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Review

Metabolic engineering of volatile isoprenoids in plants and microbes

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ABSTRACT

The chemical properties and diversity of volatile isoprenoids lends them to a broad variety of biological roles. It also lends them to a host of biotechnological applications, both by taking advantage of their natural functions and by using them as industrial chemicals/chemical feedstocks. Natural functions include roles as insect attractants and repellents, abiotic stress protectants in pathogen defense, etc. Industrial applications include use as pharmaceuticals, flavours, fragrances, fuels, fuel additives, etc. Here we will examine the ways in which researchers have so far found to exploit volatile isoprenoids using biotechnology. Production and/or modification of volatiles using metabolic engineering in both plants and microorganisms are reviewed, including engineering through both mevalonate and methylerythritol diphosphate pathways. Recent advances are illustrated using several case studies (herbivores and bodyguards, isoprene, and monoterpene production in microbes). Systems and synthetic biology tools with particular utility for metabolic engineering are also reviewed. Finally, we discuss the practical realities of various applications in modern biotechnology, explore possible future applications, and examine the challenges of moving these technologies forward so that they can deliver tangible benefits. While this review focuses on volatile isoprenoids, many of the engineering approaches described here are also applicable to non-isoprenoid volatiles and to non-volatile isoprenoids.

Key-words: biotechnology; synthetic biology; systems biology; terpenes.

INTRODUCTION

Plants produce thousands of different volatile chemicals. They are emitted globally on a massive scale: equivalent to 500–1200 Tg (5^8–12^8 tonnes) carbon per year (Guenther *et al*. 2006), representing 2–3% of the total carbon exchange between the biota and the atmosphere (Kesselmeier & Staudt 1999). Volatiles are emitted from both aerial and subterranean tissues. They may belong to several different classes of biomolecules – including isoprenoids (also known as terpenoids/terpenes), fatty acid derivatives, phenylpropanoids/benzenoids, and amino acid derivatives – and they play a host of different roles in plant ecophysiology (Dudareva *et al*. 2006). The roles of volatiles as semiochemicals in biotic interactions are well characterized, and include attractants for pollinators, seed disseminators, natural enemies of herbivores (e.g. tritrophic interactions), pathogens and pests; deterrents for herbivores; intra-plant and inter-plant signalling molecules; direct defence (e.g. intoxicants, poisons, phytoalexins), etc. (Gershenzon & Dudareva 2007). Volatiles in the isoprenoid class have also been shown to provide protection under abiotic stress conditions (Sharkey & Singsaas 1995; Vickers *et al*. 2009a; Loreto & Schnitzler 2010). These important roles in both biotic and abiotic response physiology represent a high potential for biotechnological exploitation (Aharoni *et al*. 2005). Specifically, they contribute to survival, reproductive success, biomass yield, food quality and many other important agronomic traits (Dudareva & Pichersky 2008). In addition to more obvious applications (described below), a number of these biochemicals can also be used as industrial chemicals that can either replace petrochemicals or provide novel industrial products. The bulk of biogenic volatiles are low molecular weight isoprenoid compounds (Guenther *et al*. 2006), and members of this group are prominent with respect to biotechnological applications; this review will therefore focus on volatile isoprenoids. However, many of the engineering approaches described here are equally applicable to non-volatile isoprenoids and non-isoprenoid volatiles.

There are four generic approaches to exploiting volatile isoprenoids for biotechnological applications: (1) extract the isoprenoid from naturally producing organisms for later application; (2) synthesize the isoprenoid using synthetic chemistry; (3) breed or engineer desired traits in the target organism; and (4) engineer overproduction of the isoprenoid in a model organism for later application. One or any of these approaches may be appropriate for a given use.

Extraction from natural sources may be possible where the isoprenoid is abundant; however, natural sources rarely produce sufficient amounts for broad-scale industrial applications, and the volatility of this class of products means that harvesting may be unfeasible. Chemical synthesis has been used for almost two centuries to produce agriculturally useful chemicals; however, in the case of isoprenoids, they are often too complex or synthetic chemistry routes are too expensive for synthesis at required yields, or no synthetic pathway has been developed so production is impossible. Furthermore, biological activity often requires correct chirality, and synthetic production is often not enantio-specific. In these cases,

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production using living organisms may be the only feasible route. Using such systems, metabolic pathways can be introduced, increased production can be engineered through metabolic modification, fermentation vessels can be used to contain cultures and facilitate extraction, and correct chirality can be achieved by using enantiospecific enzymes.

Production of the desired isoprenoid in the target organism requires a thorough understanding of the biology and biochemistry in a given system, and can deliver the benefit in the most efficient way since harvesting, transport and application are not required. Selective breeding and back-crossing to introduce desirable traits can achieve this if the trait is available in the germplasm of interest. While some crop species, such as maize, have germplasm available that can contribute desirable volatile components (Schnee *et al*. 2006; Degenhardt *et al*. 2009), backcrossing to introgress these components into elite agricultural cultivars can be a lengthy process. Where such germplasm is not available within the species, genetic modification can be used to access genes from unrelated species. The latter approach may be faster for cultivar development even when germplasm is available; however, testing and regulatory requirements for genetically modified organisms (GMOs) may mean that the final product takes as long or even longer to reach the market.

Where natural sources do not provide the required traits or yield is insufficient, chemical synthesis is not possible, and/or breeding/genetic engineering are unfeasible for biological, technological or socio-political reasons, bioproduction in microbial platforms such as *Saccharomyces cerevisiae* or *Escherichia coli* may provide a solution. Production in tightly controlled bioprocesses using engineered microorganisms is emerging as a preferred synthesis route for a wide variety of different biochemicals (Vickers *et al*. 2012), and in the case of volatile isoprenoids, it may solve some of the specific problems encountered for this class of natural products.

Here we will briefly review the biosynthesis of volatile isoprenoids, discuss bioengineering approaches and targets, and illustrate recent advances in these areas using case studies. We also provide a vision of where we might go in the future with these novel and exciting technologies.

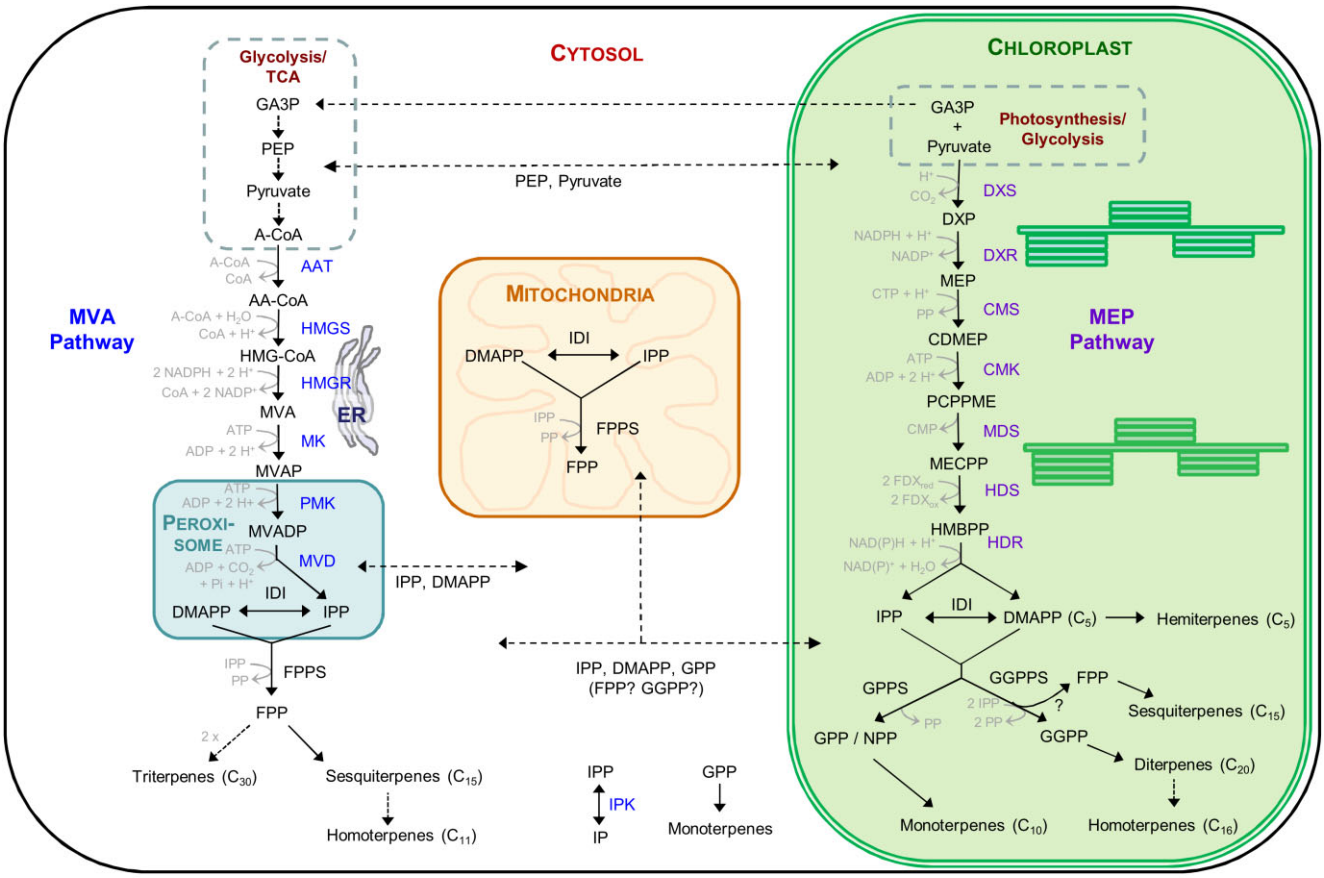
BIOSYNTHESIS OF VOLATILE ISOPRENOIDS

Isoprenoids are a large and extremely diverse group of natural products, comprising over 70 000 compounds – approximately one-third of all known natural products (The Dictionary of Natural Products Online: http://dnp.chemnetbase.com/intro/). Their diversity enables isoprenoids to fulfil a host of essential and non-essential functions in all known organisms; these roles include stabilization of cell membranes (hopanoids and sterols), cell wall biosynthesis (dolichols), modification of tRNAs, antioxidant activities, carriers for electron transport, hormones, photosynthetic and non-photosynthetic pigments, intracellular signal transduction, extracellular signalling molecules, defence molecules, vitamins, protein-targeting components, virulence factors, etc. (Holstein & Hohl 2004; Lichtenthaler *et al*. 2010).

Despite their remarkable chemical and functional diversity, all isoprenoids are derived from the same five-carbon precursors: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These isomers are produced by two unrelated metabolic pathways: the mevalonic acid (MVA) pathway, found in most eukaryotes, archea and some bacteria; and the methylerythritol phosphate (MEP) pathway [a.k.a. non-mevalonate, deoxyxylulose phosphate (DXP or DOXP) or Rohmer pathway], found in plant chloroplasts and in most bacteria (Rodríguez-Concepción & Boronat 2002; Rohmer 2003; Eisenreich *et al*. 2004; Fig. 1). Both pathways initiate from central carbon intermediates; acetyl-CoA in the case of the MVA pathway, and pyruvate and glyceraldehyde-3-phosphate in the case of the MEP pathway. Isoprenoids are classed based on the number of carbons, for example hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}) and longer-chain poly-isoprenoids. Homoterpenes (C_{16}, C_{11}) are produced by modification of larger sesquiterpene and diterpene precursors. Isoprenoid products may be modified and decorated in many different ways, such as through hydroxylation (by cytochrome P450 enzymes), oxidation (by dehydrogenases), acetylation, and glycosylation, and biosynthesis frequently involves convergence of one or more other metabolic pathways. DMAPP is used as a substrate by hemiterpene synthases; it also serves as an initial allylic substrate for prenyltransferases, which catalyse sequential condensation of IPP with growing allylic polyisoprenoid diphosphate chains (Kellogg & Poulter 1997). These reactions generate the prenyl diphosphate precursors geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP; C_{15}), and geranylgeranyl diphosphate (GGPP; C_{20}) (Fig. 1).

Plants have both an MVA pathway in the cytosol and a MEP pathway in the chloroplast. Isoprenoid precursor production is roughly compartmentalized in terms of products: FPP is primarily synthesized in the cytosol, and GPP and GGPP are primarily synthesized in the chloroplast (Fig. 1). Consequently, sesquiterpenes, triterpenes and C_{11} homoterpenes are primarily produced in the cytosol; while monoterpenes, C_{16} homoterpenes, diterpenes and tetraterpenes are primarily synthesized in the chloroplast. However, there is plenty of evidence for crosstalk between the two pathways at the prenyl diphosphate level (McCaskill & Croteau 1995; Laule *et al*. 2002; Noriko *et al*. 2002; Bick & Lange 2003; Hemmerlin *et al*. 2003; Schuhr *et al*. 2003; Dudareva *et al*. 2005; Flügge & Gao 2005; Lichtenthaler *et al*. 2010). There is also evidence that FPP can be produced in the chloroplasts in some species (Sanmiya *et al*. 1999; Aharoni *et al*. 2003; Thabet *et al*. 2011). Since plant FPP synthases (FPPS) do not possess plastid-targeting signals, it is likely that this plastidic FPP is released by the higher-order prenyltransferase GGPP synthase under some circumstances (Wu *et al*. 2006).

For an isoprenoid to be volatile, it must have specific chemical properties. Thus, volatile isoprenoids have relatively low molecular weight, are usually lipophilic, have a low boiling point and have sufficiently high vapour deficit to



MVA PATHWAY
Enzymes:
AAT, Acetyl-CoA C-acetyltransferase
HMGR, Hydroxymethylglutaryl-CoA reductase
HMGS, Hydroxymethylglutaryl-CoA synthase
MK, Mevalonate kinase
MVD, Diphosphomevalonate decarboxylase
PMK, Phosphomevalonate kinase
Metabolites:
A-CoA, Acetyl-CoA
AA-CoA, Acetoacetyl-CoA
HMG-CoA, (S)-3-hydroxy-3-methylglutaryl-CoA
MVA, (R)-mevalonate
MVAP, Mevalonate-5-phosphate
MVADP, Mevalonate diphosphate

MEP PATHWAY
Enzymes:
DXS, 1-deoxyxylulose-5-phosphate synthase
DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerasease
CMS, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase
CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
MDS, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
HDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase
HDR, Hydroxymethylbutenyl diphosphate reductase
Metabolites:
GA3P, D-glyceraldehyde 3-phosphate,
DXP, 1-deoxy-D-xylulose-5-phosphate
MEP, 2-C-methyl-D-Erythritol-4-phosphate
CDMEP, 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol
PCPPME, 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol
MECPP, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate
HMBPP, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate

PRENYL PHOSPHATE METABOLISM
Enzymes:
IDI, isopentenyl diphosphate isomerase
IPK, isopentenyl kinase
FPPS, farnesyl pyrophosphate synthase
GPPS, geranyl pyrophosphate synthase
Metabolites:
IPP, isopentenyl pyrophosphate (C_5)
DMAPP, dimethylallyl pyrophosphate (C_5)
GPP, geranyl pyrophosphate (C_{10})
NPP, neryl pyrophosphate (C_{10})
FPP, farnesyl pyrophosphate (C_{15})
GGPP, Geranylgeranyl pyrophosphate (C_{20})

COFACTORS/COFACTOR PRODUCTS
ATP, adenosine triphosphate
ATP, adenosine diphosphate
CTP, cytidine 5' triphosphate
CMP, cytidine 5'monophosphate
CoA, coenzyme A
FDX_{red}, ferrodoxin, oxidised
FDX_{ox}, ferrodoxin, reduced
NADH, Nicotinamide adenine dinucleotide, oxidised
NAD+, Nicotinamide adenine dinucleotide, reduced
NADPH, Nicotinamide adenine dinucleotide phosphate, oxidised
NADP+, Nicotinamide adenine dinucleotide phosphate, reduced

Figure 1. Volatile isoprenoid metabolism in plant cells. The cytosolic/endoplasmic reticulum (ER)/peroxisomally compartmentalized mevalonate (MVA) and chloroplastic methylerythritol pyrophsophate (MEP) pathways both produce the C_5 precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These are condensed by prenyltransferases to generate C_{10} (geranyl diphosphate, GPP), C_{15} (farnesyl diphosphate, FPP), and C_{20} (geranylgeranyl diphosphate, GGPP) precursors for isoprenoid production. Isoprenoid classes are grouped based on number of carbons, for example monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), homoterpenes (C_{11}, C_{16}). Note that only volatile isoprenoid metabolism is included here; higher-order isoprenoids including carotenoids, sterols, quinones, hormones, etc. are not included. Figure modified and updated from previous figures (Bouwmeester 2006; Vickers *et al.* 2009a; Pulido *et al.* 2012; Vranová *et al.* 2012; Dudareva *et al.* 2013) and using information from UniProt http://www.uniprot.org/uniprot/Q8S948 and (Dellas *et al.* 2013). MEP pathway nomenclature follows guidelines provided by Phillips *et al.* (2008). Enzymatic steps are represented by arrows; multiple enzymatic steps are represented by dotted lines and transport between compartments is represented by dashed lines.

volatilize at normal physiological temperatures (Dudareva *et al.* 2006). Hemiterpenes (C_5), most monoterpenes (C_{10}), many sesquiterpenes (C_{15}) and some diterpenes (C_{20}) have these properties. Volatiles are usually emitted soon after synthesis, and are only stored if specialized storage structures (e.g. glandular trichomes, resin ducts) are available.

ENGINEERING VOLATILE ISOPRENOIDS IN PLANTS

Volatility bestows on these isoprenoids the capacity to behave as signalling molecules (semiochemicals), and the most obvious biotechnological application is modification of

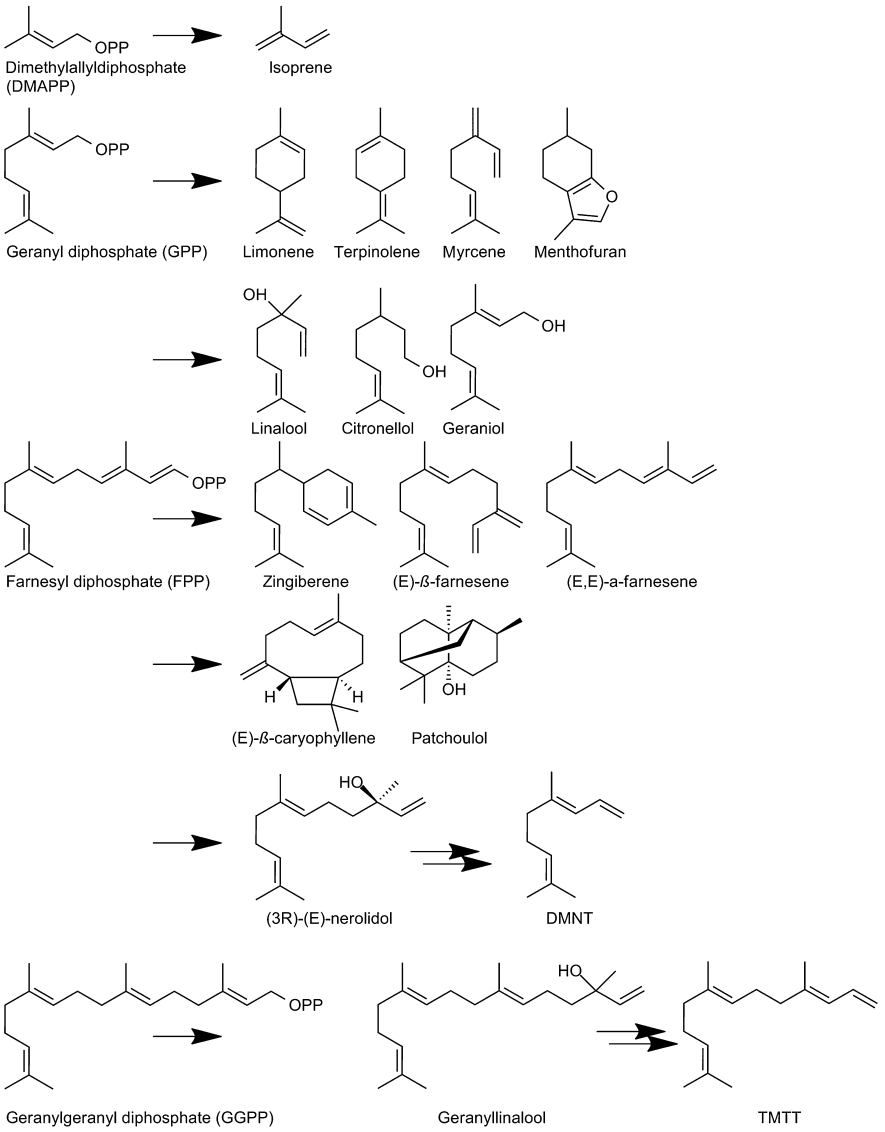


Figure 2. Structures of representative volatile isoprenoids discussed in the text.

these emissions in plants to augment or repress their activities to achieve specific outcomes. Of the many potential targets, only a few have been directly engineered in transgenic plants; they include deterring herbivores, influencing tritrophic interactions, protecting against pathogens and abiotic stress, and improving fragrances for fruits and flowers. These examples will be reviewed here and in the case studies below. Structures of representative engineered volatiles are shown in Fig. 2 and engineered interactions are shown in Fig. 3.

Insect herbivores can be deterred by volatiles that they find unattractive, or that signal danger to them (Unsicker *et al*. 2009). For example, transgenic *Arabidopsis* producing either (*E*)-*β*-farnesene (Beale *et al*. 2006) or linalool (Aharoni *et al*. 2003) repels aphids; and transgenic tobacco-producing patchoulol is less attractive to tobacco hornworm than wild-type tobacco (Wu *et al*. 2006). Volatiles may also deter oviposition in flying insects whose larvae feed on leaves – for example, transgenic *Arabidopsis* plants producing linalool deterred oviposition by *Helicoverpa armigera* moths (McCallum *et al*. 2011). The same deterrent compounds also often act as attractants for carnivorous or parasitic arthropods that prey on the herbivores (Heil 2008). Significant advances have been made in engineering these interactions recently (see Case Study: Herbivores and Bodyguards).

Many volatile isoprenoids have antimicrobial activity (Himejima *et al*. 1992), and this is one reason why they are often included in cleaning agents (e.g. *α*-pinene, limonene). They can also provide resistance to bacterial disease in plants. For example, the sesquiterpene (*E*)-*β*-caryophyllene, the main constituent of *Arabidopsis* flower scent (Tholl *et al*. 2005; Knudsen *et al*. 2006), has antibacterial activity (Muroi & Kubo 1993). *Arabidopsis* with up-regulated (*E*)-*β*-caryophyllene production has decreased floral bacterial infection during pollination and increased seed viability (Huang *et al*. 2012). Also, transgenic petunia producing linalool exhibited

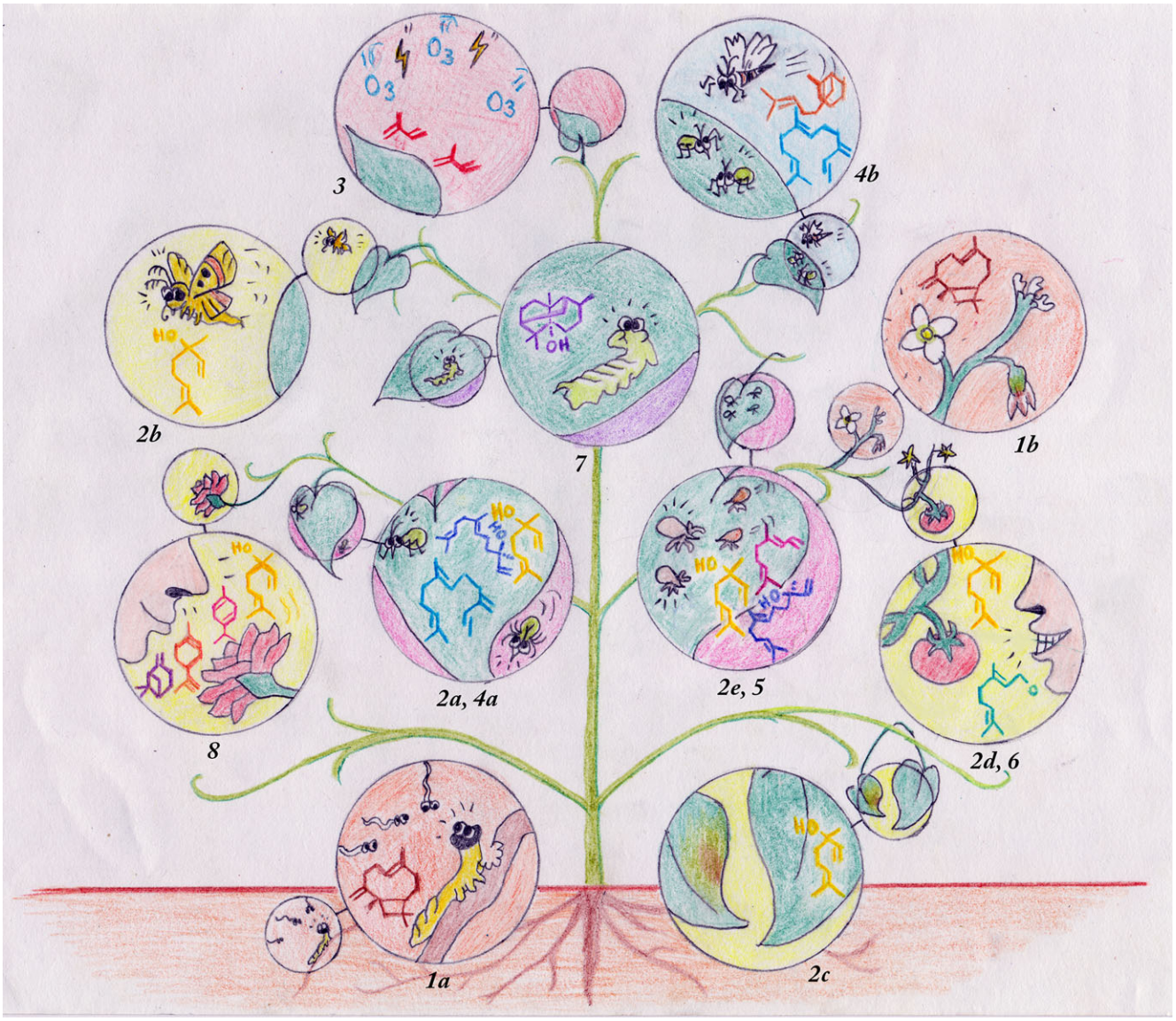


Figure 3. Various interactions that have been engineered using volatile isoprenoids in plants. 1. (*E*)-*β*-caryophyllene (a) attracts entomopathogenic nematodes (b) decreases bacterial infection in flowers during pollination. 2. Linalool (a) repels aphids (by itself and also with nerolidol) (b) deters oviposition by moths (c) provides resistance to pathogen infection (d) makes tomatoes smell/taste better (along with geraniol) (e) attracts predatory mites. 3. Isoprene protects from abiotic stress. 4. (*E*)-*β*-farnesene (a) alarms and repels aphids (deters them from visiting leaves) (b) attracts parasitic wasps [by itself and also with (*E*)-*α*-bergamotene]. 5. Nerolidol + 4,8-dimethyl-1,3,7-nonatriene (DMNT) attract predatory mites. 6. Geraniol makes tomatoes small/taste better (along with linalool). 7. Patchoulol deters tobacco hornworm. 8. Flower fragrance can be altered by production of linalool, *γ*-terpinene, limonene, and *β*-pinene. Linalool, limonene and farnesene can also be used as industrial fragrances; farnesene, limonene and cymene can be blended to make jet fuel; isoprene can be polymerized into synthetic rubbers. See text for further details. Figure kindly provided by Andrea Vickers.

resistance to mildew infection in the glasshouse (Lücker *et al*. 2001). In addition to protection against biotic agents, volatile isoprenoids can also provide protection against abiotic stress (Vickers *et al*. 2009a; see Case Study: Isoprene).

Volatile isoprenoids play a strong role in aroma and flavour of fruits, vegetables and flowers (Dudareva *et al*. 2006). However, these scents are often lost in horticultural breeding programmes focussed on other attributes (Zuker *et al*. 2002; Klee & Tieman 2013). Metabolic engineering changed floral bouquets in a number of species, for example using linalool synthase in carnations (Lavy *et al*. 2002) and *Arabidopsis* (Aharoni *et al*. 2003), and a combination of *γ*-terpinene, limonene, and *β*-pinene synthase in tobacco (Lücker *et al*. 2004). Transgenic tomatoes have also been engineered to produce the aroma/flavour compounds linalool (Lewinsohn *et al*. 2001) and geraniol (Davidovich-Rikanati *et al*. 2007) in fruits; in the latter case, the transgenic fruit tasted better than control fruit. Conversely, antisense knock-down of menthofuran synthase decreased production of menthofuran, a toxin that is also an undesirable flavour compound, in

transgenic peppermint by over 50% (Mahmoud & Croteau 2001); and down-regulation of the expression of limonene hydroxylase increased the ratio of limonene, a desirable aroma compound, in peppermint essential oil without affecting total oil yield (Mahmoud *et al*. 2004).

Optimizing volatile isoprenoid production in plants: approaches and tools

Modifying volatile emission spectra requires a detailed knowledge of the biology, biochemistry and genetics involved in production, as well as of the physiology and ecology of the function. Production of volatile isoprenoids in plants can be engineered either through modification of existing native pathways or through introduction of heterologous genes, to introduce/increase/decrease production of particular volatiles. The simplest approach is to overexpress or repress target synthase genes (as discussed above). Product ratios can also be modified to increase desired compounds by down-regulation of the subsequent step in the pathway (Mahmoud & Croteau 2001; Wang *et al*. 2001; Mahmoud *et al*. 2004) or by eliminating competing pathways. Controlling compartmentalization and increasing isoprenoid pathway flux can also be used to increase precursor availability (and ultimately product concentration). A number of useful approaches and tools for optimizing metabolic engineering outcomes are discussed below.

Increasing flux through isoprenoid pathways in plants

To provide sufficient C_5 precursors for production of novel volatiles in the context of competition from other isoprenoid products, it may be necessary to increase flux through the upstream isoprenoid pathways. However, modifying flux through core metabolic pathways such as the MVA and MEP pathways has the potential for far more extensive ramifications than increasing the availability of the immediate precursors for the desired volatile product, as many different products (essential and non-essential) are made through these pathways. Furthermore, although the two core pathways are compartmentalized, engineering through one pathway has the potential to affect downstream flux in the other because of pathway crosstalk (see above). Increasing the availability of pathway precursors is a common approach to metabolic engineering for isoprenoid production in microbes (see below), but relatively little has been done in plant isoprenoid engineering. Overexpression experiments using various MEP and MVA pathway genes in plants are reviewed in separate sections below.

MVA pathway. The cytosolic MVA pathway (Fig. 1) has been extensively studied over more than half a century, and its biochemistry and regulation are very well understood (Lombard & Moreira 2011). Regulatory control is exerted primarily through a single key rate-limiting enzyme, hydroxymethylglutaryl CoA reductase (HMGR). Post-translational feedback regulation by FPP controls degradation of HMGR (Gardner & Hampton 1999), limiting pathway flux at the HMG-CoA node. Overexpression of a native *HMGR* in *Arabidopsis* did not affect downstream isoprenoid accumulation, most likely because of its tight post-transcriptional regulation (Re *et al*. 1995). In contrast, constitutive overexpression of heterologous or truncated *HMGR* genes (both of which are presumably resistant to feedback inhibition) increased production of downstream isoprenoids including native sterols (Chappell *et al*. 1995) as well as engineered cytosolic sesquiterpenes including patchoulol (Wu *et al*. 2006) and artemisinin (an antimalarial compound) (Aquil *et al*. 2009) in stably transformed plants, and amorphadiene (an artemisinin precursor) in a transient expression system (van Herpen *et al*. 2010). Overexpression of a heterologous avian FPPS can also increase production of patchoulol (Wu *et al*. 2006); however it did not increase production of amorphadiene in the transient system where the truncated *HMGR* was already overexpressed (van Herpen *et al*. 2010).

Mevalonate kinase is also subject to feedback inhibition – not just by FPP, but by essentially all prenyl diphosphate intermediates (Dorsey & Porter 1968; Hinson *et al*. 1997). While it has not yet been examined *in planta*, overexpression improves pathway flux in engineered microbes (see below) and might also do so in plants. Other genes might also have an effect, particularly under specific conditions. For example, acetoacetyl-CoA thiolase (Fig. 1), has been shown to regulate the MVA pathway during adaptation to abiotic stress in alfalfa (Soto *et al*. 2011).

Recently, a novel approach was used to bypass compartmentalized pathway regulatory mechanisms by reconstructing a mevalonate pathway in plant chloroplasts (Kumar *et al*. 2012). The entire cytosolic MVA pathway was inserted onto the tobacco chloroplast genome and homoplastic transgenic plants were regenerated. These plants had increased levels of mevalonate, carotenoids, squalene, sterols and triacyglycerols compared with control plants, and it is reasonable to presume that these plants can also be engineered to produce high levels of volatile isoprenoids.

MEP pathway. The MEP pathway (Fig. 1) was only fully elucidated a decade ago (Lichtenthaler 2000; Rodríguez-Concepción & Boronat 2002; Eisenreich *et al*. 2004), and its regulation is less well understood. Current studies suggest that regulation is extremely complex; in contrast to the MVA pathway, it appears that most, if not all, MEP enzymes can play a role (Rodríguez-Concepción 2006). Most of these studies have been performed in *E. coli*, and are reviewed under 'MEP Pathway' in the microbial engineering section below; here, only studies in plants will be examined.

DXP synthase (DXS) appears to be a key rate-determining step (Rodríguez-Concepción 2006). It is feedback-regulated by IPP and DMAPP (Banerjee *et al*. 2013) and *DXS* transcript levels also oscillate diurnally in parallel with volatile isoprenoid production in snapdragon (Dudareva *et al*. 2005). In line with these findings, overexpression of *DXS* in engineered plants increased accumulation of a variety of isoprenoid products (Estévez *et al*. 2001; Enfissi *et al*. 2005).

DXP reductoisomerase (DXR) also appears to play a role in regulating isoprenoid accumulation in many different native systems (reviewed in Rodríguez-Concepción 2006). Overexpression of *DXR* in peppermint (Mentha × piperita L.) increased production of essential oil by 50%, without changing the ratios of different monoterpenes (Mahmoud & Croteau 2001).

The last two enzymes in the MEP pathway are both thought to be involved in pathway regulation (Rodríguez-Concepción 2006). Up-regulation of hydroxymethylbutenyl diphosphate synthase (HDS) does not increase isoprenoid accumulation in transgenic *Arabidopsis* (Flores-Péreza *et al*. 2008); however, if upstream flux bottlenecks (e.g. DXS/DXR) are released, an effect might be observed. In contrast, overexpression of hydroxymethylbutenyl diphosphate reductase (*HDR*), encoding the final enzyme in the MEP pathway, increased isoprenoid production in *Arabidopsis* (Besumbes *et al*. 2004; Botella-Pavía *et al*. 2004).

Accessing prenyl phosphate precursors: appropriate compartmentalization

The complex compartmentalization of plant cells represents a particular challenge for plant metabolic engineering, and several key issues must be addressed in experimental design for this reason. These issues include substrate availability, enzyme targeting, the role of transporters, etc. (Heinig *et al*. 2013). Some of the challenges of volatile engineering (see 'Engineering challenges: unwanted side-effects' below) could potentially be prevented by reducing the competition with primary processes for precursor availability. In order to increase monoterpene production in tomato fruits, Gutensohn *et al*. (2013) overexpressed the small subunit (SSU) of a heterodimeric GPP synthase under control of a fruit ripening-specific promoter. The SSU can dimerise with the catalytic GGPP synthase and alter product specificity from GGPP to GPP. Co-expression of the SSU in a line already overexpressing geraniol synthase resulted in a significant increase in geraniol relative to the parental line. Co-expression of a cytosolic bifunctional sesquiterpene/monoterpene synthase (alpha-zingiberene synthase, *ZIS*) also increased production of the monoterpene components compared with controls expressing only *ZIS*, suggesting that GPP can be transported from the plastid to the cytosol. Indeed there is evidence that GPP can be exported to the cytosol (Bick & Lange 2003; Gutensohn *et al*. 2013; Fig. 1).

Expression of a *Perilla frutescens* limonene synthase with different targeting signals in tobacco showed that limonene production was generally higher with plastid localization (Ohara *et al*. 2003). Early attempts to engineer sesquiterpene production were directed at the cytosol, where FPP is located, but this resulted in only low levels of the anticipated products (Hohn & Ohlrogge 1991; Wallaart *et al*. 2001; Aharoni *et al*. 2003), presumably because the synthase was unable to compete effectively with native cytosolic FPP-utilizing enzymes. However, later studies using native targeting of sesquiterpene synthases did show that detectable levels of sesquiterpenes can also be produced in the cytosol (Beale *et al*. 2006; Degenhardt *et al*. 2009). A simple explanation for these different results could be that the sesquiterpene synthases used for engineering have different affinities for the substrate FPP, but more complicated explanations such as different interactions between sesquiterpene synthase and the heterologous FPPS may also be possible.

Several studies have also attempted to overcome the apparent limitations of sesquiterpene production in the cytosol by using alternative subcellular targeting. Targeting the dual linalool/nerolidol synthase to the mitochondria resulted in the production of nerolidol, nerolidol glycosides and the nerolidol-derived 4,8-dimethyl-1,3,7-nonatriene (DMNT; C_{11}) (Kappers *et al*. 2011; Houshyani *et al*. 2013). Upon transient expression in *N. benthamiana*, targeting of the feverfew germacrene A synthase to the mitochondria increased the production of germacrene A 15-fold compared with cytosolic localization (Liu *et al*. 2011b), showing the potential of this approach. Wu *et al*. (2006) used the large isoprenoid production capacity of the chloroplasts/plastids for the production of the sesquiterpene patchoulol, by targeting both an FPPS and patchoulol synthase to the plastids. Compared with the cytosolic localized enzymes, patchoulol production in the plastids was substantially higher. Fusion constructs might also improve competition for substrate against native cytosolic enzymes (see 'Producing volatile isoprenoids in microorganisms' below).

Transcription factor engineering

The use of transcription factors to up-regulate core isoprenoid pathway (or large parts of it) has so far not been reported in plants (Lange & Ahkami 2013). However, in *Catharanthus roseus*, overexpression of the AP2/ERF transcription factor ORCA3 resulted in enhanced expression of several terpene indole-alkaloid biosynthetic genes and, consequently, in increased accumulation of the isoprenoid indole alkaloid products (van der Fits & Memelink 2000). Overexpression of ORCA2 lead to enhanced accumulation of catharanthine and vindoline in hairy roots (Liu *et al*. 2011a). In *A. annua*, overexpression of AP2/ERF transcription factors AaERF1 and AaERF2 resulted in elevated transcript levels of both *ADS* and *CYP71AV1* and consequently, in increased accumulation of artemisinic acid and artemisinin (Yu *et al*. 2012). Overexpression of *AaORA* – the expression of which could directly be linked to the expression of *ADS*, *CYP71AV1* and *DBR2* that encode enzymes of the artemisinin pathway – in transgenic *A. annua* resulted in an about 50% increase in artemisinin and 35% increase in dihydroartemisinic acid (DHAA) content (Lu *et al*. 2013).

Transient transformation: a tool for multi-gene pathway analysis

Straight-forward single-step introduction and/or modification of volatile isoprenoids in transgenic plants has so far been quite successful (as described above). The reconstruction of more complex biosynthetic pathways that involve multiple genes, in stable transformed heterologous plants,

however, can be quite complicated, and it may take years to evaluate the consequences of constructs and strategies for the anticipated product formation. A recent breakthrough in metabolic engineering of isoprenoids is the use of transient expression in *Nicotiana benthamiana*, mediated by *Agrobacterium tumefaciens*. This presents a rapid method to evaluate volatile isoprenoid metabolic engineering strategies as it allows us to express many different heterologous proteins simultaneously without the need to regenerate transgenic plants. Transient gene expression is easy to perform, and the results can be evaluated within 1 week post-agroinfiltration (van Herpen *et al*. 2010), compared with months (to years) required for stable transformation. Second, through transient expression several genes can be easily introduced into plants at the same time, and the relative expression level of the genes may be manipulated by changing the relative amount of *A. tumefaciens* strains carrying the genes (and this is difficult to achieve by stable transformation; Ting *et al*. 2013).

Metabolomics – a key technology for volatile analysis

Key in the evaluation of engineering strategies is the analysis of the anticipated products, and there are a number of excellent reviews of the methods required for trapping and analysis of isoprenoid volatiles (see e.g. Tholl *et al*. 2006; Alexander *et al*. 2013; Gaquerel & Baldwin 2013). Plant metabolomics has benefited from substantial recent improvements in collection and analysis of volatiles (including increased sensitivity, e.g. Jardine *et al*. 2013), as well as data analysis and interpretation (reviewed in Hall 2011).

It is becoming increasingly clear that targeted metabolite analysis alone is risky, as unexpected product modifications or side effects of the engineering may be overlooked. Examples include unexpected conversion of linalool to linalool glycoside in transgenic Petunia (Lücker *et al*. 2001) and the formation of glutathione and cysteine conjugates of sesquiterpene lactones (Liu *et al*. 2011b). Therefore, the use of untargeted metabolomics is increasing in both basic plant volatile research and metabolic engineering studies (Liu *et al*. 2011b; Yang *et al*. 2011; Gaquerel & Baldwin 2013; Ting *et al*. 2013). In contrast to targeted metabolite analysis, untargeted metabolomics uses statistical tools to mine large data sets for differences between samples without *a priori* bias (Hall 2011; Yang *et al*. 2011; Gaquerel & Baldwin 2013). The technological advancements in sample throughput, analytical sensitivity and data analysis capacity now allow the evaluation of differences in hundreds of metabolites between hundreds of samples (Keurentjes *et al*. 2006). This has paved the way for non-targeted approaches to assess the effect of genetic background on metabolite formation (Keurentjes *et al*. 2006; Schauer *et al*. 2006). Broad-scale non-targeted metabolomics is now being applied to volatile metabolites also; for example, Kappers *et al*. (2011) used statistical analysis of untargeted volatile profiles in combination with predatory mite behaviour to identify spider mite-induced volatiles in different cucumber genotypes that affect attraction of predatory mites. Metabolite profiling can also be combined with transcription analysis to assist in the discovery of genes involved in volatile isoprenoid biosynthesis (Goossens *et al*. 2003; Mercke *et al*. 2004).

Metabolomics methods now also extend to quantitative and semi-quantitative identification of MEP pathway intermediates in microbes (Zhang *et al*. 2011; Zhou *et al*. 2012; Banerjee *et al*. 2013) and in plants (Li & Sharkey 2013). Methods to identify and measure prenyl diphosphates are available, however separating IPP and DMAPP to determine their ratio remains a challenge. A novel approach is to convert DMAPP to isoprene using an isoprene synthase (IspS), measure the resulting isoprene, then convert the remaining IPP into DMAPP and then isoprene using IPP isomerise (IDI) + IspS (Zhou *et al*. 2013). The IspS approach might also be combined with LC-MS/MS to measure C_5 prenyl diphosphate pools more directly.

Compartmentalization in plant cells presents a problem for metabolomics analysis, especially for metabolites that are shared among compartments (e.g. prenyl diphosphates). Isolation of organelles without upsetting the metabolite levels is challenging; improved methods for extraction and analysis of organelles may help (Heinig *et al*. 2013). Use of metabolic network flux analysis (MFA) using labelled precursors is well-developed for metabolic engineering in other systems (Nielsen 2003; Quek *et al*. 2010), and is currently being developed for use in plants (Shachar-Hill 2013). While the complexity of plant metabolism does present a problem for MFA, it will likely be able to assist in dealing with compartmentalization questions in some instances (Shachar-Hill 2013).

Engineering challenges: unwanted side-effects

In comparison with microbial metabolic engineering, where metabolic systems are significantly less complex, plant metabolic engineering is relatively underdeveloped. Major advances in tools and knowledge have been made in recent years (Dudareva & DellaPenna 2013); in particular, with respect to engineering volatile isoprenoids, we now have significantly better understanding of biosynthetic pathways, regulation and compartmentalization, as well as the general biology of volatile-mediated interactions (Dudareva *et al*. 2013; Lange & Ahkami 2013). However, we are still limited, in some cases, by our understanding of the systems in which we are working and by particular challenges presented for engineering volatiles.

Non-specific catalytic activity

Isoprenoid synthases may be non-specific for both substrate and product; for example, (*E*)-*β*-farnesene synthase from peppermint (*Mentha* × *piperita*) can make (*E*)- and (*Z*)-*β*-farnesene and δ-cadinene from FPP, but it can also make limonene, terpinolene and myrcene from GPP (Crock *et al*. 1997). Furthermore, synthases may behave differently *in vivo* compared with *in vitro* where their biochemical activities are often characterized (Ginglinger *et al*. 2013), likely because of

differing availability of cofactors and other biochemical conditions. Consequently, the outcome of metabolic engineering is not always predictable because the product ratio will vary depending on the product specificity of the enzyme and the availability of precursors. The latter might change unpredictably, but can also be influenced by subcellular targeting: for example, targeting of the strawberry dual linalool/nerolidol synthase (FaNES) to the plastids resulted in mostly linalool production (Aharoni *et al*. 2003) while targeting to the mitochondria resulted in (mostly) nerolidol production (Kappers *et al*. 2005; Houshyani *et al*. 2013). Finally, enzymes from different origins may vary considerably in their catalytic efficiency, a factor that is so far hardly considered in plant metabolic engineering (in contrast to microbial engineering; see below).

Product modification

Plants have many non-specific modifying enzymes which can convert pathway intermediates/products and hence interfere with productivity of the desired compound. These competing enzymes include dehydrogenases, hydroxylases, glycosyl transferases, glutathione transferases, etc. For example, as mentioned above, when linalool synthase was overexpressed in petunia, linalool was converted into the non-volatile linalyl-β-d-glucopyranoside by an endogenous glucosyltransferase (Lücker *et al*. 2001). These issues may be particularly problematic where multi-step pathways are introduced, as metabolomics profiling has demonstrated (Yang *et al*. 2011). Transient expression of the artemisinic acid pathway in *N. benthamiana* showed that almost all pathway intermediates were partially drained from the pathway by competing enzymes (Ting *et al*. 2013). Often these side reactions are unwanted and will decrease desired product yield. However, conjugation reactions such as glycosylation may also have beneficial effects as they allow storage of the heterologous chemicals as a relatively harmless conjugate in, for example, the vacuole. This could potentially allow higher production than when the products – which are often phytotoxic – remain in the cytosol (Bouwmeester 2006). For volatiles, this has the added advantage that losses through volatilization into the atmosphere are reduced.

Upsetting the metabolic balance

Even in relatively early days, the complexity and inherent pitfalls of engineering isoprenoid metabolism in plants were recognized (McCaskill & Croteau 1998). Side effects of metabolic engineering, including growth/reproduction defects, may result from excessive diversion of isoprenoid precursors (and consequent limitation in production of essential isoprenoids) or toxicity of the engineered products (Aharoni *et al*. 2005; Dudareva & Pichersky 2008). These effects are unpredictable. There was no apparent effect from production of relatively high levels of isoprene using an heterologous chloroplast-localized IspS in transgenic tobacco under normal growth conditions (Vickers *et al*. 2009b, 2011), but under drought, reduced plant productivity was observed (Ryan *et al*. 2014; see Case Study: Isoprene). High levels of expression of a chloroplast-targeted dual linalool/nerolidol synthase correlated with growth retardation in transgenic *Arabidopsis* (Aharoni *et al*. 2003) and both retardation and leaf bleaching in transgenic potato (Aharoni *et al*. 2006). Constitutive production of (*E*)-β-caryophyllene and α-humulene in potato resulted in compromised plant growth, yield and seed germination (Robert *et al*. 2013).

Controlling gene expression in an inducible fashion or at an appropriate developmental stage can be used to avoid metabolic burden/competition. For example, several studies have used fruit-ripening-specific promoters to prevent perturbation of isoprenoid metabolism in the non-fruit (non-target) tissues. Tomato fruits producing linalool showed no obvious phenotypic perturbations, including in their isoprenoid metabolism (Lewinsohn *et al*. 2001); however, when geraniol was produced at about an order of magnitude higher levels, fruits had reduced lycopene concentrations (Davidovich-Rikanati *et al*. 2007). Gutensohn *et al*. (2013) also observed reduced carotenoids in tomato fruits overexpressing the SSU of a heterodimeric GPP synthase. Indeed, constitutive production of semiochemicals can tip the cost–benefit balance towards excessive cost (Robert *et al*. 2013) and herbivory-inducible promoters might be more effective for these applications. Effects such as these are likely to vary between species, depending on their native isoprenoid pathway flux and on the expression characteristics of the introduced genes.

Detrimental interference with complex inter-species interactions

Plants often produce volatiles to attract beneficial interactions. However, the balance is very fine, and these interactions can easily be upset. For example, increasing volatile production in wild Texas gourd (*Cucurbita pepo* var. *texana*) resulted in an increase in florivore attraction and a decrease in seed production, but no increase in pollinator attraction (Theis & Adler 2011). (*E*)-β-Caryophyllene, the primary volatile product in *Arabidopsis* under normal conditions (Huang *et al*. 2012), is currently the subject of several engineering projects for its beneficial (antibacterial and mutualist attractant) effects. However, it is an antagonist of the aphid alarm pheromone (*E*)-β-farnesene (Vet & Dicke 1992). Production of both isoprene (Loivamäki *et al*. 2008) and even (*E*)-β-farnesene at high levels (Beale *et al*. 2006) can also interfere with tritrophic interactions (see Case Study: Herbivores and Bodyguards). Appropriate control of gene expression might mitigate potential cost–benefit scenarios with these examples.

PRODUCING VOLATILE ISOPRENOIDS IN MICROORGANISMS

While engineering modifications in plants is certainly the most direct approach to modifying plant/environment interactions, there are many pitfalls to directly engineering plants (discussed above). An alternative approach is to engineer produc-

Table 1. Industrial applications of volatile isoprenoids

Application	Example	Details
Pharmaceutical	Limonene, perillyl alcohol	Anti-cancer agent
	Zerumbone	Anti-cancer/anti-HIV/anti-inflammatory
	α-Pinene	Bronchiodilator, anti-inflammatory, antibiotic
	Geraniol	Cancer chemo-prevention agent
Flavour/fragrance/ antimicrobial	Menthol, eucalyptol, α/β-pinene, limonene, nootkatone, linalool, α/β-farnesene, geroniol	Food additive/personal care/preservative/fragrance/ cleaning products
Agriculture/floriculture	Eucalyptol, limonene	Insecticide
	Nootkatone, geraniol	Insecticide/repellent
	Farnesene	Aphid repellent/parasitoid attractant
	Linalool	Insect repellent/predator attractant/pathogen resistance
	(E)-β-Caryophyllene	Predator attractant/pathogen resistance
	Isoprene	Abiotic stress protection
Industrial chemical/ chemical feedstock	Isoprene	Chemical feedstock for synthetic rubber etc.
	α/β-Pinene	Resin precursor, solvent
Fuels	Squalene	Bio-crude
	Isopentenol	Gasoline replacement
	α/β-Farnesene/limonene/p-cymene	Jet fuel replacement
	Bisabolene	Diesel replacement
Fuel additives	Isoprenoid alkenes, alkanes, alcohols	Increase octane ratings, anti-corrosives, lubricants

tion in microbes and then apply the product as required – for example, release of a volatile attractant or repellent from a reservoir in a crop field at an appropriate time. In addition, volatile isoprenoids are also of interest as industrial chemicals in the flavour, fragrance, neutraceutical and pharmaceutical industries, as well as being used as feedstocks for production of polymers, fuels and other industrial products (see Table 1). Key advantages of engineering in microbes compared with plants are the relative simplicity of their isoprenoid metabolism, ease of genetic manipulation, highly advanced engineering tools, and speed of engineering. In addition, harvesting volatiles in a controlled fermentation environment is far more feasible than harvesting from plants for bulk industrial production. Furthermore, fermentation-based microbial bioprocesses are more independent from the vagaries of season, weather, climate and pest/disease attack; hence, bioprocess conditions, including production yields (and consequently, market prices), could potentially be more stable.

Some practical realities and challenges for microbial bioprocesses

In order to be industrially competitive, a given bioprocess must be cost-competitive with alternative production methods. Prior analysis, including metabolic network analysis to determine maximum theoretical yields and techno-economic analysis to determine feasibility, should always be performed (Vickers *et al*. 2012). An appropriate target market must also be available. Cost minimization throughout the bioprocess may be needed to meet competitiveness requirements, especially for biochemicals that are required in bulk volumes. In these cases, the feedstock price is the primary cost driver (Willke & Vorlop 2008; Rude & Schirmer 2009). Sucrose is preferred over glucose as a carbon source for industrial bioprocesses (Vickers *et al*. 2012); however, industrial *E. coli* strains cannot utilize it as a carbon source. Use of wild-type strains that can utilize sucrose efficiently (Archer *et al*. 2011), engineering improved utilization in these strains (Arifin *et al*. 2011; Sabri *et al*. 2013a), or engineering lab strains for sucrose utilization (Lee *et al*. 2010; Bruschi *et al*. 2011; Sabri *et al*. 2013a) may provide a route to decreased bioprocess costs. Lignocellulosic feedstocks are also a promising bioprocess carbon source that may become more available in the near future (Blanch *et al*. 2011). Ultimately, it would be most efficient to produce volatile isoprenoids using photosynthetic organisms such as unicellular algae or the cyanobacterium *Synechocystis*. However, substantial improvements in engineering tools and approaches will be required before these organisms can compete with *E. coli* and yeasts in terms of yields.

Many technical issues still remain in using microbial cell biofactories for production of plant isoprenoids. Production of complex modified biochemicals using non-native enzymes is a significant challenge as many plant biosynthetic enzymes do not function as anticipatedin microbes. Engineering functionality of these enzymes represents a rate-limiting step in the development of biocatalysts. In addition, similarly to plants (see above), microbes have many non-specific modification enzymes that can modify intermediates or the final product, interfering with production. For example, when IspS was expressed in yeast, a number of hydroxylated derivatives were also detected (Hong *et al*. 2012). Other technical challenges, including avoiding toxicity of intermediates and end products, are addressed in the following sections.

Basic engineering requirements for making plant volatiles in microbes

When aiming to engineer a microbial cell factory to produce volatile isoprenoids, the first step is to choose a suitable pro-

duction host. Important considerations here include (1) native capacity for isoprenoid production; (2) genetic tractability ('engineerability'); (3) potential toxicity of the isoprenoid end-product; (4) competition with essential isoprenoid requirements; and (5) potential/ability to carry out (desired or detrimental) biotransformations/modifications (see above). While a wide variety of microbes can potentially be used for production of volatile isoprenoids, most engineering to date has been done in the common tractable model microorganisms, *E. coli* and *S. cerevisiae*. However, these two organisms typically do not produce volatile isoprenoids, and substantial engineering is required to generate significant titres. It should be noted that isoprenoid production is highly variable between different *E. coli* and yeast strains (Takahashi *et al*. 2007; Rodríguez-Villalón *et al*. 2008; Chae *et al*. 2010; Boghigian *et al*. 2012); we have also observed significant strain-to-strain variation in our lab (Bongers, Behrendorff, Nielsen & Vickers, unpublished data). In the case of yeast, using diploid strains with heterozygous gene deletions (haploinsufficient strains) also show promise for isoprenoid engineering (Ignea *et al*. 2011, 2012).

Once the host organism has been selected, genetic capacity must be imported. Plant genes are commonly used. For *E. coli* and yeast, introns must be removed to ensure an uninterrupted protein coding sequence. For plant chloroplast-localized proteins, the chloroplast targeting sequence must also be removed. Full-length chloroplast proteins form inclusion bodies when overexpressed in *E. coli*; this is presumably because native chaperonins recognize the targeting sequence, but do not know what to do with it (Burke & Croteau 2002a). Consequently, removing the targeting peptide increases production of the desired compound in microbes (Williams *et al*. 1998; Miller *et al*. 2001; Burke & Croteau 2002a,b; Hsiao *et al*. 2008; Vickers *et al*. 2011; Bott *et al*. 2012). The exact truncation site is critical as it can have a significant effect on enzyme activity (Burke & Croteau 2002a; Bott *et al*. 2012). Online signal prediction software can assist in identifying probable native truncation sites (Petersen *et al*. 2011); however, these tools do not always predict targeting sequences/cleavage sites in polypeptides that are known to be chloroplast localized. Sequence alignment with microbial homologs that lack targeting sequences and closely related genes that have already been characterized can be used to help choose truncation sites in these cases; however, for a completely uncharacterized gene, it is wise to test several truncations. Codon-optimizing plant isoprenoid genes to alter codon usage towards the host's preference can also improve translation (Anthony *et al*. 2009; Bott *et al*. 2012; Calabria *et al*. 2013). Finally, selection of an appropriate expression system [promoter and terminator sequences, plasmid expression systems or chromosomal integration, variable copy number (plasmid/chromosome), etc.] is required. Balancing all of these elements appropriately may have a substantial effect on the overall success of the project.

For biotechnological applications, strict product and enantiomeric specificity is often required. As discussed above, many isoprenoid synthases have relaxed substrate/product specificities, and catalytic activities of different terpene synthases can vary widely (Schomburg *et al*. 2013). Consequently, selection of an appropriate synthase forms a key part of experimental design. Screening of synthase activities, ideally *in vivo*, may be needed to identify good candidates. For example, expression of three different germacrene A synthases in yeasts showed that one was almost threefold more efficient than the other two (Liu *et al*. 2011b). High-throughput screening methods (e.g. Agresti 2012; Behrendorff *et al*. 2013) are now being widely applied for synthase selection in microbes.

Increasing flux to isoprenoids in microbes

Low-level production of volatile isoprenoids can generally be achieved in microbes simply by introduction of heterologous terpene synthases. In this case, the terpene synthases rely on the prenyl diphosphates provided by the native MVA or MEP pathway host organism. However, for bulk applications (e.g. biofuels, agricultural biochemicals, large-scale industrial feedstocks) high-level production is required in order to satisfy cost–benefit analyses (Vickers *et al*. 2012). Analogous to the challenges in plant metabolic engineering (see above), successful overproduction of these compounds in microbes requires many optimization steps. In particular, key bottlenecks are (1) increasing flux through the core isoprenoid pathway; (2) optimization of the enzymes converting prenyl diphosphates into the isoprenoid of interest; and (3) introduction of long and complex metabolic pathways (where required for complex end products). In addition, competing metabolic pathways may need to be down-regulated or removed, precursor concentrations balanced, and any toxicity of pathway intermediates/final products must be mitigated.

Microorganisms usually use either the MEP or the MVA pathway for isoprenoid production. Engineering through the native pathway is the most direct way to increase flux to isoprenoid precursors; however, as with any native metabolic pathway, organisms have evolved ways to control flux through their metabolic pathways at optimal levels, and overcoming that regulation for overproduction of specific isoprenoids can be challenging. This is especially true in organisms that have a low native flux through their isoprenoid pathways, such as *E. coli* (Ro *et al*. 2006; Ajikumar *et al*. 2008). In the case of *E. coli*, flux is probably restricted because it produces only a few isoprenoid compounds at relatively low levels under normal growth conditions. As a result of the tight metabolic control over the MEP pathway, importing a half MVA pathway and supplementing with mevalonate (Campos *et al*. 2001; Rodríguez-Villalón *et al*. 2008) or importing a full MVA pathway (Martin *et al*. 2003) has been far more successful for increasing isoprenoid titres in *E. coli*, and the highest titres to date have been achieved using this approach (Martin *et al*. 2003; Pray 2010; Beck *et al*. 2013b).

Despite this relative success with the MVA pathway, there is still interest in using the MEP pathway because its theoretical yield is 15–40% higher than the MVA pathway (Rude & Schirmer 2009; Whited *et al*. 2010; Vickers *et al*. 2012). Several groups have therefore attempted to reconstruct the MEP pathway in yeast (Partow *et al*. 2012; Carlsen *et al*. 2013;

Dietzel *et al*. 2013). However, despite significant efforts – including screening large numbers of alternative genes, engineering redox partners, complex proteomic and metabolomics analyses – little if any MEP flux could be demonstrated, suggesting that MEP pathway reconstruction in yeast will not be a viable option for bio-production. These attempts illustrate the inherent difficulties of long metabolic pathway reconstruction in heterologous organisms.

Increasing pathway flux can also result in metabolic toxicity from over-accumulation of prenyl diphosphates or toxic pathway intermediates (Martin *et al*. 2003; Pitera *et al*. 2007; Withers *et al*. 2007). Furthermore, feedback inhibition may occur when the cellular concentration of prenyl diphosphates increases (Goldstein & Brown 1990; Banerjee *et al*. 2013; Hemmerlin 2013). To minimize these effects, a downstream sink (terpene synthase or complete product pathway) should be introduced prior to engineering upstream pathway flux. To minimize build-up of toxic intermediates, balancing activities of different pathway enzymes can help (Pitera *et al*. 2007; Anthony *et al*. 2009; Ajikumar *et al*. 2010). Examples of successful engineering approaches through the core isoprenoid pathways (up to the shared IPP/DMAPP node) and engineering at the prenyl diphosphate precursor nodes are presented below in separate sections. More detailed reviews are available (Withers & Keasling 2007; Ajikumar *et al*. 2008; Kampranis & Makris 2012). Combinatorial engineering using two or more of the approaches described below can increase titres even further.

MVA pathway in yeast

S. cerevisiae is a model lab organism, and much of our knowledge of MVA pathway engineering is derived from experiments in it. As noted above, regulatory control on the mevalonate pathway is exerted primarily through a HMGR, with MK representing a secondary control point (Lombard & Moreira 2011). Engineering through HMGR has by far the most profound effect on MVA pathway flux, and as in plants (see above), it is considered to be the major engineering target. *S. cerevisiae* has two HMGR isoforms: Hmg1p, which is relatively stable, and Hmg2p, which is susceptible to FPP-mediated degradation (Gardner & Hampton 1999). Use of a truncated Hmg2p which is resistant to feedback regulation (Polakowski *et al*. 1998) results in increased accumulation of heterologous sesquiterpenes in *S. cerevisiae* (Jackson *et al*. 2003; Ro *et al*. 2006; Ohto *et al*. 2009b; Asadollahi *et al*. 2010; Rico *et al*. 2010; Westfall *et al*. 2012). A mutated *HMGR*, which is resistant to ubiquitination has a similar effect on production of downstream isoprenoids (Ignea *et al*. 2011). Overexpressing the native HMGR also increases production of heterologous plant sesquiterpene, β-sesquiphellandrene, in *Lactococcus lactis* (Song *et al*. 2012). Heterozygous deletions of genes that affect the stability of both HMG isoforms also have a significant effect on production of (*E*)-β-caryophyllene in diploid *S. cerevisiae* (Ignea *et al*. 2012). In addition, increasing cytosolic NADPH availability for HMGR also increases sesquiterpene accumulation in *S. cerevisiae* (Asadollahi *et al*. 2009).

Overexpression of core MVA pathway genes in isolation (apart from *HMGR*) has relatively little effect on isoprenoid accumulation (Ohto *et al*. 2009b), and release at the HMGR node is probably required before any further engineering steps are effective. In strains with an engineered HMGR, up-regulation of MVA pathway genes through overexpression of the *upc2-1* transcriptional regulator, which up-regulates *HMGS* (*ERG13*), MK (*ERG12*) and phosphomevalonate kinase (*PMK*; *ERG8*, as well as several genes downstream of squalene in the sterol pathway) increases sesquiterpene titres (Ro *et al*. 2006; Shiba *et al*. 2007; Westfall *et al*. 2012). Overexpressing all of the pathway genes together also produces high sesquiterpene titres (Westfall *et al*. 2012).

Finally, increasing precursor (acetyl-CoA) availability by overexpression of the native acetaldehyde dehydrogenase and a heterologous acetyl-CoA synthase also increases sesquiterpene accumulation in *S. cerevisiae* (Shiba *et al*. 2007).

MVA pathway in E. coli

While the MVA pathway is more effective at producing isoprenoids in *E. coli* than the MEP pathway (Martin *et al*. 2003), it does require optimization. When the pathway is expressed in *E. coli*, mevalonate kinase is rate-limiting and requires higher levels of expression than other pathway genes (Anthony *et al*. 2009). A later quantitative proteomics analysis identified both MK and PMK as potential bottlenecks (Redding-Johanson *et al*. 2011). Codon optimization and choice of a stronger promoter resulted in higher levels of both proteins and a threefold increase in the final sesquiterpene product (amorpha-4,11-diene, the sesquiterpene precursor of artemisinin). Genes from different organisms can also catalyse steps with greater efficiency, resulting in improved pathway flux (Newman *et al*. 2006). Balancing expression of *HMGR* and *MK* is particularly important, since build-up of HMG-CoA appears to cause cellular toxicity in *E. coli* (Martin *et al*. 2003; Pitera *et al*. 2007; Ma *et al*. 2011). By judicious selection of a HMGR with appropriate catalytic properties, combined with engineering to improve cofactor (NADPH) availability, a 120% improvement in production of amorpha-4,11-diene was achieved (Ma *et al*. 2011). Use of a feedback-insensitive MK from the archeon *Methanosarcina mazei* (Primak *et al*. 2011) also increases pathway flux (Beck *et al*. 2013a).

MEP pathway

As noted above, much of our current understanding of MEP pathway regulation comes from experiments in *E. coli*, and information to date suggests that regulation is extremely complex. Control can occur at many levels (transcription, post-transcriptional and post-translational processes, precursor supply/balance, allosteric regulation, metabolite feedback/feedforward), and is distributed across several nodes in the pathway. DXP/DOXP is an important regulatory node, and overexpression of both *DXS* and *DXR* (IspC) can increase accumulation of downstream isoprenoids (Albrecht *et al*.

1999; Harker & Bramley 1999; Wang *et al*. 1999; Farmer & Liao 2000; Matthews & Wurtzel 2000; Kim & Keasling 2001; Yuan *et al*. 2006; Rodríguez-Villalón *et al*. 2008). However, overexpression of *DXS* does not always increase production of downstream isoprenoids (Smolke *et al*. 2001). *Ex vivo* experiments suggest that methylerythritol cyclodiphosphate synthase (MCS/IspF) is subject to both feedforward control by the upstream metabolite MEP, and to feedback control by FPP, which can inhibit the MCS–MEP complex (Bitok & Meyers 2012). Synergistic effects are often observed when two or more MEP pathway genes are overexpressed, and upstream bottlenecks may require release before downstream enzymes can exert control (Albrecht *et al*. 1999; Wang *et al*. 1999; Reiling *et al*. 2004; Jin & Stephanopoulos 2007; Ajikumar *et al*. 2010). Overexpression of HDS/IspG can increase isoprenoid accumulation, but only if the upstream bottleneck at DXS is released (Yuan *et al*. 2006; Flores-Péreza *et al*. 2008). Also, if HDS/ispG is not overexpressed when upstream genes are overexpressed, methylerythritol cyclodiphosphate (MECPP) accumulates and is exported from the cell (Zhou *et al*. 2012). Similarly, while overexpression of HDR/IspH can increase isoprenoid production in *E. coli* (Cunningham *et al*. 2000), its influence depends on context (Yuan *et al*. 2006). Given the low isoprenoid yields achieved with the MEP pathway to date, it seems unlikely that precursor (glyceraldehyde 3-phosphate/pyruvate) or co-factor availability have been limiting. Still, adjusting their concentrations and inactivating competing pathways, as well as manipulating global regulators, can improve MEP pathway flux (Farmer & Liao 2001; Alper *et al*. 2005a,b; Vadali *et al*. 2005; Zhang *et al*. 2013).

Despite all of this information, the best isoprenoid yields/titres in *E. coli* using engineered MEP pathways are far less than when using an imported MVA pathway, and are nowhere near the theoretical maximum of the pathway (Rude & Schirmer 2009). Even overexpressing the entire pathway, either by chromosomal promoter replacement throughout the core pathway (Yuan *et al*. 2006) or by introducing a complete heterologous pathway onto the chromosome (Bongers, Nielsen & Vickers, unpublished results) is still limited at approximately sixfold improvement, both for higher-order (β-carotene) and lower-order (isoprene) isoprenoids. It is clear that our understanding of MEP pathway flux regulation is incomplete, and it seems likely that post-transcriptional events – for example feedback regulation, as recently demonstrated for DXS (Banerjee *et al*. 2013) – play important regulatory roles. In the case of DXS feedback regulation, IPP and DMAPP competitively inhibit binding of the DXS cofactor thiamine diphosphate; thus, engineering to avoid feedback regulation will be extremely challenging if not impossible. If protein degradation is a regulatory mechanism, as appears to be the case in plants (Flores-Perez *et al*. 2008), preventing this might also be an engineering target. Finally, phosphorylation has been shown to regulate both DXR (Jawaid *et al*. 2009) and diphosphocytidyl methylerythritol synthase (CMS/IspD) (Tsang *et al*. 2011) in the pathogenic bacterium *Francisella tularensis*. If this mechanism is also active in *E. coli*, controlling phosphorylation might also improve pathway flux.

Engineering at prenyl phosphate nodes

Balancing IPP and DMAPP through overexpression of IDI improves accumulation of isoprenoids from both the MEP and mevalonate pathway in *E. coli* (Kajiwara *et al*. 1997; Albrecht *et al*. 1999; Martin *et al*. 2003; Alper *et al*. 2005b; Vadali *et al*. 2005; Yuan *et al*. 2006; Ohto *et al*. 2009a; Yan *et al*. 2012) and from the MVA pathway in yeasts (Ignea *et al*. 2011). This is probably related to IPP : DMAPP ratio requirements for different end products.

Most natural isoprenoid products in yeast and *E. coli* are sesquiterpenes, and production of heterologous sesquiterpenes has been far more successful than production of monoterpenes and diterpenes. For monoterpene production, GPP availability must be improved significantly in yeasts and *E. coli*; a variety of other problems also manifest themselves (see Case Study: Production of Monoterpenes in Microbes). Overexpression of *FPPS* (*IspA*) increases sesquiterpene production in *E. coli* (Ohto *et al*. 2009a), and overexpression of *GGPPS* improves production of higher-order isoprenoids, including volatile diterpenes, in yeasts and *E. coli* (Reiling *et al*. 2004; DeJong *et al*. 2006). However, while FPPS (*ERG20*) appears to be rate-limiting in native yeast metabolism (Chambon *et al*. 1991), overexpression of *ERG20* does not substantially increase sesquiterpene accumulation in yeasts (Jackson *et al*. 2003; Ro *et al*. 2006). This might be due to the fact that the heterologous sesquiterpene synthases fail to compete effectively with native FPP-utilizing enzymes. A variety of different approaches have been used to down-regulate squalene synthase (*ERG9*) in yeasts and decrease conversion of FPP to squalene, thereby decreasing competition and increasing non-sterol/heterologous sesquiterpene production (Millis *et al*. 2004; Ro *et al*. 2006; Takahashi *et al*. 2007; Asadollahi *et al*. 2008; Paradise *et al*. 2008; Babiskin & Smolke 2011; Ignea *et al*. 2011; Scalcinati *et al*. 2012; Paddon *et al*. 2013). Engineering reduced dephosphorylation of prenyl diphosphate precursors also increases heterologous sesquiterpene and diterpene production (Takahashi *et al*. 2007; Scalcinati *et al*. 2012; Huang *et al*. 2013); this approach might also be applicable for other isoprenoid classes. Dellas *et al*. (2013) recently discovered that isopentenyl phosphate kinases, previously only found in archea, are widespread in all domains of life; these enzymes might provide a route to scavenging dephosphorylated isoprenoid diphosphates.

Clever tools and engineering approaches in microbes

Isoprenoid biology is becoming a test field for development of cutting-edge systems and synthetic biology techniques, and the tractability of microbial systems means that advances are rapidly made using them. Systems biology approaches have been particularly useful for identifying flux bottlenecks (proteomics and metabolomics (Redding-Johanson *et al*. 2011; Zhou *et al*. 2012); understanding toxicity effects from build-up of pathway intermediates (metabolomics; Kizer *et al*. 2008), and identifying non-obvious genes that might impact on isoprenoid biosynthesis (*in silico* metabolic

modelling; Jin & Stephanopoulos 2007; Asadollahi *et al.* 2009). As in plant engineering, metabolomics is a key technology in microbes (reviewed above).

Protein engineering of both core pathway genes and isoprenoid synthase genes to improve product yields, increase specificity and expand product range has been used frequently in isoprenoid engineering (Kampranis & Makris 2012). This includes metabolic channelling by constructing fusion enzymes (e.g. Brodelius *et al.* 2002) and protein scaffolding to co-locate core pathway proteins (Dueber *et al.* 2009). These approaches presumably improve substrate competition by introduced enzymes with native enzymes.

As in plants, appropriate regulation of gene expression is important to avoid/minimize metabolic burden/toxicity effects. One approach is to select promoters that respond dynamically to culture conditions to either overexpress or silence desired genes at an appropriate time (Scalcinati *et al.* 2012; Dahl *et al.* 2013). Another approach is to hijack the native yeast pheromone-mediated quorum-sensing system, so that production is activated at appropriate cell density (Williams *et al.* 2013).

Also as in plants, multi-gene pathway analysis is a relative bottleneck in microbes. Recently, new vectors have been made available for insertion of very long sequences encoding multiple genes onto the *E. coli* chromosome (Minaeva *et al.* 2008; Kuhlman & Cox 2010; Sabri *et al.* 2013b). Of these, a single-step method for introducing genes at well-characterized loci is the fastest and is particularly useful for metabolic engineering applications (Sabri *et al.* 2013b). For yeast, vectors are now available that allow expression of two to three genes at a time using a variety of different promoters (Partow *et al.* 2010; Vickers *et al.* 2013). Pairing these multiple expression cassettes with antibiotic resistance genes allows them to be used in prototrophic industrial strains and lab strains where auxotrophies have been exhausted through previous engineering (Vickers *et al.* 2013). Availability of these new cloning vectors, as well as rapid assembly techniques for generating large DNA constructs (Gibson *et al.* 2009; Kok *et al.* 2014) should help decrease strain generation timeframes in both yeast and *E. coli*.

CASE STUDY: HERBIVORES AND BODYGUARDS

Many plant species emit induced volatiles when they are attacked by insect herbivores to attract predators or parasites, which in turn attack the herbivores in tritrophic interactions (Dicke *et al.* 1990; Turlings *et al.* 1990). Isoprenoids are the dominant volatiles used to attract these carnivorous 'bodyguard' arthropods (Degenhardt *et al.* 2003). The potential of genetic engineering to alter tritrophic interactions through modifying volatile emissions was first demonstrated by Bouwmeester *et al.* (2003), who showed that transgenic potato producing linalool was more attractive to predatory mites than control plants. In a more elaborate study, Kappers *et al.* (2005) showed that transgenic *Arabidopsis* plants expressing the dual linalool/nerolidol synthase with mitochondrial targeting (where FPP was assumed to be available at higher concentrations than in the cytosol) emitted the homoterpene DMNT (C_{11}) and its sesquiterpene alcohol precursor (3*S*)-(*E*)-nerolidol. The transgenic plants were more attractive to predatory mites, which prey on spider mites, than to non-emitting plants. Shortly afterwards, Schnee *et al.* (2006) overexpressed a maize sesquiterpene synthase that produces a mixture of (*E*)-β-farnesene, (*E*)-α-bergamotene, and other herbivory-induced sesquiterpene hydrocarbons, in *Arabidopsis*. They showed that the wasp parasitoid *Cotesia marginiventris* could be trained to locate lepidopteran hosts by following these volatile signals. (*E*)-β-caryophyllene synthase (*TPS23*) is induced in maize roots upon feeding of Western corn root worm (*Diabrotica virgifera virgifera*) larvae (Kollner *et al.* 2008). The resulting (*E*)-β-caryophyllene recruits entomopathogenic nematodes that effectively control the larvae (Rasmann *et al.* 2005). Expression of *TPS23* is also induced in the shoot upon feeding by *Spodoptera litoralis* larvae (Kollner *et al.* 2008). Interestingly, it was less expressed in North American maize varieties, which also displayed a higher sensitivity to the western corn root worm (Degenhardt *et al.* 2009), while a maize line with a naturally high TPS23 expression level was shown to have a higher resistance to herbivores that attack the areal parts (Smith *et al.* 2012). The attractiveness of the North American maize variety to the entomopathogenic nematodes could be restored by introducing an oregano (*E*)-β-caryophyllene synthase (Degenhardt *et al.* 2009).

A slightly different approach to engineering of indirect defence is the use of insect pheromones. Many species of aphids release the sesquiterpene (*E*)-β-farnesene when they are attacked by predators or parasitoids (Beale *et al.* 2006; Dewhirst *et al.* 2010). This compound acts as an alarm pheromone, and causes the aphids to disperse, but it also acts as a kairomone that attracts aphid predators and parasitoids (Micha & Wyss 1996; Al Abassi *et al.* 2000; Kunert *et al.* 2005). Expression of a cytosolic (*E*)-β-farnesene synthase using a strong constitutive promoter successfully resulted in production of (*E*)-β-farnesene in transgenic *Arabidopsis*, and aphids were both alarmed and repelled by the plants (Beale *et al.* 2006). However, the high level of production also resulted in arrested development of the desirable parasitoid *Diaeretiella rapae*. Moreover, in a second experiment using the same plants, the same aphid species was not repelled; neither did it suffer reproductive penalties after living on the engineered plants (Kunert *et al.* 2010). The authors suggested that the aphids might be habituated to the constitutive emissions.

Clearly, as in natural systems, production of volatiles involved in tritrophic interactions should be controlled in an inducible/regulatable manner and at an appropriate level to avoid metabolic cost and unnecessary interference with normal metabolism/interactions/ecology. Production of the semiochemicals in microbes (see above) for application when required might be an alternative approach. In addition to these considerations, engineering of tritrophic interactions to protect crop species has a number of other requirements (Bouwmeester *et al.* 2003; Degenhardt *et al.* 2003). These include identification of an appropriate bodyguard species

that (1) can be attracted by engineering a known semiochemical; (2) is present in the cultivation area; and (3) can effectively control herbivore populations/effects of herbivory. On top of this, the engineered emissions should not attract other herbivores.

CASE STUDY: ISOPRENE

Isoprene production in plants

Isoprene (2-methyl-1,3-butadiene; C_5H_8) forms 40% (440–660 Tg C per year) of all biogenic isoprenoid emissions (Guenther *et al*. 2006). Production of isoprene by plants provides a protective effect under abiotic stress conditions (high light, drought, ozone, etc.) leading to oxidative stress and inhibition of photosynthesis (Sharkey & Singsaas 1995; Loreto *et al*. 2001; Vickers *et al*. 2009a; Ryan *et al*. 2014). Most agricultural species to not produce isoprene (Hewitt *et al*. 1997); engineering non-emitting plants might therefore provide protection against abiotic stress. Transgenic tobacco plants engineered to produce isoprene show resistance to ozone stress (Vickers *et al*. 2009b); similarly, transgenic isoprene-emitting *Arabidopsis* plants show resistance to thermal stress (Loivamäki *et al*. 2007; Sasaki *et al*. 2007). In the transgenic tobacco, isoprene emission also deters herbivory by *Manduca sexta* caterpillars (Laothawornkitkul *et al*. 2008a,b). The transgenic tobacco plants were engineered with a single constitutively expressed *IspS* gene, and they showed classical isoprene emission patterns with no obvious phenotypic effects (Vickers *et al*. 2009b, 2011). However, *IspS* genes are present as a small gene family, the members of which are differentially regulated (Vickers *et al*. 2010). It is unclear how this affects isoprene emission and stress responses in a naturally emitting species. Isoprene emission can also affect complex biotic interactions: in the case of transgenic *Arabidopsis*, isoprene production interfered with attraction of herbivore parasites, upsetting tritrophic interactions (Loivamäki *et al*. 2008). The effect of isoprene in stress responses appears to be mediated through alteration of the reactive oxygen species (ROS) response system (Vickers *et al*. 2009b), and it seems that the ROS system in isoprene-emitting plants is almost primed for stress, so that it can react more quickly and more effectively to oxidative stress (Vickers *et al*. 2009a). This might have unpredictable outcomes in a variety of different biotic and abiotic interactions where the reactive oxygen response system is involved in mediation of the interaction.

The distribution of isoprene among plant species is highly variable – even within a single species, there can be individuals that emit isoprene and individuals that do not (Loreto *et al*. 1998). The ability to produce isoprene has been lost and gained multiple times throughout evolutionary history (Loreto *et al*. 1998; Harley *et al*. 1999; Sharkey *et al*. 2005; Monson *et al*. 2013). It is possible that there are multiple adaptive roles, and the patchy taxonomic distribution of emitting species might be a result of isoprene emission being advantageous only in a narrow range of environment and phenotypic conditions (Monson *et al*. 2013). Given these vagaries, combined with known pitfalls described above, attempts to engineer isoprene emission in crop plants for stress protection should be approached with caution, and thorough experimental cost–benefit analysis should be performed. One approach might be to control isoprene emission tightly through appropriate stress-responsive promoters, so that isoprene is only produced when it is needed. However, this might not provide the priming response that seems to be involved with the phenotype (Vickers *et al*. 2009a,b). Another alternative might be to fumigate crops with isoprene prior to expected stress events; this might provide priming for a more effective response (human toxicity of isoprene will need to be considered in this scenario). Priming allows plants to respond rapidly with minimal metabolic burden prior to stress, and there is evidence that the benefits of priming outweigh the costs – at least in the case of biotic stress (Degenhardt *et al*. 2003).

Isoprene as an industrial chemical: production in microbes

Isoprene can also be used to make a wide variety of products, most notably, synthetic rubbers (for production of, e.g. vehicle tires), as well as co-block polymers, elastomers, adhesives etc. (reviewed in Whited *et al*. 2010; Bott *et al*. 2012; Beck *et al*. 2013b). It is normally produced industrially from petroleum by cracking of naptha or gas oil. The process is relatively expensive and energy-intensive, and yields may be insufficient for future demand (Beck *et al*. 2013b). Harvesting from plants is not feasible; however, microbial bioprocesses are currently being pursued as an alternative production route. Isoprene production at the scales required for crop fumigation might also be done this way.

Isoprene production has been engineered in a wide variety of microbes by introduction of a plant *IspS*. Removing the chloroplast targeting peptide increases production in microbes (Miller *et al*. 2001; Vickers *et al*. 2011; Bott *et al*. 2012), as does codon optimizing of the gene (Bott *et al*. 2012; Calabria *et al*. 2013). Activity of the *Populus alba* IspS enzyme can also be increased by introducing an L70R amino acid mutation to improve solubility (Bott *et al*. 2012).

E. coli is most widely used organism for isoprene production and has yielded the highest titres. The primary limitation on isoprene production in *E. coli* is availability of the precursor DMAPP, and various engineering approaches have been used to increase flux through the *E. coli* MEP pathway to overcome this limitation. Overexpressing enzymes that catalyse rate-limiting steps in the pathway (encoded by *DXS*, *DXR* and *IDI*) can increase yields of isoprene (Miller *et al*. 2001; Zhao *et al*. 2011; Bott *et al*. 2012; Calabria *et al*. 2013). As with other isoprenoids, MVA-based production is also more successful for isoprene than MEP-based production (Whited *et al*. 2010; Zurbriggen *et al*. 2012; Beck *et al*. 2013a; Calabria *et al*. 2013). Improving carbon flux through the pentose phosphate pathway by overexpressing phosphogluconolactonase can also increase

yields (Whited *et al*. 2010), as can improving precursor availability/balance by overexpressing phosphoketolase to redirect carbon from xylulose-5-phosphate into isoprenoid pathway precursors (Beck *et al*. 2013c). A wide variety of other gene targets and engineering approaches that have been shown to influence production of other isoprenoid compounds might also increase flux towards isoprene (see main text). The best titres to date achieved by the green biotechnology company Genencor (Paulo Alto, CA, USA; now a subsidiary of DuPont) are ~80 g L^{-1}; this involves using the *P. alba* IspS(L70R), an engineered MVA pathway with feedback-resistant MK, overexpression of PGL and an optimized fed-batch fermentation/extraction bioprocess (Beck *et al*. 2013b). With the exception of the sesquiterpene (*E*)-*β*-farnesene (used as a fuel precursor), where titres of just over 100 g L^{-1} have been reported (Pray 2010), this is the highest titre of any isoprenoid reported to date in an industrial microbial bioprocess.

MVA-based production in yeasts has also been tested for isoprene (Melis 2011; Hong *et al*. 2012); however, the low yield combined with the ability of the yeast culture to bioconvert isoprene into hydroxylated derivatives makes yeasts an undesirable candidate as an industrial isoprene production organism. Isoprene production has also been tested in the cyanobacterium *Synechocystis*, with the aim of using photosynthesis for direct conversion of carbon dioxide into isoprene; however, the reported yields were also very low compared with the *E. coli* process (Lindberg *et al*. 2010; Bentley & Melis 2012).

CASE STUDY: PRODUCTION OF MONOTERPENES IN YEASTS AND *E. COLI*

Monoterpenes are key industrial biochemicals (see Table 1). However, engineering microbes for the production of monoterpenes imposes several challenges in addition to increasing carbon flux towards IPP and DMAPP. Difficulties arise mostly from the fact that the two most commonly used microbial hosts for isoprenoid production, *E. coli* and *S. cerevisiae*, do not naturally produce monoterpenes, or their immediate precursor GPP, in considerable amounts (Reiling *et al*. 2004; Carrau *et al*. 2005; Oswald *et al*. 2007). Overproduction of this class of compounds therefore requires (1) generating a sufficient intracellular GPP pool; (2) expressing a highly active and product-specific monoterpene synthase; and (3) balancing flux towards monoterpenes with endogenous, essential FPP/isoprenoid biosynthesis. As a consequence of these constraints, published monoterpene titres from microbial fermentations to date have not exceeded 1 g L^{-1} (Ignea *et al*. 2011; Yang *et al*. 2013). The far higher hemiterpene and sesquiterpene titres achieved thus far (Pray 2010; Beck *et al*. 2013b) suggest that either GPP or monoterpene synthesis are current bottlenecks. Thus far, the only GPPS genes tested in a metabolic engineering context were either mutated versions of the microbial FPPSs (Reiling *et al*. 2004; Oswald *et al*. 2007; Fischer *et al*. 2011; Liu *et al*. 2013) or genes derived from *Abies grandis* and *Picea abies* (Carter *et al*. 2003; Bokinsky *et al*. 2011; Dunlop *et al*. 2011; Ignea *et al*. 2011; Alonso-Gutierrez *et al*. 2013; Yang *et al*. 2013). Expanding the search for a suitable GPPSs and MTSs as well as extensive metabolic and protein engineering around the GPP node should increase monoterpene yields in the future.

As monoterpene titres from microbial fermentations continue to rise, another challenge will need to be addressed, namely that monoterpenes are generally highly toxic towards microorganisms (Dunlop *et al*. 2011; Brennan *et al*. 2012, 2013). Even current best titres of 1 g L^{-1} (Ignea *et al*. 2011) are above minimum inhibitory concentration for monoterpenes in yeasts (typically less than 0.3 g L^{-1}; Brennan *et al*. 2012); therefore, engineering improved resistance to monoterpenes might lead to increased titres. Two-phase reactor systems, where the toxic product is sequestered into a non-toxic organic solvent phase, can also alleviate this problem (Brennan *et al*. 2012). There is also some evidence that *E. coli* is more resistant to monoterpenes than yeast (Himejima *et al*. 1992), and may consequently be a preferable production organism.

SUMMARY AND FUTURE DIRECTIONS

As we have described, rapid and significant recent advances in metabolic engineering tools and approaches for both plants and microbes has opened up new possibilities for engineering volatile isoprenoids. In addition to the examples reviewed here (repelling herbivores, attracting mutualists, protecting against pathogens and abiotic stress), there are a number of other potential targets for engineering plant volatile isoprenoids. These include enhancing or *de novo* engineering for pollinators/seed disseminators, weed control by production of alleopathic compounds, engineering resistance to parasitic angiosperms, modifying plant–plant communication, priming plants for more rapid and effective responses to biotic and abiotic stresses, etc. In the latter application, volatiles produced using engineered microbes might be particularly useful. Moreover, new industrial applications for volatile isoprenoids are continually being identified; microbial engineering may ultimately provide the most feasible route for production of these compounds.

Engineering of core isoprenoid pathways in plants has been relatively limited. However, the very large amount of information obtained through engineering microbial isoprenoid pathways (reviewed above) may ultimately be applicable to engineering through plant pathways. For example, overexpression of the common IPP isomerase (IDI) has also been used to improve accumulation of certain isoprenoids in both *E. coli* (MEP) and yeasts (MVA), and may also improve production in plants. Furthermore, much basic knowledge about regulatory controls has yet to be applied in an engineering sense. It is known that genes outside the MEP pathway can influence MEP pathway flux in both plants and microbes, and multi-level regulation at post-transcriptional levels (including feedback, allosteric regulation, protein degradation, redox control, etc.) is common in isoprenoid pathways (Flores-Perez *et al*. 2008; Pulido *et al*. 2012; Hemmerlin 2013). This may confound attempts to

engineer very high pathway flux by simply overexpressing genes. Overcoming this regulation may be required to produce high yields, especially in the MEP pathway. One approach in plants might be to regulate the plastidial Clp protease complex, which appears to mediate post-translational degradation of MEP pathway proteins (Flores-Perez *et al*. 2008).

Clearly, as in natural systems, production of volatiles in plants that are involved in biotic interactions should be controlled in an inducible/regulatable manner and at an appropriate level to avoid metabolic cost or unnecessary interference with normal metabolism/interactions. For example, predators/parasites will be wasting their time following constitutively produced messengers that are not ultimately associated with their prey (Kappers *et al*. 2005). Such mistakes could be quite costly to the natural enemies, and might affect their environmental populations in the short term. Furthermore, they might ultimately disassociate a given semiochemical with their target prey if they repeatedly cannot find it after following the signal (Kunert *et al*. 2010), rendering the engineering redundant. Regulating expression under the control of herbivory-inducible promoters might be a more effective strategy in the long term (Bouwmeester *et al*. 2003). Production of the semiochemicals in microbes (see above) for application when required might be an alternative approach.

Volatile isoprenoids might also be used as non-invasive plant diagnostic tools to track pest/pathogen infestations or nutrient deficiencies (Jansen *et al*. 2011; Alexander *et al*. 2013). Where a given biotic or abiotic stress does not elicit production of volatiles, stress-responsive promoters could be used to engineer volatile production – thus providing a rapid, sensitive and generic method to track all kinds of plant stresses. In essence, we would be engineering plants to communicate with us in a direct sense.

Further use of new plant tools, including chloroplast transformation (Kumar *et al*. 2012) and *in silico* plant metabolic modelling (de Oliveira Dal'Molin & Nielsen 2013), will also be useful for engineering isoprenoid metabolism in plants – in particular, for dealing with challenges of compartmentalization. New tools developed for microbes are rapidly accelerating the rate of discover in these systems (see above) and ultimately, where possible, will be applied to plants. Microbes are also particularly important for high throughput screening to identify useful genes and for testing fusion constructs and other synthetic biology approaches for later application in plants, although transient expression in plants is also a promising tool to this end (reviewed above).

Genetic modification is of course the most direct way to alter plant volatile profiles. However, any review of these approaches should also at least touch on the complex issues associated with GMOs. Although it has the potential to save the lives of a million children a year (Nash 2000), more than a decade after Golden Rice [a GMO with increased β-carotene (provitamin A) content] was developed, availability where it is most needed is still hampered by GMO regulatory constraints and complex socio-political issues driven by the anti-GMO movement (Potrykus 2012). Despite this, both researchers and funding organizations believe that both conventional technologies and biotechnologies will be required to combat the growing global problems of adequate nutrition in developing countries, and as global population continues to grow and global climate conditions change (Borlaug 2000; Alberts *et al*. 2013; Dudareva & DellaPenna 2013). As discussed here, volatile isoprenoids have potential to play a prominent role in future food production. While definite pitfalls regarding engineering volatile emissions in plants have been identified, it is thought that ecologically and economically sound approaches can be devised for pest control (Turlings & Ton 2006); this extends to other applications. Even where socio-political or technological barriers prevent engineering in plant species, volatiles might be produced in engineered microorganisms for biotechnological applications. To achieve these lofty goals, a better understanding of the biology of various interactions is still required.

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