

Review article

Metabolic engineering of plant monoterpenes, sesquiterpenes and diterpenes—current status and future opportunities

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Summary

Terpenoids (a.k.a. isoprenoids) represent the most diverse class of natural products found in plants, with tens of thousands of reported structures. Plant-derived terpenoids have a multitude of pharmaceutical and industrial applications, but the natural resources for their extraction are often limited and, in many cases, synthetic routes are not commercially viable. Some of the most valuable terpenoids are not accumulated in model plants or crops, and genetic resources for breeding of terpenoid natural product traits are thus poorly developed. At present, metabolic engineering, either in the native producer or a heterologous host, is the only realistic alternative to improve yield and accessibility. In this review article, we will evaluate the state of the art of modulating the biosynthetic pathways for the production of mono-, sesqui- and diterpenes in plants.

Introduction

The development of successful metabolic engineering approaches for terpenoid production requires an in-depth understanding of the pathways that contribute to their biosynthesis. The biosynthesis of terpenoids can be conceptually divided into four different stages. All terpenoids are derived from dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), and the formation of these universal C₅ building blocks constitutes stage 1 (Fig. 1). In plants, IPP and DMAPP can be synthesized via two compartmentalized pathways. The mevalonic acid (MVA) pathway operates in the cytosol (early steps), the endoplasmic reticulum (3-hydroxy-3-methylglutaryl-CoA reductase) and peroxisomes (late steps) (Carrie *et al.*, 2007; Denbow *et al.*, 1996; Leivar *et al.*, 2005; Merret *et al.*, 2007; Nagegowda *et al.*, 2005; Sapir-Mir *et al.*, 2008; Simkin *et al.*, 2011; Vollack *et al.*, 1994). In contrast, all enzymes of the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway are localized to plastids (Hsieh *et al.*, 2008; Suire *et al.*, 2000). The relative contribution of these pathways to specific terpenoid end products can vary substantially in different plants or tissues. Stage 2 of terpenoid biosynthesis involves condensation reactions of DMAPP and IPP catalysed by chain length-specific prenyltransferases (Fig. 1). The condensation of one molecule of DMAPP and one molecule of IPP leads to geranyl diphosphate (GPP), the C₁₀ precursor of most monoterpenes. This reaction is catalysed by geranyl diphosphate synthase, which is active as a homodimer in some species (a representative example is Grand fir (*Abies grandis*); Tholl *et al.*, 2001; Burke and Croteau, 2002) or a heterodimer in other species (a representative example is peppermint (*Mentha × piperita*); Burke *et al.*, 1999). The N-terminus of geranyl diphosphate synthase in most species has the general features of a plastidial targeting sequence, and the mature protein (or its activity) has been localized to plastids in various plants (Soler *et al.*, 1992; Suire *et al.*, 2000; Turner and Croteau, 2004). Recently, a *cis*-prenyltransferase, neryl

diphosphate synthase, was shown to provide precursors for monoterpene biosynthesis in several species of the genus *Solanum* (Schillmiller *et al.*, 2009). A condensation of one molecule of DMAPP with two molecules of IPP generates farnesyl diphosphate (FPP) (C₁₅), the direct precursor of most sesquiterpenes, which is catalysed by farnesyl diphosphate synthase (FPS). Plant genomes appear to encode various FPS isoforms that localize to the cytosol, plastids, mitochondria or peroxisomes (Cunillera *et al.*, 1997; Hemmerlin *et al.*, 2003; Sanmiya *et al.*, 1999; Thabet *et al.*, 2011). In tomato, a *cis*-prenyltransferase, Z, Z-farnesyl diphosphate synthase (zFPS), is localized to plastids of glandular trichomes, where it is involved in the biosynthesis of sesquiterpene volatiles (Sallaud *et al.*, 2009). Diterpenes are formed from geranylgeranyl diphosphate (GGPP) (C₂₀), which itself is synthesized, by catalysis of geranylgeranyl diphosphate synthase, from DMAPP and three molecules of IPP. Isoforms of this enzyme have been reported to occur in plastids, the endoplasmic reticulum (ER) and mitochondria (Cheniclet *et al.*, 1992; Okada *et al.*, 2000; Sitthithaworn *et al.*, 2001; Thabet *et al.*, 2012). In stage 3 of terpenoid biosynthesis, reactions catalysed by terpene synthases result in the assembly of the structural core of each terpenoid class (Fig. 1). This class of enzymes is responsible for the synthesis of hundreds of structurally diverse hydrocarbon skeletons. Terpene synthases often catalyse the formation of multiple products from a prenyl diphosphate substrate, resulting from a catalytic mechanism that involves highly reactive carbocation intermediates (Degenhardt *et al.*, 2009). In general, monoterpene synthases are localized to plastids, whereas sesquiterpene synthases are found in the cytosol (Chen *et al.*, 2011b). However, exceptions to this rule have been reported, in particular the localization of monoterpene synthases in the cytosol (Aharoni *et al.*, 2003) and sesquiterpene synthases in plastids (Sallaud *et al.*, 2009). A mitochondrial localization was determined for a terpene synthase in tomato (*Solanum lycopersicum*), but the *in vivo* substrate is currently

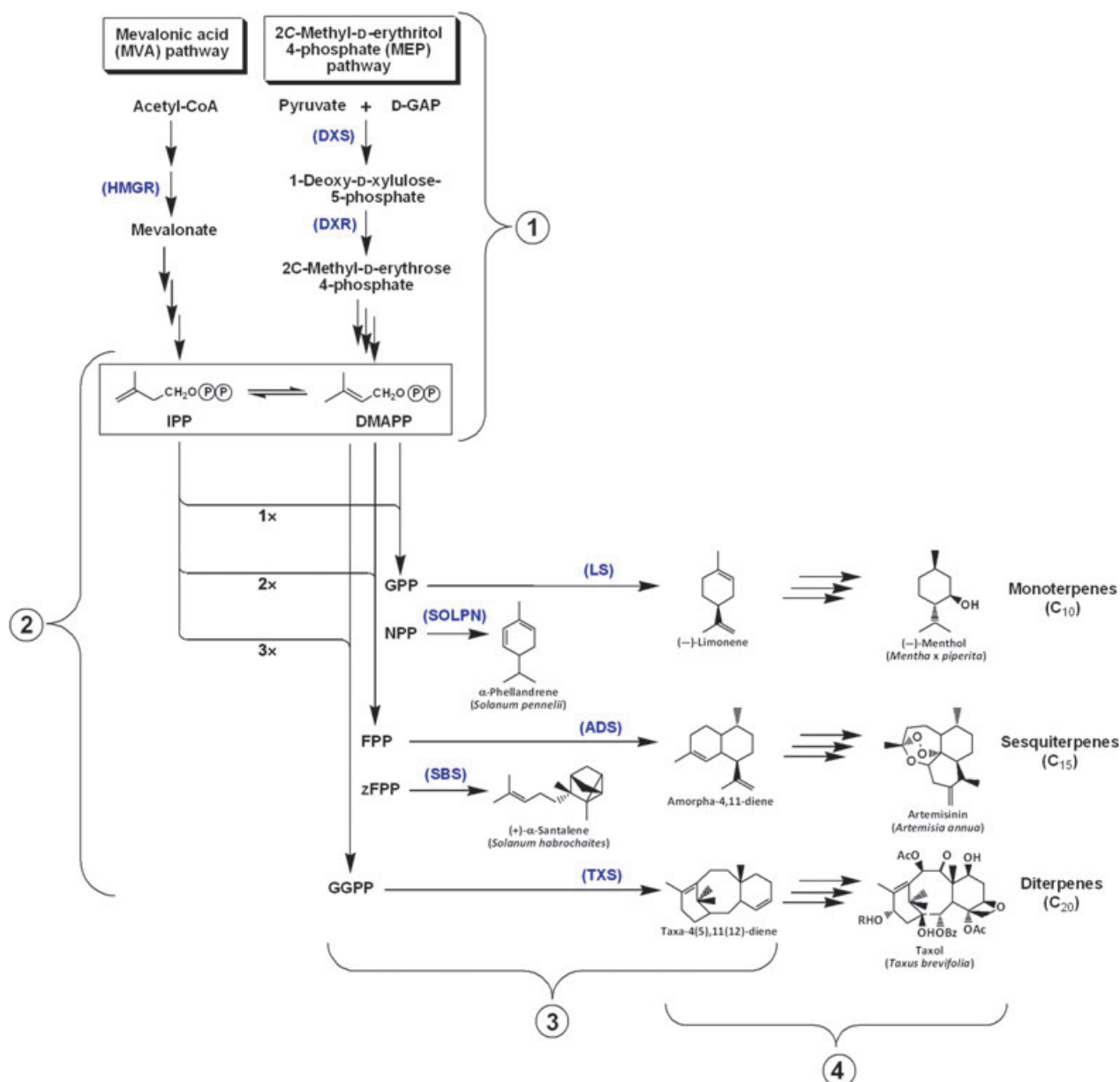


Figure 1 Overview of the biosynthesis of mono-, sesqui- and diterpenes. Stage 1: formation of the universal C₅ building blocks isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) via the mevalonic acid or 2C-methyl-D-erythritol 4-phosphate pathways. Stage 2: condensation of IPP and DMAPP, catalysed by chain length-specific prenyltransferases, to geranyl diphosphate (GPP; C₁₀), neryl diphosphate (NPP; C₁₀), *E,E*-farnesyl diphosphate (FPP; C₁₅), *Z,Z*-farnesyl diphosphate (zFPP; C₁₅) or geranylgeranyl diphosphate (GGPP; C₂₀). Stage 3: formation of the terpenoid core structure, catalysed by terpene synthases. Acronyms for selected enzymes are shown next to reaction arrows in blue colour: ADS, amorpho-4,11-diene synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; LS, limonene synthase; SBS, santalene/bergamotene synthase; SOLPN, α-phellandrene synthase; TXS, taxadiene synthase. Stage 4: modification of the parent terpenoid core by redox, substitution and conjugation reactions; chemical structures of (-)-menthol, α-phellandrene, artemisinin, α-santalene and taxol are shown as representative examples of terpenoid end products.

unknown (Falara *et al.*, 2011). Lastly, in stage 4 of the terpenoid pathway, terpenoid skeletons are further functionalized, through redox, conjugation and other modifying reactions, to yield a wide range of end products (Fig. 1).

While some terpenoids are synthesized in all plant cells, many mono-, sesqui- and diterpenes accumulate in distinct anatomical structures and cell types. Such structures include resin ducts or resin canals, which are common in conifers (Zulak and Bohlmann, 2010). Secondary phloem of conifers

also contains clusters of phloem parenchyma cells that accumulate resinous defence metabolites (Krokene *et al.*, 2008), including the diterpene taxol (generic name paclitaxel), one of the world's best-selling anticancer drugs (Croteau *et al.*, 2006; Russin *et al.*, 1995). Laticifers are elongated epithelial cells that produce a chemically complex latex, which can consist of polyterpenoids such as natural rubber (Beilen and Poirier, 2007). Glandular trichomes are epidermal protuberances that are fairly common in the angiosperm lineages and generally

store mixtures of terpenoids and/or phenolics often referred to as essential oils (Lange and Turner, 2012).

Successful terpenoid metabolic engineering approaches need to consider several levels of regulatory complexity. Intracellularly, the biosynthesis of different terpenoids is highly compartmentalized and enzymes need to be targeted to the appropriate location. In addition, the expression of genes and accumulation of enzymes involved in mono-, sesqui- and diterpene biosynthesis are often limited to certain cell types or tissues. Many of these terpenes are not accumulated in plant model species, and research is often conducted with limited community resources. Lastly, the native producer of a terpenoid of interest may not be amenable to high efficiency transformation, and an entire biosynthetic pathway may have to be transferred to a heterologous host. Despite these challenges, considerable progress has been made in using metabolic engineering for the production of target terpenoids in plants, and we will highlight a few groundbreaking studies in the upcoming sections. We have recalculated the concentrations of terpenoids reported in all papers to weight percentage, which allows direct comparisons of the outcomes of metabolic engineering strategies when yields were originally reported in different units. Due to space constraints, we will not be able to cover the production of plant terpenoids in plant tissue cultures or microbial hosts.

Terpenoid scents

By domesticating wild species and using classical breeding techniques, the floricultural industry has developed many new varieties with desirable traits, including enhanced agronomic performance, plant and flower architecture, petal colouring patterns and vase life. More recently, metabolic engineering has been used to generate novel flower colours, and some of these varieties have already been commercialized (Chandler and Tanaka, 2007). Another important factor for consumer preference, in addition to the traits mentioned above, is floral scent, which is determined by blends of emitted volatiles (Klee, 2010). In this section, we are assessing the status of metabolic engineering efforts aimed at modulating terpenoid scent profiles.

Flowers of *Petunia × hybrida* (Solanaceae) emit a blend of volatiles that is dominated by phenylpropanoids and benzenoids, with small amounts of fatty acid derivatives and sesquiterpenes (Verdonk *et al.*, 2003). To evaluate the potential of introducing a novel volatile component, Lückner *et al.* (2001) transformed *P. × hybrida* W115 with the gene encoding *S*-linalool synthase from *Clarkia breweri* under the control of the constitutive cauliflower mosaic virus (CMV) 35S promoter. The resulting transgenic lines produced trace amounts of 3S-(–)-linalool in flowers, while this compound was undetectable in leaves (Table 1). Interestingly, when leaf tissue was homogenized in the presence of saturated calcium chloride, *S*-linalool and α -terpineol were readily detectable. Follow-up experiments established that both of these compounds were derived from *S*-linalyl- β -D-glucopyranoside, which was found to be present in stems, leaves and some flower parts (corolla, sepals and ovary), but not in roots, pollen, styles or nectarines (Lückner *et al.*, 2001) (Fig. 2). The accumulation level of this glucoside in leaves reached up to 0.001% of fresh weight biomass. The genomes of plants contain large gene families coding for small molecule glycosyltransferases (Wang, 2009), some of which are promiscuous with regard to their substrate specificity, and it appears that such

an activity was expressed in the *P. × hybrida* host plants, thus resulting in the undesirable turnover of the target metabolite.

The constitutive expression of the *S*-linalool synthase gene from *C. breweri* in carnation (*Dianthus caryophyllus* cv. Eilat; Caryophyllaceae), which emits mostly phenylpropanoids and benzenoids from flowers, resulted in the formation of *S*-linalool (up to 6% of total volatiles) but also *cis*- and *trans*-*S*-linalool oxides (up to 4.5% of total volatiles) in flowers (Lavy *et al.*, 2002) (Fig. 2), indicating that oxidation is another (in addition to glycosylation) unplanned outcome of metabolic engineering efforts aimed at the synthesis of novel terpenoid volatiles. To avoid complications resulting from the constitutive expression of transgenes, such as those outlined above, organ- or tissue-specific promoters have been tested for terpenoid volatile production. Lewinsohn *et al.* (2001) introduced the *C. breweri* linalool synthase gene (including a plastidial targeting sequence) into tomato (*Solanum lycopersicum* Mill.; Solanaceae) under the control of the fruit-specific E8 promoter (Table 1). The authors reported an increased production of various volatiles, including *S*-linalool and its oxidation product, 8-hydroxylinalool, in ripening fruit (Fig. 2). The concentrations of other (nonvolatile) metabolites derived from plastidial terpenoid precursors, such as α - and γ -tocopherol, lycopene and β -carotene, were not affected by the introduction of the transgene (Lewinsohn *et al.*, 2001). In this context, it is important to note that terpenoid volatiles are only very minor components of tomato fruit (<0.1% of terpenoids derived from plastidial precursors in fruit of control plants). The proportion of terpenoid volatiles in elite transgenic lines was increased to about 3% of total plastid-derived terpenoids, which is substantial in terms of the increases in terpenoid volatiles, but the overall flux distribution within the fruit terpenoid pathway was obviously not altered significantly.

In an independent study, Davidovich-Rikanati *et al.* (2007) introduced a geraniol synthase gene from sweet basil (*Ocimum basilicum*) into tomato (preceded by a plastidial targeting sequence), which was placed under the control of a tomato fruit ripening-specific polygalacturonase promoter (Table 1). Transgenic plants released a whole range of terpenoid volatiles metabolically derived from geraniol, including oxidation products (e.g. geranial and geranic acid), C-C double bond-reduced metabolites (citronellol and its oxidation products), C-C double bond *cis/trans* isomerization products (neral and its oxidized and reduced forms) and acetylated conjugates (geranyl and citronellyl acetates). Interestingly, various monoterpenes that are not derived from geraniol, most prominently myrcene, limonene and β -ocimene, were also produced at high rates in transgenic lines. The production levels of terpenoid volatiles were fairly high in transgenic plants (~4 μ g/g or 0.0004% of fresh weight biomass), which resulted in significant (40–50%) decreases in the amounts of other terpenoids derived from plastidial precursor pools (phytoene, lycopene, β -carotene and norisoprenes that are derived from carotenoid turnover) (Davidovich-Rikanati *et al.*, 2007). As the red coloration of tomato fruit at maturity is determined largely by the accumulation of lycopene, the transgenic plants in this study had a paler yellowish/reddish appearance. In an organoleptic evaluation, untrained panellists apparently preferred the scent properties of transgenic fruit over controls (Davidovich-Rikanati *et al.*, 2007), indicating the potential of using metabolic engineering for positively affecting consumer traits (in this case scent).

Transgenic tomato plants expressing a multiproduct sesquiterpene synthase gene (termed α -zingiberene synthase (ZIS) based

Table 1 Metabolic engineering of the biosynthesis of volatilized plant terpenoids

Engineered plant species	Gene(s)	Gene source	Strategy	Promoter	Targeting	Alterations/Consequences	References
<i>Petunia</i> × <i>hybrida</i> W115	Linalool synthase (LIS)	<i>Clarkia breweri</i> (A. Gray) Greene	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	S-Linalyl-6-D-glucopyranoside ↑	Lücker <i>et al.</i> (2001)
Carnation (<i>Dianthus caryophyllus</i> L. cv. Ellat)	Linalool synthase (LIS)	<i>Clarkia breweri</i> (A. Gray) Greene	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	Linalool ↑; cis-linalool oxide ↑; trans-linalool oxide ↑	Lavy <i>et al.</i> (2002)
Tomato (<i>Solanum lycopersicum</i> Mill)	Linalool synthase (LIS)	<i>Clarkia breweri</i> (A. Gray) Greene	Ox	E8 (late-ripening-specific)	Organ: fruit; Cell: plastids	S-Linalool ↑; 8-hydroxylinalool ↑; other volatiles ↑; unchanged: α-tocopherol, γ-tocopherol, lycopene, β-carotene, lutein	Lewinsohn <i>et al.</i> (2001)
Tomato (<i>Solanum lycopersicum</i> L.)	Geraniol synthase (GES)	Basil (<i>Ocimum basilicum</i> L. cv. Sweet Dani)	Ox	Polygalacturonase (ripening-specific)	Organ: fruit; Cell: plastids	Geraniol ↑; geraniol ↑; geranic acid ↑; nerol ↑; neral ↑; neric acid ↑; citronellol ↑; citronellal ↑; citronellic acid ↑; phytoene ↓; lycopene ↓	Davidovich-Rikanati <i>et al.</i> (2007)
Tomato (<i>Solanum lycopersicum</i> L.)	Multiproduct zingiberene synthase (ZIS)	Basil (<i>Ocimum basilicum</i> L. cv. Sweet Dani)	Ox	Polygalacturonase (ripening-specific)	Organ: fruit; Cell: cytosol	α-Zingiberene ↑; α-bergamotene ↑; 7-epi sesquithujene ↑; β-bisabolene ↑; β-curcumenene ↑; α-thujene ↑; α-pinene ↑; β-phellandrene ↑; γ-terpinene ↑	Davidovich-Rikanati <i>et al.</i> (2008)
Potato (<i>Solanum tuberosum</i> L. cv. Desiree)	α-Copaene synthase	Potato (<i>Solanum tuberosum</i> L. cv. Desiree)	Ox	Potato patatin (tuber-specific)	Organ: tuber; Cell: plastids	α-Copaene ↑	Morris <i>et al.</i> (2011)
Tobacco (<i>Nicotiana tabacum</i> L. cv. Samsun NN)	Limonene synthase	<i>Perilla frutescens</i> (L.) Britt. var. Crispa	Ox	CMV35S; E12 enhancer	Organ: ubiquitous; Cell: plastids	Limonene ↑	Ohara <i>et al.</i> (2003)
Tobacco (<i>Nicotiana tabacum</i> L. cv. Samsun NN)	Limonene synthase	<i>Perilla frutescens</i> (L.) Britt. var. Crispa	Ox	CMV35S; E12 enhancer	Organ: ubiquitous; Cell: cytosol	Limonene ↑	Ohara <i>et al.</i> (2003)
Tobacco (<i>Nicotiana tabacum</i> L. cv. Samsun NN)	Limonene synthase	<i>Perilla frutescens</i> (L.) Britt. var. Crispa	Ox	CMV35S; E12 enhancer	Organ: ubiquitous; Cell: ER	No change	Ohara <i>et al.</i> (2003)
Tobacco (<i>Nicotiana tabacum</i> L. cv. Petit Havana SR11)	γ-Terpinene synthase (TER); limonene synthase (LIM); β-pinene synthase (PIN)	Lemon (<i>Citrus limon</i> L. Burm. F.)	Ox	CMV35S	Organ: ubiquitous; Cell: plastids	β-Phenene ↑; limonene ↑; γ-terpinene ↑	Lücker <i>et al.</i> (2004a)
Tobacco (<i>Nicotiana tabacum</i> L. cv. Petit Havana SR11)	γ-Terpinene synthase (TER); limonene synthase (LIM); β-pinene synthase (PIN); limonene-3-hydroxylase LIM3H)	Lemon (<i>Citrus limon</i> L. Burm. F.) (TER, LIM, PIN); Curly mint (<i>Mentha spicata</i> L. var. Crispa) (LIM3H)	Ox	CMV35S	Organ: ubiquitous; Cell: plastids (TER, LIM, PIN); Cell: ER (LIM3H)	(+)-Isopipitenol ↑; 1,3,8-p-menthatriene ↑; 1,5,8-p-TER, menthatriene ↑; p-cymene ↑; cis-isopipitenone ↑; LIM, PIN) trans-isopipitenone ↑	Lücker <i>et al.</i> (2004b); El Tamer <i>et al.</i> (2003)

Table 1 Continued

Engineered plant species	Gene(s)	Gene source	Strategy	Promoter	Targeting	Alterations/Consequences	References
Tobacco (<i>Nicotiana tabacum</i>) L. cv. Xanthi	β -Glucosidase (unspecific)	<i>Aspergillus niger</i>	Ox	CMV 35S	Organ: ubiquitous; Cell: cytosol	No change	Wei et al. (2004)
Tobacco (<i>Nicotiana tabacum</i>) L. cv. Xanthi	β -Glucosidase (unspecific)	<i>Aspergillus niger</i>	Ox	CMV 35S	Organ: ubiquitous; Cell: cell wall	β -Caryophyllene $\uparrow$	Wei et al. (2004)
Tobacco (<i>Nicotiana tabacum</i>) L. cv. Xanthi	β -Glucosidase (unspecific)	<i>Aspergillus niger</i>	Ox	CMV 35S	Organ: ubiquitous; Cell: lytic vacuole	Cembrene $\uparrow$	Wei et al. (2004)
Tobacco (<i>Nicotiana tabacum</i>) L. cv. Xanthi	β -Glucosidase (unspecific)	<i>Aspergillus niger</i>	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	No change	Wei et al. (2004)
Tobacco (<i>Nicotiana tabacum</i>) L. cv. Xanthi	β -Glucosidase (unspecific)	<i>Aspergillus niger</i>	Ox	CMV 35S	Organ: ubiquitous; Cell: ER	Cembrene $\uparrow$	Wei et al. (2004)

CMV 35S, cauliflower mosaic virus promoter; ER, endoplasmic reticulum; Ox, overexpression.

on the main product formed by the encoded enzyme) from sweet basil, under control of the fruit ripening-specific tomato polygalacturonase promoter, accumulated high levels of α -zingiberene (up to $\sim 1 \mu\text{g/g}$ or 0.0001% of fresh weight biomass), but also many other mono- and sesquiterpenes that were only found in minute amounts in controls or were entirely undetectable (Davidovich-Rikanati et al., 2008) (Table 1). In *in vitro* assays, recombinant ZIS, expressed in *E. coli*, converted FPP to sesquiterpenes (α -zingiberene as main product), but was also found capable of converting GPP to monoterpenes (Iijima et al., 2004), which partially explains the fact that transgenic plants expressing the ZIS gene accumulated both mono- and sesquiterpenes. However, this raises interesting questions about precursor availability. Most geranyl diphosphate synthases are localized to plastids, while farnesyl diphosphate synthases are mostly cytosolic (with some exceptions mentioned above). The ZIS cDNA used for transformation did not contain a recognizable targeting sequence and would thus be expected to localize to the cytosol (Iijima et al., 2004). This means that a cytosolic pool of GPP for further conversion into monoterpenes by ZIS has to be present in ripening tomato fruit. This precursor pool could be generated by export of GPP from plastids to the cytosol or through a cytosolic GPP synthase activity. Alternatively, it is conceivable that cytosolic GPP is released by FPP synthase. However, the catalytic efficiency, ($K_{\text{cat}}/K_{\text{m}}$), for the first half reaction catalysed by FPP synthase ($\text{DMAPP} + \text{IPP} \rightarrow \text{GPP}$) is about five times lower than that of the second half reaction ($\text{GPP} + \text{IPP} \rightarrow \text{FPP}$), and only a mutated version of the enzyme releases notable amounts of GPP (Stanley Fernandez et al., 2000). Further experiments are needed to discover the source(s) of cytosolic GPP in tomato fruit.

A gene encoding an α -copaene synthase was cloned from tubers of a fragrant potato variety (*Solanum tuberosum* L. cv. Phureja; Solanaceae) by Ducreux et al. (2008) and subsequently introduced into a nonfragrant potato cultivar (Desiree) (Morris et al., 2011) (Table 1). Tuber-specific over-expression of this cDNA resulted in an up to 15-fold enhanced α -copaene concentration in tubers, but an organoleptic evaluation by an untrained panel did not reveal any significant differences in the aroma of tubers from transgenic plants when compared to controls. The authors thus concluded that α -copaene was not a major contributor to potato aroma.

Metabolic engineering efforts towards manipulating terpenoid volatile production have attempted to address the issue of different pool sizes for the terpenoid precursors GPP and FPP in different subcellular compartments. Ohara et al. (2003) expressed a limonene synthase gene from *Perilla frutescens* in tobacco (*Nicotiana tabacum* cv. Samsun NN; Solanaceae) under control of the CMV 35S promoter with E12 enhancer (Table 1). In one construct, the limonene synthase cDNA (containing a plastidial targeting sequence) was left unchanged, while the putative plastidial targeting sequence was removed for a second construct (cytosolic targeting expected), and an endoplasmic reticulum (ER) sorting signal was added in a third construct. The specific activity of limonene synthase was generally higher in transgenics expressing the plastid localization construct compared to those containing the cytosolic enzyme. Transgenic tobacco plants harboring limonene synthase in plastids accumulated small amounts of limonene in leaves (143 ng/g or 0.0000143% of fresh weight biomass), which was even lower in those bearing a cytosolic limonene synthase (40 ng/g or 0.000004% of fresh weight biomass). The ER-localized limonene synthase was found to be inactive, and plants were not further evaluated (Ohara et al., 2003).

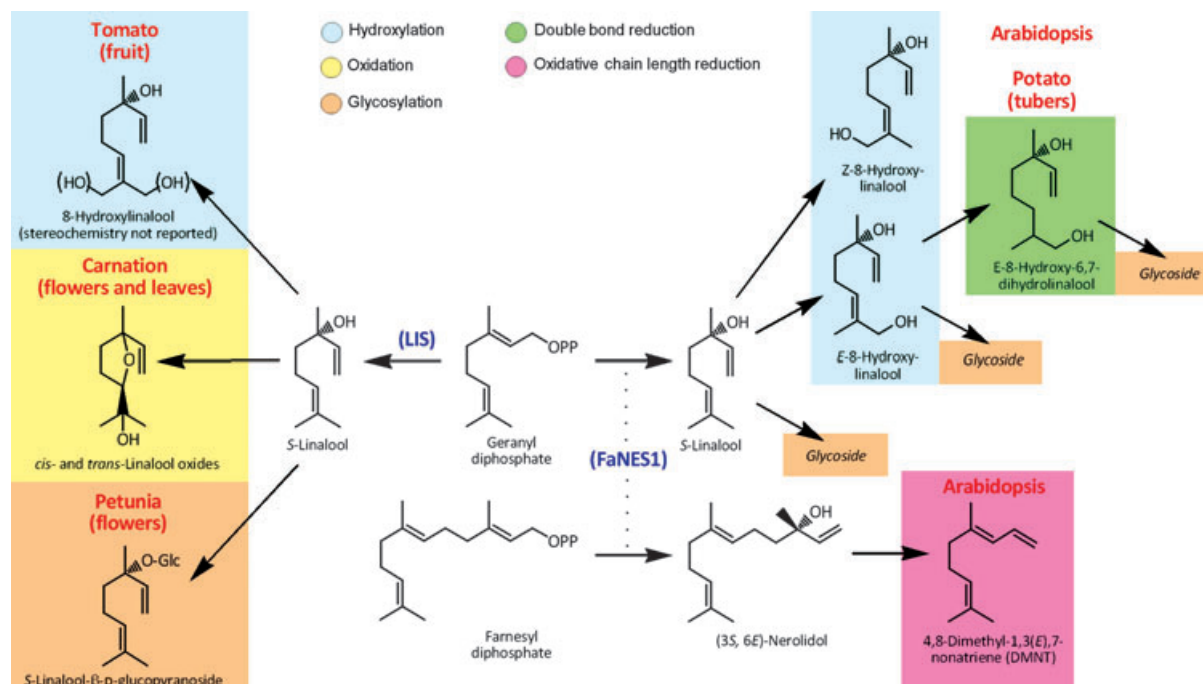


Figure 2 Metabolism of S-linalool and 3S-(E)-nerolidol in transgenic plants. After genomic integration of a linalool synthase (LIS) gene (from *Clarkia breweri*), S-linalool was further metabolized to 8-hydroxylinalool in tomato fruit (Lewinsohn *et al.*, 2001), *cis*- and *trans*-linalool oxide in carnation flowers and leaves (Lavy *et al.*, 2002) or S-linalool-β-D-glucopyranoside in *Petunia* flowers (Lücker *et al.*, 2001). Following transformation of *Arabidopsis* with a bifunctional linalool/nerolidol synthase (FaNES1) gene (from *Fragaria* × *ananassa*), S-linalool was oxidized to (Z)-8-hydroxylinalool, (E)-8-hydroxylinalool and (E)-8-hydroxy-6,7-dihydroxylinalool, which were then further metabolized to the corresponding glycosides (Aharoni *et al.*, 2003). (3S,6E)-Nerolidol accumulated as well. Oxidized and glycosylated metabolites derived from linalool were also detected in tubers of transgenic potato transformed with the FaNES1 gene. When the FaNES1 gene product was targeted to mitochondria of transgenic *Arabidopsis* plants, a metabolic conversion of (3S,6E)-nerolidol to 4,8-dimethyl-1,3(E),7-nonatriene was reported (Kappers *et al.*, 2005).

Monoterpene production levels in tobacco leaves are generally low, and several studies have demonstrated that metabolic engineering can be employed to generate transgenic plants with humanly detectable alterations in the emission of monoterpene volatile blends (Table 1). When three monoterpene synthase genes from lemon (*Citrus limon* L. Burm. F.), which code for γ-terpinene synthase, (+)-limonene synthase and β-pinene synthase, were expressed in a single transgenic tobacco (*N. tabacum* cv. Petit Havana SR1) line under control of the CMV 35S promoter, emissions of γ-terpinene, (+)-limonene and β-pinene, as well as other monoterpenes generated by the introduced monoterpene synthases as side products (Lücker *et al.*, 2002), were detected from flowers in the ng to μg g⁻¹ d⁻¹ range (Lücker *et al.*, 2004a). At the early stages of flower development, the rate of terpenoid emission was ~9-fold higher from flowers of the transgenic lines compared to wild-type controls, which then decreased over time and eventually reached wild-type levels. At later stages of flower development, monoterpene emission appeared to occur at the expense of decreased sesquiterpene emission (Lücker *et al.*, 2004a). Human panellists were able to distinguish the differences in emitted volatiles from flowers of the transgenic tobacco line in comparison with controls (El Tamer *et al.*, 2003). These studies established that tobacco, which is readily transformable and has a short regeneration time, can be used as an experimental model system to test metabolic engineering strategies for monoterpenoid volatile production. In a follow-up investigation, the transgenic tobacco line expressing three monoterpene synthase genes was additionally transformed with a limonene 3-hydroxylase (LIM3H) cDNA from curly mint

(*Mentha spicata* L. var. Crispa) (Lücker *et al.*, 2004b). Although the enzymes encoded by the transgenes localized to different compartments (genes coding for monoterpene synthases contain plastidial targeting sequences, while LIM3H has the typical features of an ER-localized cytochrome P450-dependent monooxygenase), the authors reported the detection of (+)-*trans*-isopiperitenol, the product of LIM3H when (+)-limonene is available as a substrate, in headspace volatiles. Interestingly, this novel metabolite was further converted oxidatively into (+)-*trans*-isopiperitenone, 1,3,8-*p*-menthatriene, 1,5,8-*p*-menthatriene and *p*-cymene (Lücker *et al.*, 2004b). A novel terpenoid, introduced by metabolic engineering, was again metabolized by endogenous enzymatic activities, thus generating metabolites that neither occur naturally in the source organism of the transgene nor in the host plant used in the transformation.

Many natural products can accumulate as glycosides. To test whether the hydrolysis of the glycosidic bond would lead to an increased release of terpenoid volatiles, Wei *et al.* (2004) introduced a nonspecific β-glucosidase gene (BGL1; from the fungus *Aspergillus niger*) into transgenic tobacco (*N. tabacum* L. cv. Xanthi) plants. The authors used constructs to target the gene product to different subcellular locations. A localization of BGL1 to the cytosol or plastids did not result in any changes of volatile emissions (Table 1). However, when targeted to the cell wall or ER, the presence of BGL1 led to the increased volatilization of the diterpene cembrene, while a cell wall localization caused trans-caryophyllene emissions (Wei *et al.*, 2004) (Table 1). This approach could potentially be further developed to release volatiles with the aim of modulating plant–plant, plant–microbe or plant–insect

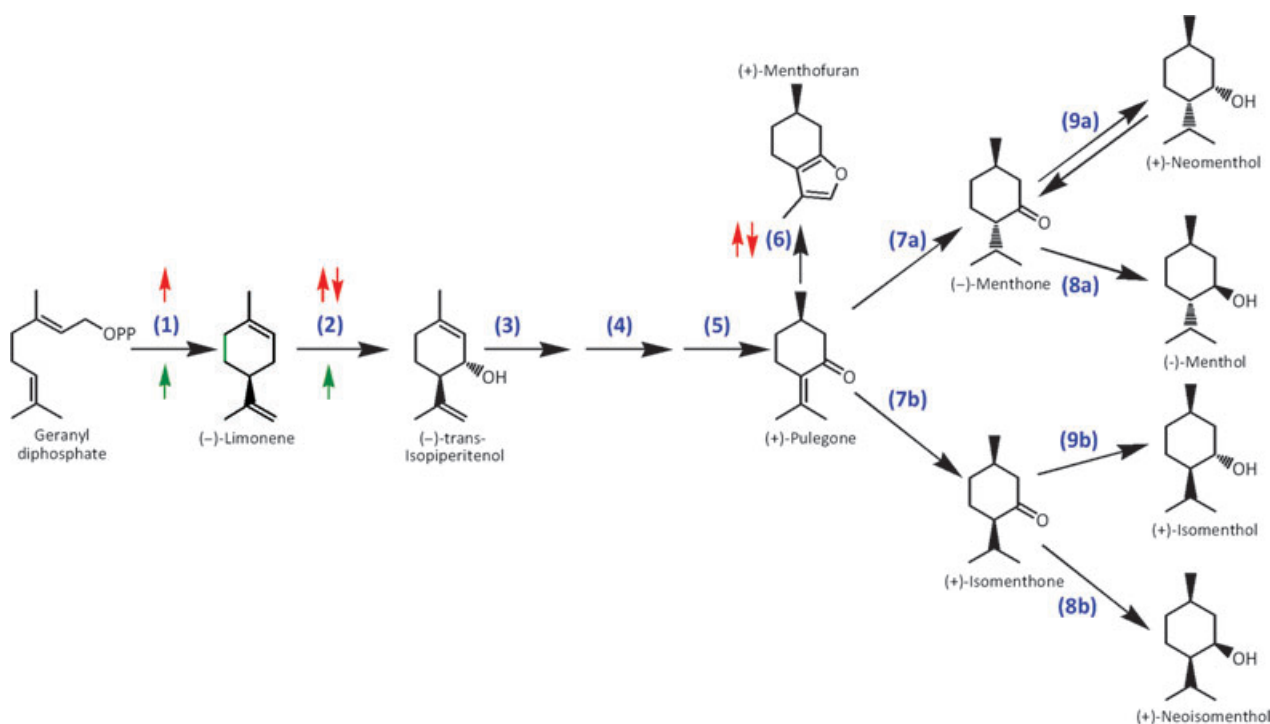


Figure 3 Overview of the p-menthane monoterpene biosynthetic pathway in peppermint glandular trichomes. The enzymes involved in this pathway are as follows: (1), (–)-limonene synthase; (2), (–)-limonene 3-hydroxylase; (3), (–)-trans-isopiperitenol dehydrogenase; (4), (–)-trans-isopiperitenone reductase; (5), (+)-cis-isopulegone isomerase; (6), (+)-menthofuran synthase; (7a and 7b), (+)-pulegone reductase; (8a), (–)-menthone: (–)-menthol reductase; (8b), (–)-menthone: (–)-menthol reductase; (9a), (–)-menthone: (–)-neomenthol reductase; (9b), (–)-menthone: (–)-neomenthol reductase. Red arrows are used to indicate which transcripts have been up- or down-regulated in transgenic peppermint plants. Green arrows indicate which mint genes have been expressed in transgenic tobacco plants.

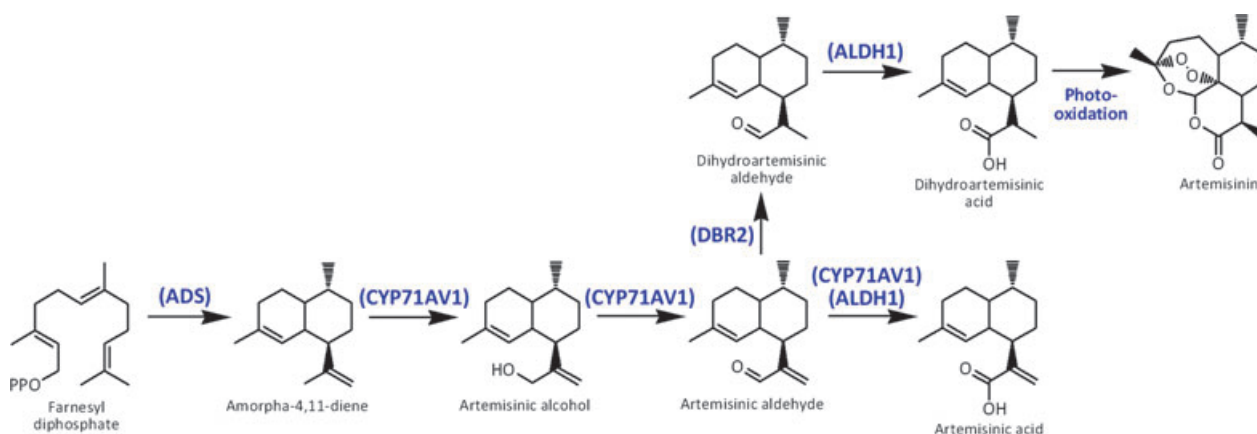


Figure 4 Overview of the artemisinin biosynthetic pathway in *Artemisia annua* glandular trichomes. Acronyms: ADS, amorpha-4,11-diene synthase; CYP71AV1, amorpha-4,11-diene monooxygenase; DBR2, artemisinic aldehyde $\Delta 11(13)$ double bond reductase; ALDH1, aldehyde dehydrogenase 1.

communications, thus increasing disease resistance. Examples of similar applications are provided in the next section.

Terpenoid volatiles in plant defence

Volatilized terpenoids play various important roles in plant–environment interactions and plant–plant communication. For example, terpenoids emitted by flowers can attract pollinating

insects (Van Schie *et al.*, 2006). Airborne terpenoids are also critical components of plant defence responses to abiotic and biotic stresses (Unsicker *et al.*, 2009; Vickers *et al.*, 2009), signalling among plant organs (Heil and Silva Bueno, 2007) and plant–plant communication (Baldwin *et al.*, 2006). From an agronomic perspective, crop losses due to insect infestation are a significant issue (El-Wakeil *et al.*, 2010). Application of insecticides is a common and effective insect management strategy,

but some of these agrochemicals have undesirable side effects on beneficial insects and can pose long-term risks to the environment (Dedryver *et al.*, 2010). Modifications in the quantities of emitted terpenoid volatiles and their composition through metabolic engineering have the potential to enable more sustainable agricultural practices by decreasing chemical inputs.

Hohn and Ohlrogge (1991) expressed trichodiene synthase, a sesquiterpene synthase from the fungal plant pathogen *Fusarium sporotrichioides*, in transgenic tobacco (*N. tabacum* cv. Petite Havana) under control of the CMV 35S promoter. The authors reported the production of trace levels of trichodiene (in the low ng/g fresh weight range) in this proof-of-concept study but did not present data on biological effects of this manipulation (Table 2).

Aharoni *et al.* (2003) expressed a multifunctional linalool/nerolidol synthase (FaNES1) gene from strawberry (*Fragaria × ananassa* cv. Elsanta) in *Arabidopsis thaliana* L. (Col-0 ecotype). The terpene synthase gene was expressed with its native plastidial targeting sequence under control of the CMV 35S promoter. Plants with high expression levels of FaNES1 emitted the monoterpene *S*-linalool, but also accumulated several hydroxylated and subsequently glycosylated linalool derivatives (up to ~ 140 µg/g or 0.014% of fresh weight biomass) (Table 2) (Fig. 2). The sesquiterpene 3*S*-(*E*)-nerolidol was produced only at trace levels, indicating that FPP, the direct precursor of sesquiterpene formation via FaNES1, was limiting in plastids (Aharoni *et al.*, 2003). The authors demonstrated that aphid colonization in dual-choice assays was diminished in transgenic plants (compared to untransformed controls), but the transgene expression also resulted in a severe growth retardation. The authors speculated that the stunted phenotype in transgenic plants accumulating *S*-linalool derivatives may have been caused by a dramatically reduced availability of plastidial pools of substrates for other terpenoids, such as chlorophylls, carotenoids and terpenoid growth regulators (Aharoni *et al.*, 2003). The same study also evaluated the effects of expressing a heterologous sesquiterpene synthase gene in the cytosol (Table 2). A germacrene A synthase gene from chicory (*Cichorium intybus*) was expressed in *Arabidopsis* under control of the CMV 35S promoter, but only trace amounts of the expected product, germacrene A, were produced (Aharoni *et al.*, 2003), which is indirect evidence for tight control of FPP pools in the cytosol. In follow-up work, the FaNES1 gene (including a plastidial targeting sequence) was expressed constitutively (this time using the *Chrysanthemum* Rubisco small subunit promoter) in potato, which also resulted in the production of *S*-linalool and its hydroxylated and glycosylated derivatives (Aharoni *et al.*, 2006) (Table 2). The FaNES1 gene (once again containing a plastidial targeting sequence) was also transformed into *Arabidopsis* under control of a wound-inducible potato PI2 promoter (Yang *et al.*, 2008) (Table 2). Transgenic plants were phenotypically indistinguishable from wild-type controls (Yang *et al.*, 2008), which is an advantage over transgenics that expressed the FaNES1 gene under control of a constitutive promoter and were severely impacted by growth retardation (Aharoni *et al.*, 2003, 2006). After induction with methyl jasmonate, linalool production was demonstrated (albeit without providing quantitative data) (Yang *et al.*, 2008). Diamondback moth feeding, however, did not trigger linalool emission. Kappers *et al.* (2005) modified the FaNES1 expression construct to target the gene product to mitochondria in *Arabidopsis*. Interestingly, in contrast to the results obtained with the construct that targeted FaNES1 to

plastids, a mitochondrial localization of the enzyme enabled the production of substantial quantities of the sesquiterpene 3*S*-(*E*)-nerolidol, whereas no formation of the monoterpene *S*-linalool was reported (Table 2). Based on these results, it appears that FPP pools in mitochondria were sufficient to support sesquiterpene biosynthesis, while GPP must have been all but absent. Transgenic plants generated as part of this effort also synthesized the C11-homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (Fig. 2), which has been demonstrated to be formed from 3*S*-(*E*)-nerolidol by CYP82G1, a ER-localized cytochrome P450 monooxygenase (Lee *et al.*, 2010). The emission of two novel terpenoids from transgenic plants was shown to attract predatory mites, which could potentially act as a biological control agent to address arthropod infestations (Kappers *et al.*, 2005).

Wu *et al.* (2006) completed one of the most comprehensive studies investigating the targeting of gene products to specific subcellular locations and the effects of such experimental modifications (all constructs used promoters for the constitutive expression of transgenes). When patchoulol synthase (PTS), a sesquiterpene synthase from *Pogostemon cabli* L., was targeted to the cytosol in transgenic tobacco (*N. tabacum* L. cv. Xanthi), only small amounts of the expected product, patchoulol, were detected (up to 0.0001% of fresh weight biomass) (Table 1). When the same gene was expressed coordinately with an additional copy of farnesyl diphosphate synthase (FPS), the patchoulol accumulation in transgenic tobacco also remained very low (Wu *et al.*, 2006). However, when both gene products (PTS and FPS) were targeted to plastids, a patchoulol accumulation of up to 0.003% of fresh weight biomass was reported, the majority of which appeared to be volatilized (Wu *et al.*, 2006). Interestingly, the authors also demonstrated that the volatiles emitted from these transgenic plants significantly deterred tobacco hornworms from feeding on leaves (Wu *et al.*, 2006) (Table 2).

Several sesquiterpenes have been implicated in tritrophic (plant–herbivore and herbivore–predator) interactions, with demonstrated negative effects of plant volatile emissions on herbivore colonization and/or positive effects on the attraction of carnivorous natural enemies (also referred to as ‘bodyguards’) of insect pests (Mumm and Dicke, 2010; Vandermoten *et al.*, 2012). When lepidopteran larvae feed on leaves, maize plants release a mixture of volatiles that attracts parasitic wasps (Turlings *et al.*, 1990). Schnee *et al.* (2006) identified and characterized a multiproduct sesquiterpene synthase (TPS10) from maize (*Zea mays* L. B73) that forms a blend of sesquiterpenes that is reminiscent of that induced by herbivory. When the TPS10 gene was introduced into *Arabidopsis* (Col-0 ecotype), the emitted volatiles were identical to those released by maize (Table 2). Herbivore parasitoids were attracted by the sesquiterpenes emitted by the transgenic plants (Schnee *et al.*, 2006), indicating the potential of using metabolic engineering to reduce herbivore attacks. Beale *et al.* (2006) expressed an (*E*)-β-farnesene synthase gene (Eβf; cytosolic targeting of encoded enzyme) from peppermint (*Mentha × piperita* L. cv. Black Mitcham) ubiquitously in *Arabidopsis* (Col-0 ecotype) and observed (*E*)-β-farnesene emission, with a concomitant decrease in the volatilization of the endogenous sesquiterpene β-caryophyllene (Table 2). Aphid colonization was significantly diminished in transgenic plants when compared to untransformed controls, while at the same time aphid predators were attracted by the volatile emissions (Beale *et al.*, 2006). Diminished aphid colonization and increased attraction of aphid predators were also observed when an Eβf

Table 2 Metabolic engineering of terpenoid volatiles as a defense against insect feeding

Gene(s)	Engineered plant species	Gene source(s)	Strategy	Promoter	Targeting	Alterations/Consequences	References
Trichodiene synthase	Tobacco (<i>Nicotiana tabacum</i> L. cv. Petite Havana)	<i>Fusarium sporotrichioides</i>	Ox	CMV35S	Organ: ubiquitous; Cell: cytosol	Trichodiene ↑	Hohn and Ohlrogge (1991)
Linalool/nerolidol synthase (FaNESI)	Thale cress (<i>Arabidopsis thaliana</i> (L.) Heynh.; Col-0 ecotype)	Strawberry (<i>Fragaria × ananassa</i> cv. Elsanta)	Ox	CMV 35S (doubled)	Organ: ubiquitous; Cell: plastids	(S)-Linalool ↑; (Z)-8-hydroxylinalool ↑; (E)-8-hydroxy-6,7-dihydroxylinalool ↑; glycosylated terpene alcohols ↑; (3S)- <i>E</i> -nerolidol ↑; diminished aphid colonization; severe growth retardation;	Aharoni <i>et al.</i> (2003)
Linalool/nerolidol synthase (FaNESI)	Potato (<i>Solanum tuberosum</i> L.)	Strawberry (<i>Fragaria × ananassa</i> cv. Elsanta)	Ox	<i>Chrysanthemum</i> Rubisco small subunit	Organ: ubiquitous; Cell: plastids	(S)-Linalool ↑; (Z)-8-hydroxylinalool ↑; (E)-8-hydroxy-6,7-dihydroxylinalool ↑; glycosylated terpene alcohols ↑; (3S)- <i>E</i> -nerolidol ↑; severe growth retardation	Aharoni <i>et al.</i> (2006)
Linalool/nerolidol synthase (FaNESI)	Thale cress (<i>Arabidopsis thaliana</i> (L.) Heynh.; Col-0 ecotype)	Strawberry (<i>Fragaria × ananassa</i> cv. Elsanta)	Ox	Potato PI 2 (wound-inducible)	Organ: ubiquitous; Cell: plastids	(S)-Linalool ↑ (no quantification!)	Yang <i>et al.</i> (2008)
Linalool/nerolidol synthase (FaNESI)	Thale cress (<i>Arabidopsis thaliana</i> (L.) Heynh.; Col-0 ecotype)	Strawberry (<i>Fragaria × ananassa</i> cv. Elsanta)	Ox	CMV35S	Organ: ubiquitous; Cell: mitochondria	(3S)- <i>E</i> -Nerolidol; ↑ 4,8-dimethyl-1,3(E),7-nonatriene ↑; attraction of aphid predatory mites	Kappers <i>et al.</i> (2005)
Germacrene A synthase (CiGAS10)	Thale cress (<i>Arabidopsis thaliana</i> (L.) Heynh.; Col-0 ecotype)	Chicory (<i>Cichorium intybus</i> L.)	Ox	CMV35S	Organ: ubiquitous; Cell: cytosol	Germacrene A ↑ (trace amounts)	Aharoni <i>et al.</i> (2003)
Patchoulol synthase (PTS)	Tobacco (<i>Nicotiana tabacum</i> L. cv. Xanthi)	Patchouli (<i>Pogostemon cablin</i> (Blanco) Benth) (PTS)	Ox	CMV	Organ: ubiquitous; Cell: cytosol	Patchoulol ↑	Wu <i>et al.</i> (2006)
Patchoulol synthase (PTS); farnesyl diphosphate synthase (FPS)	Tobacco (<i>Nicotiana tabacum</i> L. cv. Xanthi)	Patchouli (<i>Pogostemon cablin</i> (Blanco) Benth) (PTS); chicken (<i>Gallus gallus domesticus</i>) (FPS)	Ox	CMV(PTS); CMV 35S(FPS)	Organ: ubiquitous; Cell: cytosol	Patchoulol ↑	Wu <i>et al.</i> (2006)
Patchoulol synthase (PTS)	Tobacco (<i>Nicotiana tabacum</i> L. cv. Xanthi)	Patchouli (<i>Pogostemon cablin</i> (Blanco) Benth) (PTS)	Ox	CMV	Organ: ubiquitous; Cell: plastids	Patchoulol (traces)	Wu <i>et al.</i> (2006)
Patchoulol synthase (PTS); farnesyl diphosphate synthase (FPS)	Tobacco (<i>Nicotiana tabacum</i> L. cv. Xanthi)	Patchouli (<i>Pogostemon cablin</i> (Blanco) Benth) (PTS); chicken (<i>Gallus gallus domesticus</i>) (FPS)	Ox	CMV (PTS); CMV 35S(FPS)	Organ: ubiquitous; Cell: plastids	Patchoulol ↑ ↑; reduced hornworm feeding growth retardation; reduced fertility	Wu <i>et al.</i> (2006)
Multifunct. sesquiterpene synthase (TPS10)	Thale cress (<i>Arabidopsis thaliana</i> Heynh.; Col-0 ecotype)	Maize (<i>Zea mays</i> L. B73Zea) (L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: cytosol	α-Bergamotene ↑; (E)-β-farnesene ↑; other sesquiterpenes ↑; attraction of lepidopteran predatory wasps	Schnee <i>et al.</i> (2006)
(E)-β-Farnesene synthase (Eβf)	Thale cress (<i>Arabidopsis thaliana</i> (L.) Heynh.; Col-0 ecotype)	Peppermint (<i>Mentha × piperita</i> L. cv. Black Mitcham)	Ox	CMV35S	Organ: ubiquitous; Cell: cytosol	(E)-β-Farnesene ↑; β-caryophyllene ↓; diminished aphid colonization; attraction of aphid predators	Beale <i>et al.</i> (2006)

Table 2 Continued

Gene(s)	Engineered plant species	Gene source(s)	Strategy	Promoter	Targeting	Alterations/Consequences	References
(E)- β -Farnesene synthase (E β F)	Tobacco (<i>Nicotiana tabacum</i> L. cv. W38)	Sweet wormwood (<i>Artemisia annua</i> L. strain 025)	Ox	CMV35S	Organ: ubiquitous; Cell: cytosol	(E)- β -Farnesene $\uparrow$; diminished aphid colonization; attraction of aphid predators (caution: preliminary data)	Yu <i>et al.</i> (2012a)
Geraniol synthase	Maize (<i>Zea mays</i> L. PHWEE (Pioneer HiBred))	Mexican lippia (<i>Lippia dulcis</i>)	Ox	CMV 35S enhancer, maize ubiquitin	Organ: ubiquitous; Cell: plastids	Geranyl acetate $\uparrow$; hydroxylated and glycosylated geraniol derivatives (no structure elucidation) $\uparrow$; no change in fungal resistance	Yang <i>et al.</i> (2011)
Cytochrome P450-dependent monooxygenase	Tobacco (<i>Nicotiana tabacum</i> L. cv. Xanthi)	Tobacco (<i>Nicotiana tabacum</i> L. cv. Xanthi)	Anti	CMV 35S	Organ: ubiquitous; Cell: n.a.	Cembratriene-diol (CBT-diol); $\downarrow$ Cembratriene (CBT-ol) $\uparrow$; diminished aphid colonization	Wang <i>et al.</i> (2001)
Limonene synthase	Orange (<i>Citrus sinensis</i> (L.) Osbeck)	Satsuma mandarin (<i>Citrus unshiu</i> (Swingle) Marcow)	Anti	CMV 35S	Organ: ubiquitous; Cell: n.a.	Limonene (peel) $\downarrow$; increased resistance against fungus and bacterium; diminished fruit fly colonization	Rodríguez <i>et al.</i> (2011)

Anti, antisense repression; CMV, cauliflower mosaic virus promoter; CVMV, Cassava vein mosaic virus promoter; ER, endoplasmic reticulum; n.a., not applicable; Ox, overexpression.

gene from sweet wormwood (*Artemisia annua* L. strain 025) was expressed in tobacco (*N. tabacum* L. cv. W38) (Yu *et al.*, 2012a) (Table 2).

To test if the accumulation of geranic acid, which had been demonstrated to be an effective growth inhibitor for certain fungi, could be used to increase disease resistance, Yang *et al.* (2011) transformed maize (*Zea mays* L.; genotype PHWWE (Pioneer HiBred)) with a geraniol synthase gene from Mexican lippia (*Lippia dulcis*) under control of the constitutive ubiquitin promoter (Table 2). Transgenic plants accumulated geranyl acetate and several hydroxylated and glycosylated derivatives of geranic acid, but bioassays indicated that there was no improved resistance to fungi, possibly because of the further metabolism of geranic acid into inactive derivatives.

As part of a gene discovery effort, Wang *et al.* (2001) used an antisense approach to knock-down the expression of a putative cytochrome P450 monooxygenase gene, using a constitutive promoter, in transgenic *N. tabacum* cv. T.I. 1068 plants (Table 2). Transgenics accumulated ~ 19-fold higher concentrations (compared to wild-type controls) of cembratrienol (up to 4.3% of dry leaf biomass) in glandular trichomes. Exudate harvested from antisense-expressing plants had increased activity (compared to wild-type controls) against aphids, which demonstrates the utility of antisense approaches in the metabolic engineering of disease-related traits. An antisense strategy was also used to reduce the expression levels of the limonene synthase gene in orange (*Citrus sinensis* (L.) Osbeck) (Rodríguez *et al.*, 2011) (Table 2). The peel of fruit from transgenic orange trees had dramatically reduced limonene concentrations (no absolute quantitation data provided). When challenged with a fungus or a bacterium, transgenic fruit showed increased resistance (compared to fruit peel of wild-type controls). In addition, fruit flies were less attracted to fruit accumulating less limonene compared to those with wild-type limonene levels. As no other phenotypic differences between transgenic plants and controls were observed, the antisense approach could potentially be used to improve pest resistance in the field.

In summary, transgenic plants that emitted enhanced levels of certain terpenoids due to the expression of heterologous terpene synthases were found to have a reduced aphid colonization (Aharoni *et al.*, 2003; Beale *et al.*, 2006; Yu *et al.*, 2012a), deter the feeding of hornworms (Wu *et al.*, 2006) or attract predatory mites as 'bodyguards' against arthropod infestation (Kappers *et al.*, 2005). Interestingly, the downregulation of the expression levels of genes involved in terpenoid biosynthesis has also been used successfully to increase disease resistance against aphids or fruit flies (Rodríguez *et al.*, 2011; Wang *et al.*, 2001). Taken at face value, these results may appear to be contradictory. However, it is important to note that the chemical ecology of terpenoid volatiles is very complex, and changes in terpenoid emissions might have deterrent effects on one species while other species might be attracted (Unsicker *et al.*, 2009). When designing metabolic engineering strategies, it is thus of utmost importance to consider interactions of plants with herbivores and herbivore enemies, and potentially other trophic levels.

Extractable essential oil terpenoids

On plant surfaces, glandular trichomes often synthesize large quantities of metabolic products, including terpenoid essential oils (Wagner, 1991). Transformation protocols have been published for many different essential oil species. However, the

transformation rates currently achievable with the majority of species that accumulate terpenoid oils are fairly low and, in some cases, the generation cycles are quite long (years), which has precluded rapid phenotypic screens (Pitzschke and Hirt, 2010). To date, progress in engineering terpenoid essential oil yield and composition has been confined to mint, lavender and *Eucalyptus*. Over the last two decades, peppermint has become an experimental model system for furthering our understanding of essential oil biosynthesis. The developmentally controlled distribution of glandular trichomes has been studied in detail (Turner *et al.*, 2000a,b), and the p-menthane biosynthetic pathway is completely defined (Croteau *et al.*, 2005) (Fig. 3). Combining high oil yields and desirable oil composition (high levels of (–)-menthol, moderate levels of (–)-menthone and low levels of (+)-pulegone and (+)-menthofuran) is the main goal of mint biotechnology investigations. Additionally, weed control and fungal disease control in peppermint are of high interest but have not yet been addressed experimentally (Croteau *et al.*, 2005).

Arguably the most obvious approach for enhancing essential oil yield is to increase the availability of biosynthetic precursors. As mentioned before, precursors for plant terpenoids can be derived from the MVA pathway (localized to the cytosol, ER and peroxisomes), the MEP pathway (localized to plastids) or a combination of the two. Feeding studies with isotope-labelled precursors established that monoterpenes in peppermint (*Mentha × piperita* L. cv. Black Mitcham; Lamiaceae) are derived exclusively from the MEP pathway (Eisenreich *et al.*, 1997), whereas the MVA pathway is inactive in gland cells (McCaskill and Croteau, 1995). The constitutive overexpression of the gene encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS; note that DXPS is also used as an acronym in the literature), which catalyses the entry step into the MEP pathway (note that this is not the committed first step as the thiamin and pyridoxol biosynthetic pathways also involve DXS), did not result in significant yield increases in transgenic peppermint plants (Lange *et al.*, 2011) (Table 3). The first documented success at increasing essential oil yield was reported by Mahmoud and Croteau (2001), who overexpressed the endogenous gene coding for 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), which catalyses the first committed step in the MEP pathway. The authors obtained two classes of transgenic lines: one set of lines had an abnormal pigmentation with lighter green leaves and low oil yields, which was demonstrated to be due to co-suppression effects that resulted in decreases of DXR transcript levels; a second set of lines, in which DXR was truly overexpressed, had up to 44% increased oil yields when compared with wild-type controls (Mahmoud and Croteau, 2001) (Table 3). Field testing over several growth seasons indicated that, after a period that was required to establish a strong root system, transgenic peppermint plants overexpressing DXR had up to 18% higher oil yields than wild-type controls (Lange *et al.*, 2011).

A second essential oil crop that was subjected to genetic manipulation for oil yield improvements is spike lavender (*Lavandula latifolia* Medik.; Lamiaceae). The monoterpenoid essential oil of this species differs from that of the more common English lavender (*Lavandula angustifolia*) by higher relative amounts of camphor and 1,8-cineole (at the expense of linalyl acetate), which results in a more pungent scent (Lis-Balchin, 2002). The constitutive overexpression of an *Arabidopsis* DXS cDNA in spike lavender led to the most highly increased essential oil yields reported thus far (up to 359% in leaves and up to 74% in

flowers), with essentially no negative effects on oil composition (Muñoz-Bertomeu *et al.*, 2006) (Table 3), which might be interpreted as an indication for the importance of the MEP pathway for precursor supply.

Interestingly, the constitutive expression of a truncated gene encoding only the catalytic domain of the *Arabidopsis* 3-hydroxy-3-methylglutaryl-CoA reductase, an enzyme of the MVA pathway (Friesen and Rodwell, 2004), in spike lavender also resulted in significant increases in oil yields (up to ~2-fold higher than in leaves and flowers of control plants) (Muñoz-Bertomeu *et al.*, 2007) (Table 3). Other terpenoid end products primarily derived from the MVA pathway (sterols) were also increased (up to 2.7-fold higher than in leaves of control plants), while no effects were reported on the accumulation of carotenoids and chlorophylls, which are primarily derived from the MEP pathway. These results indicate that the MVA pathway in spike lavender glandular trichomes, in contrast to peppermint (McCaskill and Croteau, 1995), is operational for essential oil biosynthesis. Muñoz-Bertomeu *et al.* (2007) suggested that metabolic crosstalk between the MVA and MEP pathways in spike lavender enables the uptake of MVA-derived terpenoid intermediates from the cytosol into plastids to overcome limitations in precursor supply via the MEP pathway. Besides precursor availability, the spatial regulation of enzyme activities was suggested as an additional mechanism for controlling the biosynthesis of essential oils in spike lavender (Muñoz-Bertomeu *et al.*, 2006), but these intriguing possibilities need further investigation.

In transgenic peppermint lines overexpressing the gene encoding a native (plastidial) isoform of isopentenyl diphosphate isomerase under control of a constitutive promoter, oil yield improvements of up to 26% (compared to wild-type controls) were reported, without significant variation of the monoterpene profile (Lange *et al.*, 2011) (Table 3). Moreover, transgenic peppermint lines overexpressing an *Abies grandis* GPP synthase gene showed oil yield increases up to 18%, with a favourable oil composition (<5% (+)-menthofuran and <2% (+)-pulegone) when compared with wild-type controls (Lange *et al.*, 2011) (Table 3).

Conflicting results have been obtained with transgenic lines constitutively overexpressing the gene coding for (–)-limonene synthase (LS), which catalyses the first committed step of the p-menthane pathway of monoterpene biosynthesis. Krasnyanski *et al.* (1999) constitutively overexpressed a spearmint LS gene in peppermint. The transgenic lines accumulated mostly lower levels of (–)-menthol and higher levels of (+)-pulegone, (+)-menthofuran and (–)-menthone compared to wild type, while no yield data were reported (Krasnyanski *et al.*, 1999) (Table 3). These monoterpene patterns could be indicative of stress reactions in transgenic plants (Burbott and Loomis, 1967; Clark and Menary, 1980; Court *et al.*, 1993; Lange *et al.*, 2011; Rios-Esteva *et al.*, 2008). Mahmoud *et al.* (2004) reported the constitutive overexpression of the spearmint LS gene, as evidenced by high transcript abundance, protein concentration and enzyme activity, in glandular trichomes of transgenic peppermint plants (Table 3). However, the oil yield and monoterpene composition were the same as in wild-type controls. In yet another study assessing the effects of expressing the spearmint LS gene in transgenic peppermint plants, Diemer *et al.* (2001) reported no changes in (–)-limonene levels but, surprisingly, large variations in the concentrations of other monoterpenes and increased oil yields for four of these lines (Table 3). The authors also reported dramatically enhanced (up to 200%) oil yields in transgenic plants

Table 3 Metabolic engineering of extractable essential oils

Engineered plant species	Gene(s)	Gene Source(s)	Strategy	Promoter(s)	Targeting site(s)	Alterations/Consequences	References
Peppermint (<i>Mentha × piperita</i> L. cv. Black Mitcham)	1-Deoxy-D-xylulose 5-phosphate synthase (DXS)	Peppermint (<i>Mentha × piperita</i> L. cv. Black Mitcham)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	Essential oil yield unchanged; (+)-menthofuran ↓; (+)-pulegone ↓	Lange et al. (2011)
	1-Deoxy-D-xylulose-5-phosphate reductoisomerase (DXR)	Peppermint (<i>Mentha × piperita</i> L. cv. Black Mitcham)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	Essential oil yield ↑↑	Mahmoud and Croteau (2001)
	Isopentenyl diphosphate isomerase	Peppermint (<i>Mentha × piperita</i> L. cv. Black Mitcham)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	Essential oil yield ↑	Lange et al. (2011)
	Geranyl diphosphate synthase	Grand fir (<i>Abies grandis</i> (Douglas ex D. Don) Lindley)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	Essential oil yield ↑; menthofuran ↓; pulegone ↓	Lange et al. (2011)
	(-)-Limonene synthase (LS)	Spearmint (<i>Mentha spicata</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	(-)-Menthone ↑; (+)-menthofuran ↑; (+)-pulegone ↑; (-)-menthol ↓	Krasnyanski et al. (1999)
	(-)-Limonene synthase (LS)	Spearmint (<i>Mentha spicata</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	Essential oil yield ↑↑↑	Diemer et al. (2001)
	(-)-Limonene synthase (LS)	Spearmint (<i>Mentha spicata</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	No effects on essential oil yield or composition	Mahmoud et al. (2004)
	(-)-Limonene-3-hydroxylase (L3H)	Peppermint (<i>Mentha × piperita</i> L. cv. Black Mitcham)	Ox	CMV 35S	Organ: ubiquitous; Cell: ER	No effects on essential oil yield or composition; (-)-limonene ↑ in co-suppressed lines	Mahmoud et al. (2004)
	(+)-Menthofuran synthase (MFS)	Peppermint (<i>Mentha × piperita</i> L. cv. Black Mitcham)	Anti	CMV 35S	Organ: ubiquitous; Cell: n.a.	(+)-Menthofuran ↓; (+)-pulegone ↓	Mahmoud and Croteau (2001)
	(+)-Menthofuran synthase (MFS)	Peppermint (<i>Mentha × piperita</i> L. cv. Black Mitcham)	Ox	CMV 35S	Organ: ubiquitous; Cell: ER	Menthofuran ↑ in immature leaves; no effects on essential oil composition in mature leaves	Mahmoud and Croteau (2003)
	1-Deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) and (+)-menthofuran synthase (MFS)	Peppermint (<i>Mentha × piperita</i> L. cv. Black Mitcham)	Ox (DXR); Anti (MFS)	CMV 35S	Organ: ubiquitous; Cell: plastids (DXR) Cell: n.a. (MFS)	Essential oil yield ↑; (+)-menthofuran ↓; (+)-pulegone ↓	Lange et al. (2011)
Corn mint (<i>Mentha arvensis</i> L.)	(-)-Limonene synthase (LS)	Spearmint (<i>Mentha spicata</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	No effects on essential oil yield	Diemer et al. (2001)
Spike lavender (<i>Lavandula latifolia</i> Medik.)	1-Deoxy-D-xylulose-5-phosphate synthase (DXS)	Thale cress (<i>Arabidopsis thaliana</i> (L.) Heynh., Col-0 ecotype)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	Essential oil yield ↑↑↑; various changes in monoterpene comp. (depending on plant line)	Muñoz-Bertomeu et al. (2006)
	(-)-Limonene synthase (LS)	Spearmint (<i>Mentha spicata</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	Limonene ↑ (in developing leaves)	Muñoz-Bertomeu et al. (2008)
	3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR1S)	Thale cress (<i>Arabidopsis thaliana</i> (L.) Heynh., Col-0 ecotype)	Ox	CMV 35S	Organ: ubiquitous; Cell: ER		Muñoz-Bertomeu et al. (2007)

Table 3 Continued

Engineered plant species	Gene(s)	Gene Source(s)	Strategy	Promoter(s)	Targeting site(s)	Alterations/Consequences	References
River red gum (<i>Eucalyptus camaldulensis</i> Dehnh.)	(–)-Limonene synthase (LS)	<i>Perilla frutescens</i> (L.) Britton	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	Essential oil yield ↑; sterols ↑; chlorophylls and carotenoids unchanged Limonene ↑; α-pinene ↑; 1,8-cineole ↑	Ohara et al. (2010)
	(–)-Limonene synthase (LS)	<i>Perilla frutescens</i> (L.) Britton	Ox	CMV 35S	Organ: ubiquitous; Cell: cytosol	Limonene ↑; α-pinene ↑; 1,8-cineole ↑	Ohara et al. (2010)

Anti, antisense repression; CMV 35S, cauliflower mosaic virus promoter; ER, endoplasmic reticulum; n.a., not applicable (no functional protein generated); Ox, overexpression.

when compared to wild-type controls (Diemer *et al.*, 2001). In a separate set of transformations, in which spearmint LS was expressed in corn mint (*Mentha arvensis* L.), the oil yields were similar to or lower than in wild-type controls (Diemer *et al.*, 2001) (Table 3).

The constitutive overexpression of an LS gene in spike lavender, in which (–)-limonene is only a minor component (0.5–2.0% of the total monoterpenes), led to significant increases in the accumulation of (–)-limonene (compared to wild-type controls), but also dramatic changes in the levels of other monoterpenes (Muñoz-Bertomeu *et al.*, 2008) (Table 3). A developmental time course study indicated that the differences between transgenic and control plants were most pronounced in young leaves. In general, the authors reported a good correlation between LS transcript levels and (–)-limonene accumulation (Muñoz-Bertomeu *et al.*, 2008).

The first metabolic engineering of a woody essential oil plant was published by Ohara *et al.* (2010). The authors transformed the River Red Gum tree (*Eucalyptus camaldulensis* Dehnh.), which accumulates essential oil in sub-dermal secretory cavities of leaves, with constructs for the expression (ubiquitous in terms of tissue level) of an LS gene from *Perilla frutescens* to accumulate the encoded enzyme either in the cytosol or in plastids. This study had several unexpected outcomes. A cytosolic accumulation of LS resulted in higher amounts of extractable limonene than the expression of LS in plastids (Ohara *et al.*, 2010) (Table 3), although the availability of GPP, the substrate for LS, is generally considered to be much lower in the cytosol compared to plastids. Furthermore, it was also reported that limonene accumulation and LS transcript levels did not correlate. The authors also found an induction of the accumulation of 1,8-cineole and α-pinene in LS-expressing transgenics, which is surprising because the formation of these monoterpenes is catalysed by independent monoterpene synthases. There are interesting questions arising from these findings. For example, do *Eucalyptus* secretory cavities have unusually high pools of cytosolic GPP or can this intermediate be imported from plastids when needed? Can the ubiquitous expression of a transgene (here LS) induce the activities of various monoterpene synthases in secretory cavities? Methods for the isolation of these cavities in high purity have been reported recently by Goodger *et al.* (2010), and it should thus be possible to address these interesting issues experimentally.

As a second step in the biosynthetic pathway leading to p-menthane monoterpenes in peppermint, (–)-limonene is converted to (–)-trans-isopiperitenol by the activity of limonene-3-hydroxylase (L3H). The attempt to overexpress the endogenous L3H gene in transgenic peppermint plants was unsuccessful, as none of the transgenics contained elevated levels of L3H activity or increased oil yield (Mahmoud *et al.*, 2004) (Table 3). However, the authors analysed several cosuppressed transgenic lines (reduction of endogenous L3H transcript levels), which accumulated oil that was highly enriched in (–)-limonene (up to 80% of the total monoterpenes) but depleted of the downstream metabolites in the pathway (Mahmoud *et al.*, 2004) (Table 3).

The metabolic engineering efforts with peppermint discussed above were aimed at increasing essential oil yield. Now, we will highlight examples for purposefully modulating oil composition. For commercial purposes, it is desirable to keep (+)-pulegone levels below 2% and (+)-menthofuran levels at 1–4% of the total monoterpenes. The relative abundance of these intermediates can increase to 15–20% under stress conditions like high night temperatures, low day light intensities, and nutrient and water

deficiency (Burbott and Loomis, 1967; Clark and Menary, 1980; Court *et al.*, 1993). To decrease (+)-menthofuran levels, an antisense construct of the (+)-menthofuran synthase (MFS) cDNA was expressed constitutively in transgenic peppermint plants (Mahmoud and Croteau, 2001) (Table 3). Surprisingly, some of the transgenic lines did not only have desirably low (+)-menthofuran contents, but the relative amounts of (+)-pulegone were also decreased when compared to wild-type controls. Using a combination of mathematical modelling and experimental hypothesis testing, it was possible to elucidate the mechanisms for the unexpected decreases in (+)-pulegone amounts in MFS antisense lines. It was demonstrated that (+)-menthofuran is a weak competitive inhibitor of (+)-pulegone reductase, the enzyme that converts (+)-pulegone to (–)-menthone and (+)-isomenthone in peppermint glandular trichomes (Rios-Esteva *et al.*, 2008). To assess if (+)-menthofuran concentrations in the essential oil biosynthetic cells were high enough to cause an inhibition of (+)-pulegone reductase, these cells were isolated and analysed for monoterpene contents. It was shown that (+)-menthofuran was selectively retained in these specialized cells of glandular trichomes and that, when peppermint plants were exposed to abiotic stresses, the concentration of (+)-menthofuran rose up to the millimolar range, thus resulting in a significant (+)-pulegone reductase inhibition (Rios-Esteva *et al.*, 2008). As another surprise, MFS antisense plants accumulated significantly more essential oil (35% yield increase) compared to wild-type controls (Mahmoud and Croteau, 2001). Follow-up studies, once again integrating mathematical modelling with further experimental testing, established that the yield increase was due to a shift in the developmental program regulating glandular trichome development, such that the oil secretion began earlier in MFS antisense plants (higher percentage of mature glands on leaves of the same age) and leaves generally contained a higher number of glandular trichomes when compared to wild-type controls (Rios-Esteva *et al.*, 2010). We hypothesized that a 'push–pull' mechanism (elevated transcript levels of a biosynthetic gene induce the initiation of glandular trichomes) might be responsible for the increased oil yields in some transgenic plants (Rios-Esteva *et al.*, 2008). However, since no direct correlation between transgene expression levels and essential oil yield was reported in the studies published thus far, this hypothesis needs further experimental testing. An elite transgenic line (designated MFS7A) of this study was further examined in multi-year field trials, which confirmed yield increases and favourable composition of distilled essential oil (up to 69% higher yield compared to wild-type controls, with low amounts of (+)-menthofuran and (+)-pulegone) (Lange *et al.*, 2011).

A logical next step was to combine high essential oil yields with desirable composition in a single peppermint line. The elite MFS7A line, which harbours an antisense version of the (+)-menthofuran synthase gene (Mahmoud and Croteau, 2001), was transformed with an additional construct for the overexpression of the DXR gene. Several of the resulting transgenic lines had significantly increased oil yields (up to 78% higher than wild-type controls in field trials with BD7A-4 line) and a favourable composition (low (+)-menthofuran (1–5%) and (+)-pulegone ($\leq 0.5\%$), and high (–)-menthone and (–)-menthol levels) (Lange *et al.*, 2011), indicating that metabolic engineering was used successfully to modulate two commercially relevant traits.

Thus far, all efforts to modulate peppermint essential oil composition and yield have relied on constructs that result in a constitutive expression of transgenes. However, the

cell-type-specific expression of transgenes in essential oil plants could potentially be achieved by using gland cell-specific promoters. An excellent recent review (Tissier, 2012) highlights progress in identifying such promoters, and we will discuss successful metabolic engineering efforts with tobacco glandular trichomes below (Table 6). Another option would be to modulate the expression of genes encoding regulatory proteins that control the formation of glandular trichomes. Various transcription factors involved in trichome hair initiation and development in *Arabidopsis* have been characterized (Ishida *et al.*, 2008). However, currently available evidence suggests that these regulators may induce the formation of glandular trichomes in certain plants (e.g. cotton seeds; Wang *et al.*, 2004) but not in others (e.g. tobacco; Payne *et al.*, 1999). Further experimental evidence is needed to evaluate the potential of using regulatory factors in engineering glandular trichome biology for enhanced essential oil accumulation.

Terpenoid pharmaceuticals

Case study 1: artemisinin

Artemisinin, a sesquiterpene lactone with an unusual endoperoxide bridge (Fig. 4), has been extracted from sweet wormwood (*Artemisia annua* L.), a shrub known to Chinese herbalists as Qinghaosu (Chinese: 青蒿素) since ancient times. The World Health Organization has endorsed the use of artemisinin-based combination therapies to treat drug-resistant strains of the malaria-causing agent, *Plasmodium falciparum* (derivatives are sold under the brand names Riamet, Coartem, Artemotil and artesunate). However, the artemisinin yields from the natural producer are fairly low (ranging from 0.01% to 2% of dry weight biomass), which might be partially explained by the fact that the biosynthesis of this metabolite is restricted to glandular trichomes on the surfaces of leaves and floral bracts (Duke and Paul, 1993; Duke *et al.*, 1994; Olsson *et al.*, 2009; Pu *et al.*, 2012). Increased demand for artemisinin-based combination therapies has led to supply shortages (Covello, 2008). As the total synthesis of artemisinin is not a commercially viable option (Schmid and Hofheinz, 1983), research efforts aimed at developing alternative production processes, using advanced breeding (Graham *et al.*, 2010) and metabolic engineering (Covello, 2008), were initiated in the early 2000s. Considerable progress has been made with expressing parts of the artemisinin biosynthetic pathway in microbial hosts, followed by semi-synthetic conversion to the desired end product (Westfall *et al.*, 2012). Alternatively, the production of artemisinin has also been achieved in transgenic plants (*A. annua* and tobacco).

The first published metabolic engineering studies aimed at increasing the accumulation of artemisinin in the native producer, *A. annua*, overexpressed genes involved in precursor supply pathways for sesquiterpenoid biosynthesis. Based on experiments with specific inhibitors of the MVA and MEP pathways, Towler and Weathers (2007) demonstrated that both pathways contribute to artemisinin formation in *A. annua*. While follow-up studies by Schramek *et al.* (2010) indicated a predominant synthesis of artemisinin via the MEP pathway, Ram *et al.* (2010) concluded that the MVA pathway, in particular the activity of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), was a limiting factor for artemisinin accumulation. These discrepancies notwithstanding, the expression of an HMGR gene from Madagascar periwinkle (*Catharanthus roseus* (L.) G. Don.) in transgenic *A. annua*, under control of a constitutive promoter, led to modest increases

(22.5%) in artemisinin levels in shoots when compared with control plants (Aquil *et al.*, 2009) (Table 4). The same experiment was later repeated by Nafis *et al.* (2011) with essentially identical results. The overexpression of a cotton (*Gossypium arboreum* L.) FPS gene, which encodes the enzyme that catalyses the reaction providing the ultimate precursor for sesquiterpene biosynthesis, in a fairly low-yielding *A. annua* strain (artemisinin content around 0.3% of dry weight biomass) led to a two- to threefold increase in the concentration of artemisinin (Chen *et al.*, 2000) (Table 4). When an endogenous FPS gene was overexpressed in a high-yielding *A. annua* strain (artemisinin content approximately 0.65% of dry weight biomass), a roughly 34% increase (compared to wild-type controls) was reported for transgenic lines (Han *et al.*, 2006) (Table 4). Banyai *et al.* (2010) achieved a 2.5-fold increase in artemisinin yield (from 0.5% to 1.3% of dry weight biomass) using the same approach (Table 4).

Ma *et al.* (2009a) reported on changes in the composition of various metabolites following the transformation of *A. annua* with an endogenous amorpho-4,11-diene synthase (ADS) gene, which encodes the enzyme that catalyses the first committed step in the artemisinin biosynthetic pathway (Fig. 4). An overexpression resulted in the accumulation of increased amounts of artemisinin, borneol and phytol, while the levels of the sesquiterpenes (*E*)- β -farnesene and germacrene D decreased (Ma *et al.*, 2009a) (Table 4). Conversely, when ADS expression levels were decreased using an antisense approach, the amounts of the sesquiterpenes (*E*)- β -farnesene and germacrene D increased (Table 4) (Ma *et al.*, 2009a). As a note of caution, the authors did not provide any description of molecular biological methods and data in their paper, which precludes us from discussing their results further. By overexpressing the genes encoding both HMGR (enzyme of MVA precursor supply pathway) and ADS (first committed enzyme of the artemisinin biosynthetic pathway), under the control of two different constitutive promoters, led to the production of a transgenic line that had a 7.7-fold higher artemisinin level in a very low-yielding genetic background (wild-type artemisinin content 0.02% of dry weight biomass) (Alam and Abidin, 2011).

An alternative to the overexpression of artemisinin pathway-specific genes is the downregulation of enzyme activities involved in pathways that compete for precursors. The suppression of the expression of the squalene synthase gene, which codes for an early enzyme of the sterol biosynthetic pathway, by means of hairpin-RNA-mediated RNA interference (RNAi), led to significant increases (> 3-fold compared to wild-type controls) of the artemisinin concentration (up to 3.1% of dry weight biomass) in greenhouse-grown transgenic *A. annua* plants (Zhang *et al.*, 2009) (Table 4). Despite notable decreases in sterol levels, the transgenic plants apparently had a growth performance similar to wild-type controls. Transgenic lines were also evaluated in field trials, for which a 2-fold increase of artemisinin yield (from 0.45% to 0.9% of dry weight biomass) was reported (Zhang *et al.*, 2009). Similar results were obtained with an antisense approach for decreasing squalene synthase transcripts, for which significant increases in artemisinin yield (3.7-fold higher) were reported for transgenic plants (when compared to wild-type controls) that were subjected to chilling before harvest (artemisinin levels increased to 1.6% of dry weight biomass) (Feng *et al.*, 2009). Another example for the downregulation of a competing pathway was the antisense suppression of the transcript for β -caryophyllene synthase, a sesquiterpene synthase that competes with ADS for FPP (Chen *et al.*, 2011a). This approach led to

a 55% increase in artemisinin content compared with wild-type controls.

Targeting the artemisinin biosynthetic pathway might be the most obvious metabolic engineering approach to increase yield. However, as a complementary concept, several groups have also attempted to modulate the expression of genes not directly involved in the biosynthesis but with potential to afford increases in artemisinin yield as a secondary benefit. For example, Sa *et al.* (2001) transformed *A. annua* with an isopentenyl transferase (from *Agrobacterium tumefaciens*) that is involved in cytokinin biosynthesis. It was known that cytokinin addition increased artemisinin yields in tissue cultures (Whipkey *et al.*, 1992), and the rationale was to increase endogenous cytokinin levels. Interestingly, transgenic isopentenyl transferase-overexpressing *A. annua* plants accumulated elevated levels of cytokinins, chlorophylls and artemisinin (up to 1.6% of the dry weight biomass). Two papers reported on the effects of an earlier flowering time on artemisinin levels. However, the constitutive expressions of a flowering promoting factor gene or the CONSTANS gene (both from *Arabidopsis*) in *A. annua* did not have notable effects on artemisinin yields (Wang *et al.*, 2004, 2007). The expression of the cryptochrome photoreceptor gene from *Arabidopsis* in *A. annua* resulted in the regeneration of transgenic plants with various phenotypic irregularities, particularly when grown under blue light (Hong *et al.*, 2009). The artemisinin content in greenhouse-grown transgenic plants was increased by up to 40% compared to wild-type controls (0.15% of dry weight biomass), but these plants were severely stunted in growth (Hong *et al.*, 2009). Ma *et al.* (2009b) noticed that the transcript of a transcription factor containing a WRKY motif (designated AaWRKY1) was represented at high abundance in a glandular trichome-specific cDNA library from *A. annua*. The authors demonstrated that AaWRKY1 interacted with specific *cis*-acting elements in the ADS promoter and, when expressed transiently in *A. annua*, activated several genes involved in artemisinin biosynthesis. Although the utility of such a transcription factor for increasing artemisinin levels has not yet been demonstrated, it is conceivable that the concept of a global pathway regulator is a viable strategy, as previously for several pathways involved in the formation of specialized plant metabolites (Kliebenstein and Osbourn, 2012). Based on the observation that artemisinin biosynthesis is induced by jasmonic acid, Yu *et al.* (2012b) identified two *A. annua* jasmonic acid-responsive transcription factors (termed AaERF1 and AaERF2) that were capable of binding to both the ADS and CYP71AV1 promoters. An overexpression of the gene encoding either of these transcription factors in *A. annua* led to a 60% increased artemisinin yield (up to 0.8% of dry weight biomass) in transgenic lines when compared to wild-type controls.

Taken together, the studies discussed above indicate that dramatic (> 3-fold) increases in artemisinin yield were achieved mostly with low-yielding *A. annua* cultivars. Currently, the artemisinin yield limit appears to be around 3% of the dry weight under ideal growth conditions (Zhang *et al.*, 2009) and approximately 2% in the field (Graham *et al.*, 2010). An extensive breeding program evaluated the distribution of traits that contribute to artemisinin yield (Graham *et al.*, 2010). Not surprisingly, a balance between various factors, including glandular trichome density, plant height and stature, leaf area, artemisinin concentration and agronomic performance, needed to be maintained to achieve high yields in the field.

Table 4 Metabolic engineering of artemisinin biosynthesis in *Artemisia annua* (L)

Gene(s)	Gene Source(s)	Strategy	Promoter(s)	Targeting site(s)	Alterations/Consequences	References
<i>Engineering of pathways directly related to artemisinin biosynthesis</i>						
3-Hydroxy-3-methylglutaryl-CoA reductase(HMGR)	Madagascar periwinkle (<i>Catharanthus roseus</i> (L.) G. Don)	Ox	CMV 35S	Organ: ubiquitous; Cell: ER	Artemisinin ↑	Aquil <i>et al.</i> (2009)
3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR)	Madagascar periwinkle (<i>Catharanthus roseus</i> (L.) G. Don)	Ox	CMV 35S	Organ: ubiquitous; Cell: ER	Artemisinin ↑	Nafis <i>et al.</i> (2011)
Farnesyl diphosphate synthase (FPS)	Cotton (<i>Gossypium arboreum</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: cytosol	Artemisinin ↑↑	Chen <i>et al.</i> (2000)
Farnesyl diphosphate synthase (FPS)	Sweet wormwood (<i>Artemisia annua</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: cytosol	Artemisinin ↑	Han <i>et al.</i> (2006)
Farnesyl diphosphate synthase (FPS)	Sweet wormwood (<i>Artemisia annua</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: cytosol	Artemisinin ↑↑; Stunted growth in co-suppressed lines	Banyai <i>et al.</i> (2010)
Amorpha-4,11-diene synthase (ADS)	Sweet wormwood (<i>Artemisia annua</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: cytosol	Artemisinin ↑; borneol ↑; phytol ↑; (E)-β-farnesene ↓; germacrene D ↓	Ma <i>et al.</i> (2009a)
Amorpha-4,11-diene synthase (ADS)	Sweet wormwood (<i>Artemisia annua</i> L.)	Anti	CMV 35S	Organ: ubiquitous; Cell: n.a.	(E)-β-Farnesene ↑; germacrene D ↑	Ma <i>et al.</i> (2009a)
Amorpha-4,11-diene synthase (ADS); 3-Hydroxy-3-methylglutaryl-CoA reductase(HMGR)	Madagascar periwinkle (<i>Catharanthus roseus</i> (L.) G. Don) (HMGR); Sweet wormwood (<i>Artemisia annua</i> L.) (ADS)	Ox	Ubiquitin (HMGR) and CMV 35S (ADS)	Organ: ubiquitous; Cell: ER (HMGR) Cell: cytosol (ADS)	Artemisinin ↑↑↑	Alam and Abidin (2011)
Squalene synthase (SQS)	Sweet wormwood (<i>Artemisia annua</i> L.)	RNAi	CMV 35S	Organ: ubiquitous; Cell: n.a.	Artemisinin ↑; sterols ↓	Zhang <i>et al.</i> (2009)
Squalene synthase (SS)	Sweet wormwood (<i>Artemisia annua</i> L.)	Anti	CMV 35S	Organ: ubiquitous; Cell: n.a.	Artemisinin ↑↑↑ (under chilling conditions); sterols ↓	Feng <i>et al.</i> (2009)
β-Caryophyllene synthase (CPS)	Sweet wormwood (<i>Artemisia annua</i> L.)	Anti	CMV 35S	Organ: ubiquitous; Cell: n.a.	Artemisinin ↑	Chen <i>et al.</i> (2011a)
<i>Engineering of various pathways and processes with effects (or not) on artemisinin content</i>						
Isopentenyl transferase (IPT)	<i>Agrobacterium tumefaciens</i>	Ox	CMV 35S	Organ: ubiquitous; Cell: cytosol	Artemisinin ↑↑; cytokinins ↑↑↑; chlorophylls ↑↑	Sa <i>et al.</i> (2001)
Flowering promoting factor (fp1)	Thale cress (<i>Arabidopsis thaliana</i> (L.) Heynh.; Col-0 ecotype)	Ox	CMV 35S	Organ: ubiquitous; Cell: unknown	No artemisinin increase	

Table 4 Continued

Gene(s)	Gene Source(s)	Strategy	Promoter(s)	Targeting site(s)	Alterations/Consequences	References
CONSTANS zinc finger transcription factor (CO)	Thale cress (<i>Arabidopsis thaliana</i> (L.) Heynh.; Col-0 ecotype)	Ox	CMV 35S	Organ: ubiquitous; Cell: nuclei	No artemisinin increase	Wang <i>et al.</i> (2007)
Cryptochrome blue light receptor (CRY1)	Thale cress (<i>Arabidopsis thaliana</i> (L.) Heynh.; Col-0 ecotype)	Ox	CMV 35S	Organ: ubiquitous; Cell: cytosol and nuclei	Artemisinin ↑; anthocyanins ↑; Stunted growth	Hong <i>et al.</i> (2009)
WRKY domain transcription factor (AaWRKY1)	Sweet wormwood (<i>Artemisia annua</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: nuclei	Transient expression of AaWRKY1 in transgenic <i>A. annua</i> leaves activates expression of artemisinin pathway genes	Ma <i>et al.</i> (2009b)
Jasmonate-responsive AP2 family transcription factors (AaERF1 and AaERF2)	Sweet wormwood (<i>Artemisia annua</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: nuclei	Artemisinin ↑	Yu <i>et al.</i> (2012b)

Anti, antisense repression; CMV 35S, cauliflower mosaic virus promoter; ER, endoplasmic reticulum; n.a., not applicable (no functional protein generated); Ox, overexpression; RNAi, RNA interference-based suppression.

A suitable non-native host for artemisinin production would be a fast-growing crop plant (with high biomass yield in the field) engineered to contain the required genes to synthesize this metabolite. Research thus far has focused on tobacco, for which facile and fairly efficient transformation protocols are available. In a first preliminary study, tobacco (*Nicotiana tabacum* L. cv. Xanthi) was transformed with the ADS gene from *A. annua*, which resulted in the accumulation of amorpha-4,11-diene, the product of ADS catalysis, in trace amounts (below 0.0000002% of fresh weight biomass) (Wallaart *et al.*, 2001) (Table 5). As the cytosolic FPP supply appeared to be highly regulated, the overexpression of a heterologous sesquiterpene synthase gene alone was not sufficient for the production of high levels of sesquiterpenes in tobacco (Hohn and Ohlrogge, 1991; Wallaart *et al.*, 2001; Wu *et al.*, 2006). As an alternative, Wu *et al.* (2006) co-expressed genes coding for plastid-targeted ADS (from *A. annua*) and FPS (from chicken) in transgenic tobacco plants and achieved an approximately 5,000-fold yield increase of amorpha-4,11-diene (up to 0.0025% of fresh weight biomass) compared to transgenics accumulating ADS in the cytosol (Table 5). Encouraged by these early successes, additional artemisinin pathway genes were transferred to tobacco. Constitutive co-expression of the ADS gene from *A. annua* (gene product targeted to plastids), an FPS gene from chicken (gene product targeted to plastids) and the CYP71AV1 gene from *A. annua* (codes for cytochrome P450-dependent monooxygenase responsible for oxidation of amorpha-1,4-diene) in tobacco resulted in the production of amorpha-1,4-diene and artemisinic alcohol (up to 0.0006% of fresh weight biomass) (Zhang *et al.*, 2011) (Table 5). Interestingly, the main product of CYP71AV1, when accumulated recombinantly in yeast, was artemisinic acid (Ro *et al.*, 2006; Teoh *et al.*, 2006), which was not detected in the transgenic tobacco lines mentioned above. Possible explanations would be that the activity of CYP71AV1 was modulated in transgenic tobacco plants (possibly because of the lack of the appropriate cytochrome P450 reductase, which is required for electron transfer to cytochrome P450 in ER membranes) or that an endogenous alcohol dehydrogenase/aldehyde reductase was preventing the formation of artemisinic aldehyde and artemisinic acid. When the transformation construct was expanded to include an additional artemisinic aldehyde $\Delta 11(13)$ double bond reductase (DBR2) gene (cytosolic targeting of gene product), amorpha-1,4-diene, artemisinic alcohol and dihydroartemisinic alcohol were produced (total amorphane sesquiterpenoids at ~0.0005% of fresh weight biomass) (Zhang *et al.*, 2011) (Table 5). Once again, the corresponding amorphane acids were not detected. The transformation construct was then expanded further to include an aldehyde dehydrogenase from *A. annua*. Unexpectedly, this strategy increased the yield of amorphane sesquiterpenoids to ~0.002% of fresh weight biomass, but no amorphane acids or artemisinin were detectable (Zhang *et al.*, 2011) (Table 5).

Previous work with *Arabidopsis* had demonstrated that a mitochondrial localization of a heterologous sesquiterpene synthase led to higher sesquiterpenoid accumulation levels compared to transgenics in which the gene product was expressed cytosolically, probably due to a higher plasticity of the FPP pool in mitochondria (Kappers *et al.*, 2005). van Herpen *et al.* (2010) first tested a construct that constitutively expressed an *A. annua* ADS, targeted to mitochondria, in infiltrated transgenic tobacco (*Nicotiana benthamiana* Domin.). Amorpha-1,4-diene was accumulated to 0.0009% of fresh weight biomass. The use of a triple-

Table 5 Metabolic engineering of artemisinin biosynthesis in tobacco

Engineered cultivar	Gene(s)	Gene Source(s)	Strategy	Promoter(s)	Targeting site(s)	Alterations/Consequences	References
<i>Nicotiana tabacum</i> L. cv. Petite Havana SR1	Amorpha-4,11-diene synthase (ADS)	Sweet wormwood (<i>Artemisia annua</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: cytosol	Amorpha-4,11-diene (traces)	Wallaart et al. (2001)
<i>Nicotiana tabacum</i> L. cv. Xanthi	Amorpha-4,11-diene synthase (ADS)	Sweet wormwood (<i>Artemisia annua</i> L.)	Ox	CMV	Organ: ubiquitous; Cell: cytosol	Amorpha-4,11-diene (traces)	Wu et al. (2006)
	Amorpha-4,11-diene synthase (ADS); farnesyl diphosphate synthase (FPS)	Sweet wormwood (<i>Artemisia annua</i> L.)	Ox	CMV (ADS) and CMV 35S FPS	Organ: ubiquitous; Cell: plastids (ADS and FPS)	Amorpha-4,11-diene ↑	Wu et al. (2006)
	Amorpha-4,11-diene synthase (ADS); farnesyl diphosphate synthase (FPS)	Sweet wormwood (<i>Artemisia annua</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids (ADS and FPS)	Amorpha-4,11-diene ↑	Zhang et al. (2011)
	Amorpha-4,11-diene synthase (ADS); farnesyl diphosphate synthase (FPS); amorphaadiene oxidase (CYP71AV1)	Sweet wormwood (<i>Artemisia annua</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids (ADS and FPS) Cell: ER (CYP71AV1)	Amorpha-4,11-diene ↑; artemisinic alcohol ↑	Zhang et al. (2011)
	Amorpha-4,11-diene synthase (ADS); farnesyl diphosphate synthase (FPS); amorphaadiene oxidase (CYP71AV1); artemisinic aldehyde Δ ¹¹ (13) double-bond reductase (DBR2)	Sweet wormwood (<i>Artemisia annua</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids (ADS and FPS) Cell: ER (CYP71AV1) Cell: Cytosol (DBR2)	Amorpha-4,11-diene ↑; artemisinic alcohol ↑; dihydroartemisinic alcohol ↑	Zhang et al. (2011)
	Amorpha-4,11-diene synthase (ADS); farnesyl diphosphate synthase (FPS); amorphaadiene oxidase (CYP71AV1); artemisinic aldehyde Δ ¹¹ (13) double-bond reductase (DBR2); aldehyde dehydrogenase (ALDH1)	Sweet wormwood (<i>Artemisia annua</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids (ADS and FPS) Cell: ER (CYP71AV1) Cell: Cytosol (DBR2, ALDH1)	Amorpha-4,11-diene ↑; artemisinic alcohol ↑; dihydroartemisinic alcohol ↑	Zhang et al. (2011)
<i>Nicotiana benthamiana</i> Domin	Amorpha-4,11-diene synthase (ADS)	Sweet wormwood (<i>Artemisia annua</i> L.)	Ox (infiltration)	CMV 35S	Organ: ubiquitous; Cell: mitochondria	Amorpha-4,11-diene ↑; unknown compound ↑	van Herpen et al. (2010)
	Amorpha-4,11-diene synthase (ADS); truncated 3-hydroxy-3-methylglutaryl- CoA reductase (tHMGR); farnesyl diphosphate synthase (FPS)	Sweet wormwood (<i>Artemisia annua</i> L.) (ADS); Thale cress (<i>Arabidopsis thaliana</i> (L.) Heynh.; Col-0 ecotype)(tHMGR, FPS)	Ox (infiltration)	CMV 35S (genes in single ORF, separated by 2A)	Organ: ubiquitous; Cell: mitochondria (ADS, FPS) Cell: cytosol (tHMGR)	Amorpha-4,11-diene ↑; unknown compound ↑↑	van Herpen et al. (2010)
	Amorpha-4,11-diene synthase (ADS); amorphaadiene oxidase (CYP71AV1); truncated 3- hydroxy-3-methylglutaryl-CoA	Sweet wormwood (<i>Artemisia annua</i> L.) (ADS, CYP71AV1); Thale cress	Ox (infiltration)	CMV 35S (ADS, FPS and tHMGR in single ORF, separated by 2A)	Organ: ubiquitous; Cell: mitochondria (ADS, FPS) Cell:	Amorphaadiene ↑; unknown compound ↑; artemisinic acid-12-β-digluconide ↑↑	van Herpen et al. (2010)

Table 5 Continued

Engineered cultivar	Gene(s)	Gene Source(s)	Strategy	Promoter(s)	Targeting site(s)	Alterations/ Consequences	References
<i>Nicotiana tabacum</i> L. cv. Xanthi	reductase (tHMGR); farnesyl diphosphate synthase (FPS)	(<i>Arabidopsis thaliana</i> (L.) Heynh.; Col-0 ecotype) (tHMGR, FPS)			cytosol (tHMGR) Cell: ER (CYP71AV1)		
	Amorpha-4,11-diene synthase (ADS); amorphadiene oxidase (CYP71AV1); cytochrome P450 reductase (CPR); artemisinic aldehyde Δ 11(13) double-bond reductase (DBR2); truncated 3-hydroxy-3-methylglutaryl-CoA reductase (tHMGR)	Sweet wormwood (<i>Artemisia annua</i> L.) ADS, CYP71AV1, CPR, DBR2; yeast (<i>Saccharomyces cerevisiae</i>) (tHMGR)	Ox	CMV 35S (ADS), HS (CYP71AV1), RBC (CPR), NOS (tHMGR), SUP (DBR2)	Organ: ubiquitous; Cell: cytosol (tHMGR, ADS, DBR2) Cell: ER (CYP71AV1, CPR))	Artemisinin $\uparrow$	Farhi et al. (2011)
	Amorpha-4,11-diene synthase (ADS); amorphadiene oxidase (CYP71AV1); cytochrome P450 reductase (CPR); artemisinic aldehyde Δ 11(13) double-bond reductase (DBR2); truncated 3-hydroxy-3-methylglutaryl-CoA reductase (tHMGR)	Sweet wormwood (<i>Artemisia annua</i> L.) ADS, CYP71AV1, CPR, DBR2; yeast (<i>Saccharomyces cerevisiae</i>) (tHMGR)	Ox	CMV 35S (ADS), HS (CYP71AV1), RBC (CPR), NOS (tHMGR), SUP (DBR2)	Organ: ubiquitous; Cell: mitochondria (ADS) Cell: cytosol (tHMGR, DBR2) Cell: ER (CYP71AV1, CPR))	Artemisinin $\uparrow$	Farhi et al. (2011)
	Amorpha-4,11-diene synthase (ADS); amorphadiene oxidase (CYP71AV1); cytochrome P450 reductase (CPR); artemisinic aldehyde Δ 11(13) double-bond reductase (DBR2); truncated 3-hydroxy-3-methylglutaryl-CoA reductase (tHMGR)	Sweet wormwood (<i>Artemisia annua</i> L.) ADS, CYP71AV1, CPR, DBR2; yeast (<i>Saccharomyces cerevisiae</i>) (tHMGR)	Ox	CMV 35S (ADS), HS (CYP71AV1), RBC (CPR), NOS (tHMGR), SUP (DBR2)	Organ: ubiquitous; Cell: mitochondria (ADS) Cell: cytosol (tHMGR, DBR2) Cell: ER (CYP71AV1, CPR))	Artemisinin $\uparrow$	Farhi et al. (2011)

2A, ribosomal skipping sequence; CMV 35S, cauliflower mosaic virus promoter; CVMV, Cassava vein mosaic virus promoter; ER, endoplasmic reticulum; HS, hps18.1 promoter; NOS, nopaline synthase promoter; Ox, overexpression; RBC, Rubisco small subunit promoter; SUP, super promoter.

gene construct containing ADS (from *A. annua*; mitochondrial targeting), a truncated HMGR (from *Arabidopsis*; cytosolic targeting) and an FPS (from *Arabidopsis*; mitochondrial targeting) led to significant (7-fold) yield increases of extractable amorpha-1,4-diene (up to 0.006% of fresh weight biomass) (van Herpen *et al.*, 2010). While some researchers use different constitutive promoters to avoid known issues with promoter silencing in multi-gene constructs, this study took advantage of ribosomal skipping technology, which allows multiple genes to be expressed from a single promoter (Donnelly *et al.*, 2001). A potentially yield-limiting factor was the accumulation of an as yet unidentified metabolite at high levels (up to 0.02% of fresh weight biomass) in transgenic plants. The expression of a truncated HMGR gene (gene product targeted to cytosol) appeared to contribute significantly to the strong accumulation of amorpha-1,4-diene in transgenic plants (van Herpen *et al.*, 2010), suggesting that the transport of terpenoid precursors from the cytosol to mitochondria was not limiting. When the above-mentioned triple-gene construct was co-infiltrated with another plasmid harbouring the *A. annua* amorphaadiene oxidase (CYP71AV1) gene, amorpha-1,4-diene was almost completely converted to artemisinic acid and, subsequently, artemisinic acid-12- β -diglucoside (amorphane sesquiterpenoid yield of 0.04% of fresh weight biomass) (van Herpen *et al.*, 2010) (Table 5). Interestingly, the concentration of the unknown metabolite that accumulated in transgenic plants expressing the triple-gene construct alone was very low in this new set of transgenic lines. The conversion of artemisinic acid to a glycosylated metabolite is an unexpected and undesirable consequence of metabolic engineering (van Herpen *et al.*, 2010), which could potentially be addressed by suppressing the expression of the gene coding for the responsible endogenous glucosyltransferase activity.

The first example of the successful production of artemisinin in a heterologous host required a tour de force metabolic engineering approach. Farhi *et al.* (2011) transformed tobacco (*Nicotiana tabacum* L. cv. Xanthi) with a mega construct containing 5 genes with direct relevance for artemisinin

formation (plus a gene to enable selection of positive transformants), each controlled by a different constitutive promoter (Table 5). The gene products were targeted to different subcellular compartments to enhance precursor availability. ADS (from *A. annua*) was targeted to mitochondria, a truncated HMGR (from *Saccharomyces cerevisiae*) and DBR2 were targeted to the cytosol, and CYP71AV1 as well as cytochrome P450 reductase (both from *A. annua*) were targeted to the ER. This approach allowed the production of artemisinin at 0.0007% of dry weight biomass. While the groundbreaking work by Farhi *et al.* (2011) demonstrated conclusively that the artemisinin biosynthetic pathway can be expressed in a heterologous host plant, the accumulation levels of the antimalarial metabolite in tobacco are currently too low to be competitive with other production alternatives. Based on what has been learned with tobacco as a host, the University of Wageningen and Dafra Pharma International NV are cooperating on the engineering of chicory (*Cichorium intybus* L.) to produce the artemisinin precursor dihydroartemisinic acid in roots. Chicory was chosen as production platform because it already accumulates (nonartemisinin) sesquiterpene lactones and may contain enzyme activities relevant for artemisinin biosynthesis (De Kraker *et al.*, 2003).

Case study 2: taxol precursors

Taxol (generically named paclitaxel) is a natural product with a diterpenoid core (Fig. 5) that was first extracted from the Pacific Yew (*Taxus brevifolia* Peattie) tree in the early 1970s (Wani *et al.*, 1971). Following successful clinical trials, the U.S. Food and Drug Administration approved the use of taxol for the treatment of ovarian and breast cancer in the early 1990s. The limited natural supply of this biologically active metabolite, which accumulates to only 0.01–0.04% of the dry biomass of Pacific yew bark (Rao, 1993), was one of the reasons for the slow progress in the development of the drug. Various semi-synthetic routes, which were based on using more abundant taxanes in various yew species as starting materials, were developed for the commercial production of taxol and its analogues (Patel, 1998). Over the last

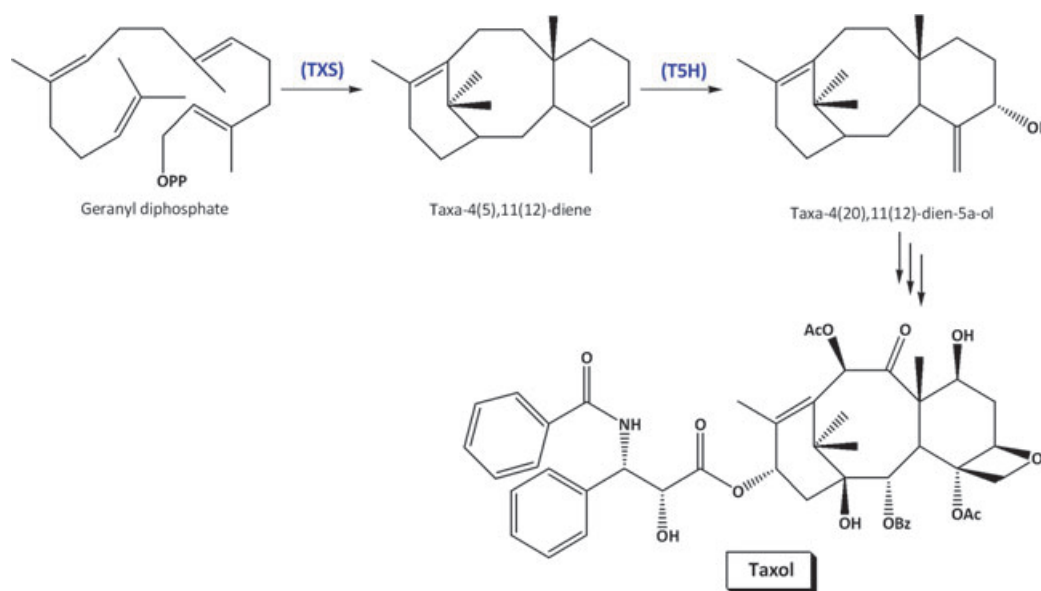


Figure 5 Overview of the taxol biosynthetic pathway in some members of the genus *Taxus*. Acronyms: TXS, taxadiene synthase; T5H, taxadien-5 α -hydroxylase.

10–15 years, yew cell suspension cultures have become the main source of taxol for pharmaceutical giant Bristol-Myers Squibb, using a process that was originally developed by Christen *et al.* (1989) and further optimized by Phyton Biotech, Inc. Heterologous high-level production (elite strains produced about 1 g/L) of taxa-4(5),11(12)-diene, the first pathway-specific intermediate in taxol biosynthesis (Fig. 5), was recently achieved in *E. coli* by expressing a taxadiene synthase (TXS) gene (after codon optimization) from *Taxus brevifolia* (Ajikumar *et al.*, 2010). After introduction of the next following gene of the taxol pathway, which codes for taxadien-5 α -ol hydroxylase (T5H), and a gene encoding an appropriate cytochrome P450 reductase from yew, a much lower yield of the expected taxane end product, taxa-4(5),11(12)-diene-5 α -ol, was reported (under 60 mg/L) (Ajikumar *et al.*, 2010), indicating that substantial strain improvements will be necessary to enable a cost-competitive synthesis of the complex, highly functionalized, natural product in *E. coli*. It should be noted that the taxol pathway alone (starting with geranylgeranyl diphosphate) requires the coordination of roughly 20 enzyme activities.

Research has also been conducted to express taxol biosynthetic genes in non-native plant hosts, and progress of these efforts will be reviewed here. A TXS gene isolated from *T. baccata* (L.) (targeted to plastids) was constitutively expressed in *Arabidopsis* (Col-3 ecotype), which resulted in taxa-4(5),11(12)-diene accumulation of about 0.000025% of leaf dry weight biomass (Besumbes *et al.*, 2004) (Table 6). Surprisingly, this low-level accumulation of taxa-4(5),11(12)-diene led to a decrease in much more abundant plastidial terpenoids such as carotenoids (43% decrease to 0.1% dry weight) and chlorophylls (40% decrease to 0.15% of dry weight), thus resulting in a pale phenotype of the transgenic plants. The authors correctly noted that the flux from the common intermediate GGPP towards taxa-4(5),11(12)-diene was negligible compared to that required for the synthesis of carotenoids and chlorophylls and hypothesized that as yet unknown regulatory mechanisms controlling the GGPP pool might be responsible for the observed phenotype (Besumbes *et al.*, 2004). When the TXS gene was expressed in *Arabidopsis* under control of a two-component glucocorticoid-inducible promoter, spraying of mature leaves with the inducer dexamethasone resulted in a more efficient recruitment of GGPP and consequently led to significant increases of taxa-4(5),11(12)-diene production up to 0.00006% (24-fold increase compared with production yields for lines constitutively expressing the transgene), indicating the potential for improving yields by using inducible promoter technology (Besumbes *et al.*, 2004) (Table 6). The *Arabidopsis* 35S::TXS line was later crossed with lines overexpressing the DXS or DXR gene (encoding the entry enzymes of the MEP pathway for terpenoid precursor biosynthesis in plastids), and for both 35S::TXS/35S::DXS and 35S::TXS/35S::DXR lines increases in taxa-4(5),11(12)-diene levels, when compared with the 35S::TXS line, were reported but no absolute quantitation was performed (Carretero-Paulet *et al.*, 2006) (Table 6). The same experiment (expression of TXS gene under control of a dexamethasone-inducible promoter) was later repeated by Khani *et al.* (2010) with a different *Arabidopsis* ecotype but, with the exception of PCR data to test for the integration of the transgene into the *Arabidopsis* genome, the authors did not provide any follow-up data.

The suitability of using a sink organ for taxane accumulation was evaluated by Kovacs *et al.* (2007) (Table 6). The TXS gene from *T. baccata* L. was also expressed in a yellow-fruited tomato

(*Solanum lycopersicum* Mill.) mutant (lacking a functional fruit phytoene synthase) under the control of either a constitutive (CMV 35S) or ripening-specific (polygalacturonase) promoter. Fruit of transgenic lines produced taxa-4(5),11(12)-diene at 0.035% (constitutive promoter) or 0.047% of dry weight biomass (fruit-specific promoter), whereas the yield of the taxane from leaves was much lower (0.015% of dry weight biomass in 35S::TXS transgenics). These data indicate that, without much optimization, transgenic tomato fruit produced taxa-4(5),11(12)-diene in quantities similar to those reported for taxol in Pacific yew bark.

Tobacco accumulates cembrane diterpenoids in leaf glandular trichomes (Keene and Wagner, 1985). The Tissier laboratory tested if the trichomes would be a suitable location for the accumulation of other diterpenes, in particular taxanes. When the TXS gene was expressed in *Nicotiana sylvestris* (Speg. & Comes) using the trichome-specific CBTS promoter, a taxa-4(5),11(12)-diene yield of 0.002% of leaf fresh weight biomass was reported (Tissier *et al.*, 2008). In subsequent studies, the TXS gene was cloned downstream of the CBTS promoter, while the T5H gene was placed downstream of the CYP71D16 promoter (Rontein *et al.*, 2008). In addition, a third construct contained a hairpin RNAi construct designed to knock-down the expression of the cembratrienol synthase gene, which codes for the enzyme catalysing the first committed step of cembrane diterpenoid biosynthesis. In transgenic plants harbouring all three constructs, the expected 5 α -hydroxy-taxa-4(20),11(12)-diene could not be detected (Rontein *et al.*, 2008). Instead, a new product, which was later found to be 5(12)-oxa-3(11)-cyclotaxane (Fig. 5), was identified as being accumulated in transgenic tobacco lines (Table 6). The yields of production of this structurally unusual taxane were not reported, and it is thus not possible to evaluate the relevance of these findings for engineering efforts that utilize the metabolic specialization of glandular trichomes.

The moss *Physcomitrella patens* (Hedw.) Bruch & Schimp. accumulates diterpenes derived from *ent*-kaurene and has been promoted as a potential host for the production of diterpenoids (Simonsen *et al.*, 2009). Anterola *et al.* (2009) expressed the TXS gene under control of a constitutive ubiquitin promoter in *Physcomitrella*. Stable moss transformants accumulated taxa-4(5),11(12)-diene at 0.05% of fresh weight biomass, apparently without significant alteration of *ent*-kaurene-derived diterpenoid levels (Anterola *et al.*, 2009) (Table 6), indicating that the moss might be developed into a production host for diterpenoids.

Conclusions and future opportunities

Exciting progress has been made with regard to engineering terpenoid metabolism in plants. Several successful strategies have been reported and some common themes are emerging, but critical challenges remain:

- 1 A localization of terpene synthases and, if necessary, the appropriate prenyl diphosphate synthases, in plastids or mitochondria is generally preferable over a cytosolic localization, because of the tight regulation of cytosolic prenyl diphosphate pools. Wu *et al.* (2006) conducted the most comprehensive evaluation of the effects of targeting different gene products to plastids or the cytosol. The most impressive example from their work is the accumulation of both FPS and ADS in plastids, which resulted in a roughly 5,000-fold increase in the concentration of amorpho-4,11-diene in transgenic tobacco plants when compared to transgenic plants expressing the unaltered ADS gene

Table 6 Metabolic engineering of taxane biosynthesis

Engineered plant species	Gene(s)	Gene Source(s)	Strategy	Promoter(s)	Targeting site(s)	Alterations/Consequences	References
Thale cress (<i>Arabidopsis thaliana</i> (L.) Heynh.; Col-3 ecotype)	Taxadiene synthase (TXS)	European yew (<i>Taxus baccata</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	Taxa-4(5),11(12)-diene ↑; carotenoids ↓↓; chlorophylls ↓↓	Besumbes et al. (2004)
	Taxadiene synthase (TXS)	European yew (<i>Taxus baccata</i> L.)	Ox	Glucocorticoid (dexamethasone (DEX))-inducible	Organ: ubiquitous; Cell: plastids	Taxa-4(5),11(12)-diene ↑	Besumbes et al. (2004)
	Taxadiene synthase (TXS); 1-deoxy-D-xylulose 5-phosphate synthase (DXS) or 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR)	European yew (<i>Taxus baccata</i> L.) (TXS); Thale cress (<i>Arabidopsis thaliana</i> (L.) Heynh.; Col-3 ecotype) (DXS, DXR)		CMV 35S	Organ: ubiquitous; Cell: plastids (TXS, DXS, DXR)	Taxa-4(5),11(12)-diene ↑	Carretero-Paulet et al. (2006)
Tomato (<i>Solanum lycopersicum</i> Mill.; yellow flesh mutant)	Taxadiene synthase	European yew (<i>Taxus baccata</i> L.)	Ox	CMV 35S	Organ: ubiquitous; Cell: plastids	Taxa-4(5),11(12)-diene ↑	Kovacs et al. (2007)
	Taxadiene synthase	European yew (<i>Taxus baccata</i> L.)	Ox	Polygalacturonase (fruit ripening-specific)	Organ: ubiquitous; Cell: plastids	Taxa-4(5),11(12)-diene ↑↑	Kovacs et al. (2007)
Tobacco (<i>Nicotiana glauca</i> Speg. & Comes)	Taxadiene synthase	Pacific yew (<i>Taxus brevifolia</i> Peattie)	Ox	Cembratrienol synthase (glandular trichome-specific)	Organ: leaf glandular trichomes; Cell: plastids	Taxa-4(5),11(12)-diene ↑	Tissier et al. (2008)
	Taxadiene synthase; taxadiene 5α-hydroxylase (CYP725A4)	Pacific yew (<i>Taxus brevifolia</i> Peattie) (TXS); Japanese yew (<i>Taxus cuspidata</i> Siebold & Zucc.) (CYP725A4)	Ox	Cembratrienol synthase (TXS); cembratrienol hydroxylase (CYP725A4) (glandular trichome-specific)	Organ: leaf glandular trichomes; Cell: plastids (TXS) Cell: ER (CYP725A4)	5(12)-Oxa-3(11)-cyclotaxane ↑	Rontein et al. (2008)
Physcomitrella patens (Hedw.) Bruch & Schimp.	Taxadiene synthase	Pacific yew (<i>Taxus brevifolia</i> Peattie)	Ox	Ubiquitin promoter	Organ: ubiquitous; Cell: plastids	Taxa-4(5),11(12)-diene ↑	Anterola et al. (2009)

CMV 35S, cauliflower mosaic virus promoter; ER, endoplasmic reticulum; HS, hsp8.1 promoter; Ox, overexpression.

(encoding a cytosolic gene product). An (unnatural) mitochondrial localization of a heterologous terpene synthase was first tested by Kappers *et al.* (2005). The authors reported that transgenic *Arabidopsis* plants expressing an FaNES1 gene from strawberry with an engineered 5' mitochondrial targeting sequence emitted two new terpenoids, which attracted predatory mites that could potentially aid in the defence against herbivores. A mitochondrial localization of terpene synthases was also used successfully for the production of precursors for the antimalarial sesquiterpene artemisinin (Farhi *et al.*, 2011; van Herpen *et al.*, 2010).

2 Constitutive promoters have been used successfully but one needs to keep in mind that the ubiquitous expression of a transgene may interfere with essential processes, thus decreasing plant fitness. For example, the constitutive expression of the FaNES1 gene from strawberry in transgenic *Arabidopsis* plants apparently depleted the plastidial precursor pools for the biosynthesis of carotenoids and chlorophylls, thus resulting in stunted growth (Aharoni *et al.*, 2003). A similar, undesirable phenotype was also reported from transgenic *Arabidopsis* plants expressing the TXS gene from European yew. Tissue- or cell-type-specific promoters can provide advantages over constitutive promoters, if the target terpenoid can be accumulated in specialized anatomical structures such as glandular trichomes or secretory cavities. There are very few examples in the literature where such promoters have been employed to drive the expression of genes involved in terpenoid biosynthesis. Glandular trichome-specific promoters were used successfully to down-regulate the expression levels of cembratrienol synthases (CBTSs) and a cembratrienol hydroxylase in tobacco (Wang and Wagner, 2003; Ennajdaoui *et al.*, 2010). In an independent study, two glandular trichome-specific promoters were fused to two target genes involved in the biosynthesis of the diterpene taxol in yew: transgenic tobacco plants transformed with CBTS promoter::TXS and CYP71D16 promoter::T5H fusion constructs accumulated a structurally unusual cyclotaxane, but production yields were not reported (Rontein *et al.*, 2008). Several promoters that drive the expression of transgenes in a glandular trichome-specific fashion have now been characterized. The utility of these promoters as tools for metabolic engineering efforts aimed at accumulating specific terpenoids in glandular trichomes remains to be evaluated.

3 Transcription factors to up-regulate the entire terpenoid pathway (or large parts of it) are currently unavailable. Following seminal discoveries in the 1990s, anthocyanin biosynthesis has been a paradigm for the coordinate control of gene expression by bHLH transcription factors that physically interact with R2R3 MYB proteins to activate essentially all genes of the pathway (reviewed in Grotewold, 2006). Such master switches are obviously a phenomenal tool for metabolic engineering. However, although transcription factors that regulate the expression of certain genes involved in terpenoid biosynthesis have been characterized (Ma *et al.*, 2009b; Yu *et al.*, 2012b), regulators with broader utility have not yet been identified. A second class of regulators of interest would be those affecting the initiation and density of glandular trichomes. Impressive progress has been made with furthering our understanding the formation of nonglandular trichomes in *Arabidopsis* (reviewed in Ishida *et al.*, 2008). The utility of such regulators for increasing the numbers of glandular trichomes has not yet been demonstrated conclusively for terpenoid producers. Given the potential for a step change in terpenoid production by 'developmental engineering', this area of research deserves more attention.

4 The potential for an undesirable metabolization of a target terpenoid is as yet not predictable but can have dramatic effects on the outcome of engineering efforts. Several studies aimed at producing certain target terpenoids in experimental model plants have resulted in the accumulation of breakdown products, apparently generated by nonspecific enzyme activities present in the host plant (Fig. 2). Glycosylation has been observed in *Arabidopsis* (Aharoni *et al.*, 2003), potato tubers (Aharoni *et al.*, 2006) and *Petunia* flowers (Lücker *et al.*, 2001), metabolic redox reactions have been reported for carnation (flowers and leaves) and tomato fruit (Davidovich-Rikanati *et al.*, 2007; Lavy *et al.*, 2002; Lewinsohn *et al.*, 2001), and a chain length reduction has been discovered in *Arabidopsis* (Kappers *et al.*, 2005). The types of metabolic conversions appear to depend on the host organism and the specific organ targeted for the accumulation of an engineered terpenoid. In addition, metabolism of target metabolites may also depend on their chemical structure. These issues need to be considered when designing metabolic engineering strategies.

5 The host organism for transformation needs to be chosen very carefully. The vast majority of plant metabolic engineering studies involving the heterologous expression of genes with relevance for terpenoid accumulation have been performed with model plants for which facile and fairly rapid transformation protocols are available. Unfortunately, the yields of engineered terpenoids in experimental model plants have been extremely low, with the highest reported accumulation levels being around 0.01% of fresh weight biomass. In typical essential oil plants, mono- and sesquiterpene concentrations generally vary between 0.5 and 5% of dry weight biomass, and tobacco diterpenes have been reported to accumulate to more than 10% of dry weight biomass (Wagner, 1991). However, even in tobacco, where diterpene production in glandular trichomes is comparatively high, the expression of heterologous genes has not yet resulted in high yields of novel diterpenoids. Higher terpenoid accumulation levels have been reported for volatiles released from engineered transgenic plants. If the aim of a metabolic engineering effort should be to modulate the trophic interaction of a crop plant with insects, terpenoid volatiles emitted from transgenic plants can have positive effects on disease resistance (Aharoni *et al.*, 2003; Beale *et al.*, 2006; Kappers *et al.*, 2005; Wu *et al.*, 2006; Yu *et al.*, 2012a). In contrast, if a collection of terpenoid(s) should be required, volatilization is undesirable. The strategic question arises if one should, rather than attempting to accumulate novel terpenoids in a heterologous plant with favourable agronomic characteristics, optimize terpenoid accumulation in the native producer. The main limitations of engineering efforts with nonmodel terpenoid producers are often the low efficiency of transformation and relatively long time periods required for the regeneration of transgenic plants. However, metabolic engineering efforts have been employed very successfully for enhancing the production of essential oils in peppermint (Lange *et al.*, 2011) and spike lavender (Muñoz-Bertomeu *et al.*, 2006), and for the increased accumulation of the antimalarial sesquiterpene artemisinin in sweet wormwood (reviewed in Covello, 2008).

6 Researchers should perform field trials with elite transgenic lines, so that the true costs of production of target terpenoids can be calculated. There is an unfortunate propensity in the literature for focusing on fold-change values when comparing transgenic lines with a reference line grown under greenhouse conditions. If the reference line contains extremely low levels of a target metabolite, one might determine a several thousand fold increase

in the concentration of that metabolite, while not achieving relevant production levels. It is also important to evaluate, in addition to terpenoid accumulation, the agronomic characteristics of elite transgenic lines, which can only be carried out in replicated field trials. We are cognizant of the fact that obtaining permission from government agencies for such trials can be a substantial burden or might even be impossible in some countries. As a research community, we need to take advantage of the existing technical expertise and available field locations worldwide to address these issues collaboratively.

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