Physiol.* 135, 2012–2024
15 Galili, G. *et al*. (2002) Genetic, molecular, and genomic approaches to improve the value of plant foods and feeds. *Crit. Rev. Plant Sci.* 21, 167–204
16 Mahmoud, S.S. and Croteau, R.B. (2002) Strategies for transgenic manipulation of monoterpene biosynthesis in plants. *Trends Plant Sci.* 7, 366–373
17 Rodriguez-Concepcion, M. and Boronat, A. (2002) Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. *Plant Physiol.* 130, 1079–1089
18 Adam, K.P. and Zapp, J. (1998) Biosynthesis of the isoprene units of chamomile sesquiterpenes. *Phytochemistry* 48, 953–959
19 Bick, J.A. and Lange, B.M. (2003) Metabolic cross talk between cytosolic and plastidial pathways of isoprenoid biosynthesis: unidirectional transport of intermediates across the chloroplast envelope membrane. *Arch. Biochem. Biophys.* 415, 146–154
20 Dudareva, N. *et al*. (2005) The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. *Proc. Natl. Acad. Sci. U. S. A.* 102, 933–938
21 Dudareva, N. *et al*. (2004) Biochemistry of plant volatiles. *Plant Physiol.* 135, 1893–1902
22 Hemmerlin, A. *et al*. (2003) Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in Tobacco Bright Yellow-2 cells. *J. Biol. Chem.* 278, 26666–26676
23 Laule, O. *et al*. (2003) Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U. S. A.* 100, 6866–6871
24 Schuhr, C.A. *et al*. (2003) Quantitative assessment of crosstalk between the two isoprenoid biosynthesis pathways in plants by NMR spectroscopy. *Phytochem. Rev.* 2, 3–16
25 Lange, B.M. *et al*. (2000) Probing essential oil biosynthesis and secretion by functional evaluation of expressed sequence tags from mint glandular trichomes. *Proc. Natl. Acad. Sci. U. S. A.* 97, 2934–2939
26 Estevez, J.M. *et al*. (2001) 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. *J. Biol. Chem.* 276, 22901–22909
27 Mahmoud, S.S. and Croteau, R. (2001) Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. *Proc. Natl. Acad. Sci. U. S. A.* 98, 8915–8920
28 Botella-Pavia, P. *et al*. (2004) Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors. *Plant J.* 40, 188–199
29 Besumbes, O. *et al*. (2004) Metabolic engineering of isoprenoid biosynthesis in *Arabidopsis* for the production of taxadiene, the first committed precursor of Taxol. *Biotechnol. Bioeng.* 88, 168–175
30 Lücker, J. *et al*. (2004) Increased and altered fragrance of tobacco plants after metabolic engineering using three monoterpene synthases from lemon. *Plant Physiol.* 134, 510–519
31 Chappell, J. *et al*. (1995) Is the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase a rate-limiting step for isoprenoid biosynthesis in plants? *Plant Physiol.* 109, 1337–1343
32 Harker, M. *et al*. (2003) Co-ordinate regulation of sterol biosynthesis enzyme activity during accumulation of sterols in developing rape and tobacco seed. *Planta* 216, 707–715
33 Schaller, H. *et al*. (1995) Expression of the *Hevea brasiliensis* (H.B.K.) Mull. Arg. 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 in tobacco results in sterol overproduction. *Plant Physiol.* 109, 761–770
34 Masferrer, A. *et al*. (2002) Overexpression of *Arabidopsis thaliana* farnesyl diphosphate synthase (FPS1S) in transgenic *Arabidopsis* induces a cell death/senescence-like response and reduced cytokinin levels. *Plant J.* 30, 123–132
35 Chen, D. *et al*. (2000) Expression of a chimeric farnesyl diphosphate synthase gene in *Artemisia annua* L. transgenic plants via *Agrobacterium tumefaciens*-mediated transformation. *Plant Sci.* 155, 179–185
36 Krasnyanski, S. *et al*. (1999) Transformation of the limonene synthase gene into peppermint (*Mentha* × *piperita* L.) and preliminary studies on the essential oil profiles of single transgenic plants. *Theor. Appl. Genet.* 99, 676–682
37 Diemer, F. *et al*. (2001) Altered monoterpene composition in transgenic mint following the introduction of 4*S*-limonene synthase. *Plant Physiol. Biochem.* 39, 603–614
38 Lücker, J. *et al*. (2001) Expression of *Clarkia S*-linalool synthase in transgenic petunia plants results in the accumulation of *S*-linalyl-β-D-glucopyranoside. *Plant J.* 27, 315–324

39 Dudareva, N. *et al*. (1996) Evolution of floral scent in *Clarkia*: novel patterns of *S*-linalool synthase gene expression in the *C. breweri* flower. *Plant Cell* 8, 1137–1148
40 Lewinsohn, E. *et al*. (2001) Enhanced levels of the aroma and flavor compound *S*-linalool by metabolic engineering of the terpenoid pathway in tomato fruits. *Plant Physiol.* 127, 1256–1265
41 Lavy, M. *et al*. (2002) Linalool and linalool oxide production in transgenic carnation flowers expressing the *Clarkia breweri* linalool synthase gene. *Mol. Breed.* 9, 103–111
42 Aharoni, A. *et al*. (2003) Terpenoid metabolism in wild-type and transgenic *Arabidopsis* plants. *Plant Cell* 15, 2866–2884
43 Chen, F. *et al*. (2003) Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers. *Plant Cell* 15, 481–494
44 Hohn, T.M. and Ohlrogge, J.B. (1991) Expression of a fungal sesquiterpene cyclase gene in transgenic tobacco. *Plant Physiol.* 97, 460–462
45 Wallaart, T.E. *et al*. (2001) Amorpha-4,11-diene synthase: cloning and functional expression of a key enzyme in the biosynthetic pathway of the novel antimalarial drug artemisinin. *Planta* 212, 460–465
46 Zook, M. *et al*. (1996) Structural characterization of 15-hydroxytrichodiene, a sesquiterpenoid produced by transformed tobacco cell suspension cultures expressing a trichodiene synthase gene from *Fusarium sporotrichioides*. *Phytochemistry* 43, 1235–1237
47 Kappers, I.F. *et al*. (2005) Genetic engineering of terpenoid metabolism attracts bodyguards to *Arabidopsis*. *Science* 309, 2070–2072
48 Jennewein, S. and Croteau, R. (2001) Taxol: biosynthesis, molecular genetics, and biotechnological applications. *Appl. Microbiol. Biotechnol.* 57, 13–19
49 Bertea, C.M. *et al*. (2001) Demonstration that menthofuran synthase of mint (*Mentha*) is a cytochrome P450 monooxygenase: cloning, functional expression, and characterization of the responsible gene. *Arch. Biochem. Biophys.* 390, 279–286
50 Mahmoud, S.S. *et al*. (2004) Cosuppression of limonene-3-hydroxylase in peppermint promotes accumulation of limonene in the essential oil. *Phytochemistry* 65, 547–554
51 Wang, E. *et al*. (2001) Suppression of a P450 hydroxylase gene in plant trichome glands enhances natural-product-based aphid resistance. *Nat. Biotechnol.* 19, 371–374
52 Lücker, J. *et al*. (2004) Metabolic engineering of monoterpene biosynthesis: two-step production of (+)-*trans*-isopiperitenol by tobacco. *Plant J.* 39, 135–145
53 Ohara, K. *et al*. (2003) Limonene production in tobacco with *Perilla* limonene synthase cDNA. *J. Exp. Bot.* 54, 2635–2642
54 Steliopoulos, P. *et al*. (2002) Biosynthesis of the sesquiterpene germacrene D in *Solidago canadensis*: ^{13}C and ^{2}H labeling studies. *Phytochemistry* 60, 13–20
55 Turner, G.W. and Croteau, R. (2004) Organization of monoterpene biosynthesis in *Mentha*. Immunocytochemical localizations of geranyl diphosphate synthase, limonene-6-hydroxylase, isopiperitenol dehydrogenase, and pulegone reductase. *Plant Physiol.* 136, 4215–4227
56 Jasinski, M. *et al*. (2001) A plant plasma membrane ATP binding cassette-type transporter is involved in antifungal terpenoid secretion. *Plant Cell* 13, 1095–1107
57 Outchkourov, N.S. *et al*. (2003) The promoter–terminator of chrysanthemum *rbcS1* directs very high expression levels in plants. *Planta* 216, 1003–1012
58 Wang, E. *et al*. (2002) Isolation and characterization of the *CYP71D16* trichome-specific promoter from *Nicotiana tabacum* L. *J. Exp. Bot.* 53, 1891–1897
59 Antonious, G.F. and Kochhar, T.S. (2003) Zingiberene and curcumene in wild tomato. *J. Environ. Sci. Health B* 38, 489–500
60 El-Tamer, M.K. *et al*. (2003) The influence of monoterpene synthase transformation on the odour of tobacco. *J. Biotechnol.* 106, 15–21
61 Winkel, B.S.J. (2004) Metabolic channeling in plants. *Annu. Rev. Plant Biol.* 55, 85–107
62 Kristensen, C. *et al*. (2005) Metabolic engineering of dhurrin in transgenic *Arabidopsis* plants with marginal inadvertent effects on the metabolome and transcriptome. *Proc. Natl. Acad. Sci. U. S. A.* 102, 1779–1784
63 Brodelius, M. *et al*. (2002) Fusion of farnesyldiphosphate synthase and *epi*-aristolochene synthase, a sesquiterpene cyclase involved in capsidiol biosynthesis in *Nicotiana tabacum*. *Eur. J. Biochem.* 269, 3570–3577
64 Xu, Y-H. *et al*. (2004) Characterization of GaWRKY1, a cotton transcription factor that regulates the sesquiterpene synthase gene (+)-δ-cadinene synthase-A. *Plant Physiol.* 135, 507–515
65 Broun, P. (2004) Transcription factors as tools for metabolic engineering in plants. *Curr. Opin. Plant Biol.* 7, 202–209
66 Verpoorte, R. and Memelink, J. (2002) Engineering secondary metabolite production in plants. *Curr. Opin. Biotechnol.* 13, 181–187