Strategies for transgenic manipulation of monoterpene biosynthesis in plants

Soheil S. Mahmoud and Rodney B. Croteau

Monoterpenes, the C_{10} isoprenoids, are a large family of natural products that are best known as constituents of the essential oils and defensive oleoresins of aromatic plants. In addition to ecological roles in pollinator attraction, allelopathy and plant defense, monoterpenes are used extensively in the food, cosmetic and pharmaceutical industries. The importance of these plant products has prompted the definition of many monoterpene biosynthetic pathways, the cloning of the relevant genes and the development of genetic transformation techniques for agronomically significant monoterpene-producing plants. Metabolic engineering of monoterpene biosynthesis in the model plant peppermint has resulted in yield increase and compositional improvement of the essential oil, and also provided strategies for manipulating flavor and fragrance production, and plant defense.

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The terpenoids, or isoprenoids, are the largest and most diverse family of natural products, ranging in structure from linear to polycyclic molecules, and in size from the five-carbon hemiterpenes to natural rubber, comprising thousands of isoprene units [1,2]. All terpenoids are synthesized through the condensation of isoprene units (dimethylallyl diphosphate and isopentenyl diphosphate) [3] and are classified by the number of five-carbon units present in the core structure. Monoterpenes are the C_{10} class of isoprenoids, encompassing nearly 1000 colorless, liphophilic and volatile metabolites. Although found throughout nature, these substances are best known as constituents of the essential oils, floral scents and defensive resins of aromatic plants, to which they impart their characteristic aromas and flavors [4–6].

The precise physiological roles of most plant monoterpenes have not been defined but it is generally agreed that they mediate plant–environment interactions by playing roles in pollinator attraction, defense and plant–plant communication [2,7,8]. For example, conifers secrete a complex mixture of monoterpenes, sesquiterpenes (C_{15}) and diterpenes (C_{20}), termed oleoresin, in response to attack by insect predators. This defensive secretion can contain >30 monoterpenes, including limonene, pinene and myrcene, that are toxic to both the insect and their symbiotic fungal associates [7,8]. Other monoterpenes, such as linalool, camphene and cineole, are involved in pollinator attraction and allelopathy [9–11]. Monoterpenes also find extensive industrial applications as flavoring agents, topical medicines, perfumes and insecticides [2,12]. There is also evidence that monoterpenes have potential use as herbicides, pesticides, antimicrobial agents and dietary anticarcinogens [13]. Because of their relative low toxicity to vertebrates, monoterpenes offer significant advantages in pest control applications compared with conventional methods. Furthermore, most monoterpenes are readily catabolized by microorganisms and do not persist in the environment [14].

In essential-oil- and oleoresin-producing plants, monoterpene production and accumulation are restricted to highly specialized anatomical structures. For example, monoterpene biosynthesis in mints originates in the secretory cells of peltate glandular trichomes, which cover the surfaces of the above-ground parts of the plant, and the essential oil is stored in the subcuticular storage cavity of these trichomes [15–17]. Similarly, constitutive oleoresin biosynthesis in conifers is localized to various types of resin ducts or blisters composed of epithelial cells with a central storage cavity [7,18,19]. Plants that lack such specialized structures typically accumulate only trace quantities of monoterpenes.

Research over the past decade has yielded substantial insight into the metabolism of monoterpenes in plants [12,20–23]. The biosynthetic pathways for many commercially important monoterpenes have been established and the genes encoding key pathway enzymes have been cloned from several species [24–29]. The availability of appropriate molecular tools and the economic importance of monoterpenes have prompted a recent surge of interest in engineering these pathways in plants. In this article, we discuss strategies for engineering monoterpene metabolism and summarize recent developments in this field.

Biosynthesis of monoterpenes

All terpenoids are synthesized through the condensation of isopentenyl diphosphate (IPP) and its allylic isomer, dimethylallyl diphosphate (DMAPP) [2]. Most terpenes, the 'regular terpenes', are produced by the sequential head-to-tail addition of DMAPP to IPP, whereas the less common 'irregular' terpenes are produced by non-head-to-tail joining of the two building units or by rearranging a regular structure [2]. This article focuses on the regular monoterpenes, which compose the vast majority of compounds in this category.

The sequential head-to-tail addition of IPP units to DMAPP initially yields geranyl diphosphate (GPP, C_{10}), farnesyl diphosphate (FPP, C_{15}) and

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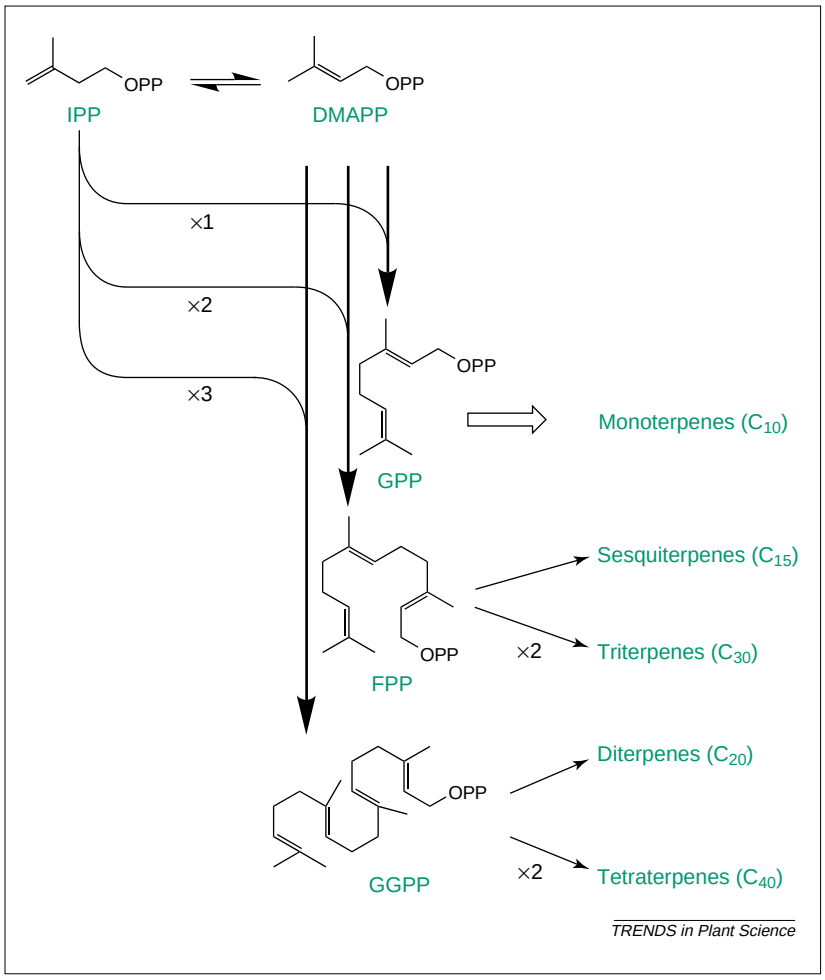

Fig. 1. Overview of isoprenoid biosynthesis and the role of prenyltransferases in higher plants. Abbreviations: DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; IPP isopentenyl diphosphate; OPP, the diphosphate moiety.

geranylgeranyl diphosphate (GGPP, C_{20}), which are the linear precursors of the major terpenoid classes (Fig. 1) [1]. Regular monoterpenes are derived exclusively from GPP, which is often cyclized to produce the backbone skeletons of the various monoterpene subfamilies; relatively few monoterpenes (e.g. geraniol, linalool and ocimene) are acyclic [12,23]. The *p*-menthane monoterpenes characteristic of mint species are cyclohexanoid, whereas all other major groups, including the bornane, camphane, fenchane, carane and thujane monoterpenes, have bicyclic carbon skeletons (Fig. 2). Comprehensive reviews on the biosynthesis of monoterpene subfamilies are available [12,23].

Most monoterpenes are produced by modification of the initial parent compound derived from GPP. For example, cyclization of GPP by the principal terpene synthase of peppermint (*Mentha*×*piperita*) yields limonene, which subsequently undergoes redox transformations to produce a range of structurally related metabolites (Fig. 3) [20,21]. Thus, the biosynthesis of monoterpenes can be described in four phases: (1) production of the terpenoid building units (IPP and DMAPP); (2) condensation of IPP and DMAPP by prenyltransferase to form GPP; (3) conversion of GPP to the monoterpene parent skeleton; and (4) transformation of the parent structure to various derivatives. Monoterpene catabolism and evaporative losses appear to play very minor roles in influencing the production yields of these natural products in plants [30,31]. In the following sections, we describe each step in the process and discuss their potential exploitation for genetic engineering.

Production of IPP and DMAPP

Until recently, the classical mevalonate (MVA) pathway of isoprenoid biosynthesis was considered to be the universal route for the production of IPP. Furthermore, it was widely accepted that DMAPP was derived from IPP via isomerization catalyzed by the enzyme IPP isomerase. However, it is now established that plant cells produce IPP via two chemically and spatially distinct, and independent, pathways [32,33] (Fig. 4). In the cytosol, IPP (and, by isomerization, DMAPP) is generated from acetyl-CoA via the MVA pathway, which supplies precursors for the biosynthesis of sesquiterpenes (C_{15}) and triterpenes (C_{30}) [32]. In plastids, the newly discovered 1-deoxy-D-xylulose-5-phosphate (DXP) pathway, which also operates in certain microorganisms [33–35], produces IPP for the biosynthesis of monoterpenes (C_{10}), diterpenes (C_{20}) and tetraterpenes (C_{40}) [36–39]. DMAPP appears to arise independently of IPP in this pathway [40].

The DXP pathway starts by the transketolase-type condensation of two carbons from pyruvate with glyceraldehyde-3-phosphate to yield DXP, and is followed by isomerization and reduction of DXP to 2-C-methyl-D-erythritol-4-phosphate, formation of the cytidyl diphosphate derivative, phosphorylation at C2 and cyclization to 2-C-methyl-erythritol-2,4-cyclodiphosphate [34,35], which ultimately gives rise to 4-hydroxy-DMAPP [41] and then IPP and DMAPP [42]. Genes encoding the enzymes of this pathway have been isolated from plants and bacteria.

Recent investigations have shown that precursor (IPP and DMAPP) supply can be limiting in the biosynthesis of terpenoids, and that overexpression of the first gene of the DXP pathway [encoding DXP synthase (DXPS)] improves the production of various types of isoprenoids in bacteria [43,44] and plants [45]. The second step of the pathway, which is catalyzed by deoxyxylulose phosphate reductoisomerase (DXR), also appears to be slow. Thus, co-overexpression of *DXR* and *DXPS* in *Escherichia coli* engineered to produce lycopene led to increased production compared with cells that overexpressed *DXPS* alone [44]. Furthermore, ectopic expression of *DXR* in peppermint, a plant that accumulates substantial quantities of *p*-menthane monoterpenes in the essential oil, increased production by 40–60% [46]. There might be additional slow steps downstream of DXR, and the identification and elimination of such bottlenecks in the DXP pathway remains an attractive goal for metabolic engineering to increase terpenoid

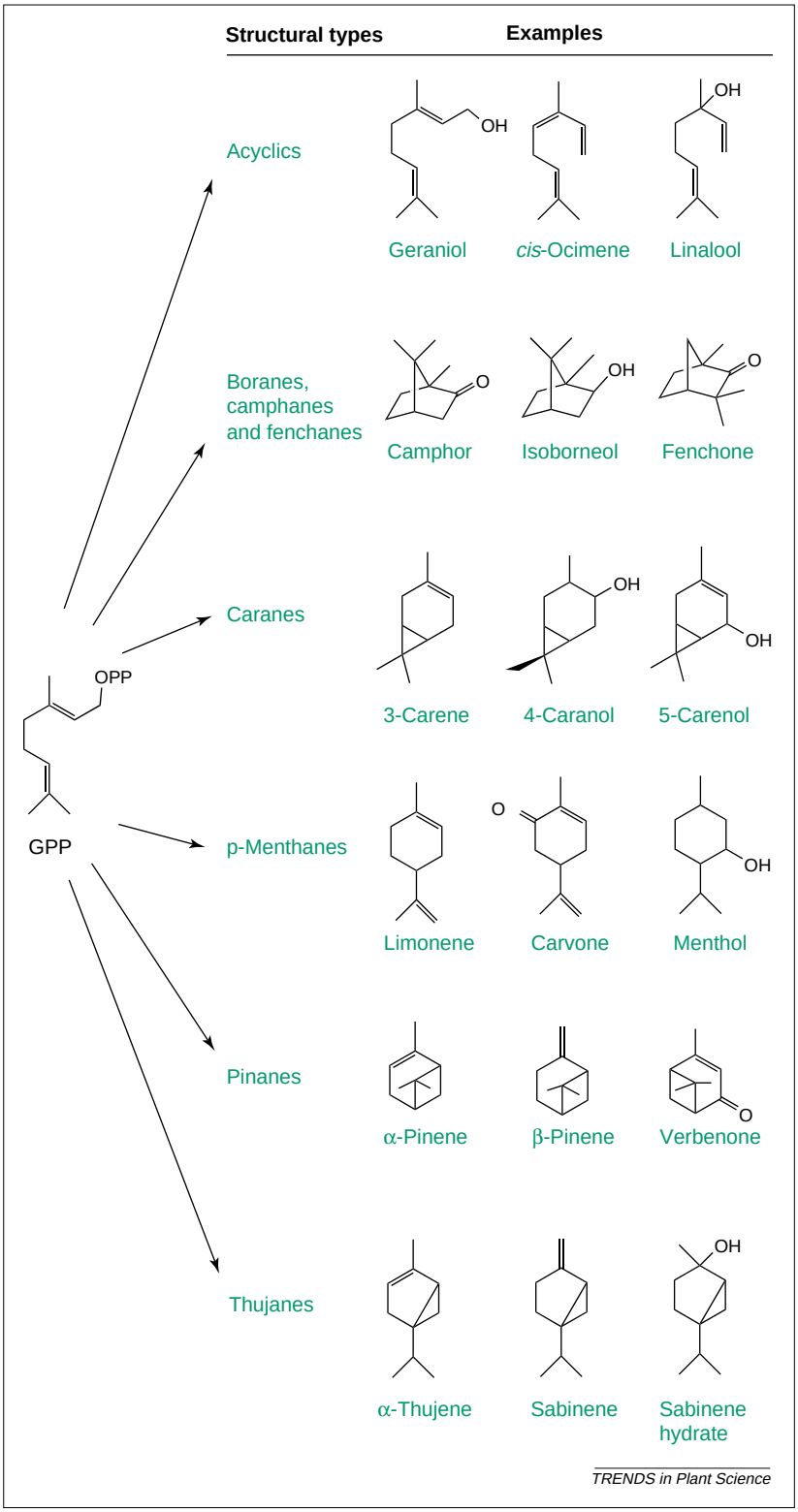

Fig. 2. Representative members of various monoterpene subfamilies. Abbreviations: GPP, geranyl diphosphate; OPP, the diphosphate moiety.

production. Because the DXP pathway gives rise to both IPP and DMAPP, the role of IPP isomerase (for the interconversion of IPP and DMAPP) in plastidial terpenoid metabolism is presently uncertain.

Formation of GPP

The biosynthesis of the terpenoid precursors GPP, FPP and GGPP from IPP and DMAPP is catalyzed by the prenyltransferases GPP synthase, FPP synthase and GGPP synthase, respectively [47]. All three enzymes can use IPP and DMAPP for the elongation steps. In addition, FPP and GGPP synthases can extend GPP and FPP to form the respective C_{15} and C_{20} products [47,48]. Because the plastidial pools of IPP and DMAPP are used for the production of both GPP (C_{10}) and GGPP (C_{20}), the control of flux of IPP and DMAPP at this branch point is probably regulated and the prenyltransferases are therefore good candidates for metabolic engineering. Overproduction of GPP synthase and/or downregulation of the production of GGPP synthase would be expected to result in increased production of GPP and, consequently, of monoterpene end products. However, this strategy must be approached cautiously because GGPP is also the precursor of essential metabolites such as gibberellins, carotenoids, prenylated quinones and chlorophyll [38], so downregulating GGPP production could have deleterious effects. To minimize such complications, genetic manipulation of GPP and GGPP production should be restricted to tissues (or cell types) that are specialized for monoterpene metabolism, which would require the use of tissue-specific promoters.

GPP synthase belongs to the family of short-chain prenyltransferases that also includes FPP synthase and GGPP synthase. Although FPP and GGPP synthases have been studied extensively [47–49], much less is known about GPP synthase. However, the enzyme has been isolated from several monoterpene-producing plants and characterized. GPP synthase resembles other prenyltransferases in its general biochemical properties [47]. Recently, a GPP synthase was cloned using a highly enriched peppermint oil gland cDNA library as a tool [24,50]. The enzyme is a heterodimer consisting of a large subunit (inactive alone) that resembles GGPP synthase in sequence, and a unique small subunit that is required for catalysis and that also determines the C_{10} chain length of the product [50,51]. GPP synthase from *Arabidopsis* has also been described recently. This enzyme resembles other prenyltransferases and appears to be monomeric [52]. The regulatory features of GPP synthase of either architecture have not yet been described but overexpression of the mint genes is now being investigated.

Synthesis of monoterpene parent skeletons and subsequent modifications

The committed step in the biosynthesis of monoterpenes is catalyzed by monoterpene synthases (sometimes called cyclases), which convert the universal precursor GPP to the parent structures of the various monoterpene families. Monoterpene synthases are obvious, attractive targets for the engineering of monoterpene metabolism, and they are essential for genetically imparting the ability to produce monoterpenes on

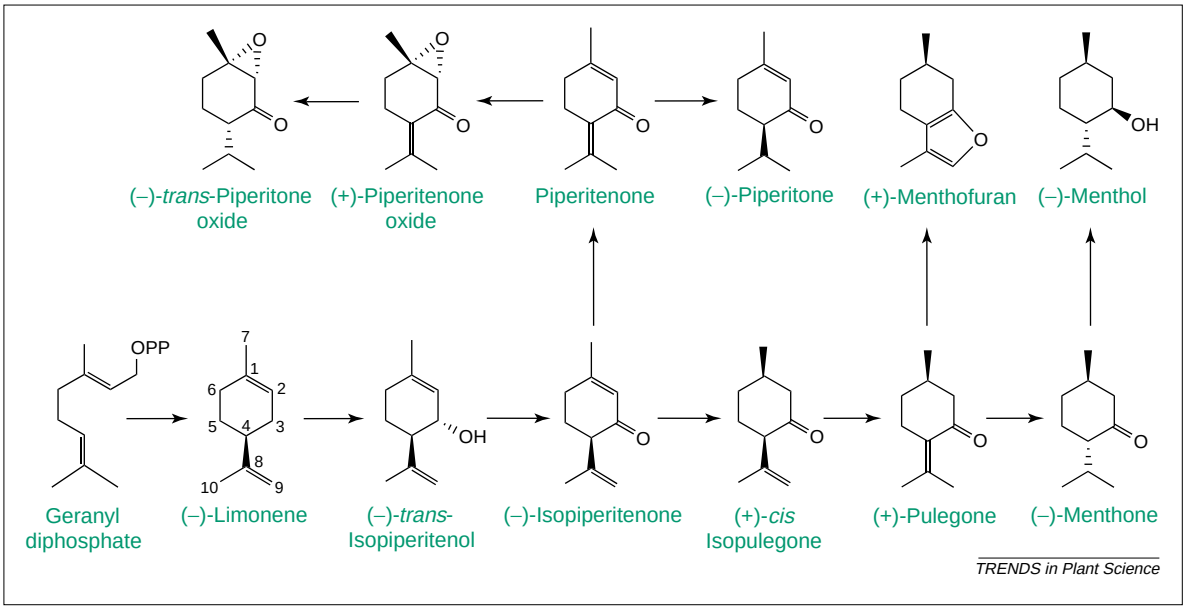

Fig. 3. Pathways of monoterpene metabolism in peppermint.

plants that do not naturally do so. Furthermore, because monoterpene synthase steps might be rate limiting [12], manipulating the expression of these genes could increase yield as well as change the monoterpene profile of the targeted plant. Ectopic expression of limonene synthase in peppermint [53,54] and cornmint (*Mentha arvensis*) [53] was reported to increase yield and to alter monoterpene composition in both species.

Peppermint is a source of a commercially important essential oil and has been developed as a model system for the study of monoterpene biosynthesis [31,55]. The essential oil of peppermint is composed mainly of *p*-menthane monoterpenes, with (–)-menthol as the main component of the oil in mature plants (Fig. 3). The oil composition of peppermint can be adversely affected by environmental conditions such as low photon flux density and high temperatures, which favor the production and accumulation of pulegone and menthofuran, both of which contribute to an 'off' odor and taste [56,57]. To minimize the production of undesired metabolites in peppermint through metabolic engineering, we are manipulating the expression of pathway genes that lead to pulegone and menthofuran accumulation, while at the same time trying to maximize the production of menthol. Reducing the expression of the cytochrome P450 menthofuran synthase (MFS) gene [which is responsible for the conversion of pulegone to menthofuran (Fig. 3)] using an antisense approach resulted in significant (50–75%) decrease in the concentration of menthofuran in the derived oil [46]; suppressing the expression of this gene resulted in virtual elimination of menthofuran from the oil (S.S. Mahmoud *et al.*, unpublished). These findings demonstrate that monoterpene biosynthetic pathways can be modified through metabolic engineering to optimize the production of desired products.

A second group of industrially important plants that are attractive targets for metabolic engineering are the conifers (*Coniferophyta*), which are used for lumber and pulp production. These plants produce a terpenoid-based oleoresin (pitch) that is of central function in defense against bark beetles and the fungal pathogens they carry, which destroy millions of trees annually [7,8,11,58]. Oleoresin is a mixture of monoterpenes and sesquiterpenes (turpentine), and diterpene resin acids (rosin), which is produced constitutively and is also induced upon beetle attack. Mobilization of oleoresin at the wound site kills the invaders and seals the injury. In spite of its insecticidal activity, the oleoresin also plays a crucial role in the chemical ecology of bark beetles [8,59]. Volatile components of the pitch are used in host recognition and colonization, and in pheromone signaling, and they also mediate tritrophic-level interactions involving the attraction of beetle predators [59,60]. These complex, terpenoid-based interactions can be exploited transgenically in several ways to develop insect-resistant trees. Thus, oleoresin composition can be altered to disguise the host, to deter the invader, to disrupt insect aggregation and reproduction or to attract predators of the beetles.

Production of new metabolites in plants

One of the most exciting areas of metabolic engineering is focused on the production of natural products in transgenic plants to improve agronomic traits such as pest resistance and competitiveness (through allelopathy), and to alter fragrance and flavor profiles. Transgenic production of the acyclic monoterpene linalool, a major constituent of the floral scent of many plant species, has yielded promising results. In a recent study, the (*S*)-linalool synthase (*Lis*) gene of *Clarkia breweri* was constitutively expressed in *Petunia hybrida* (an ornamental but

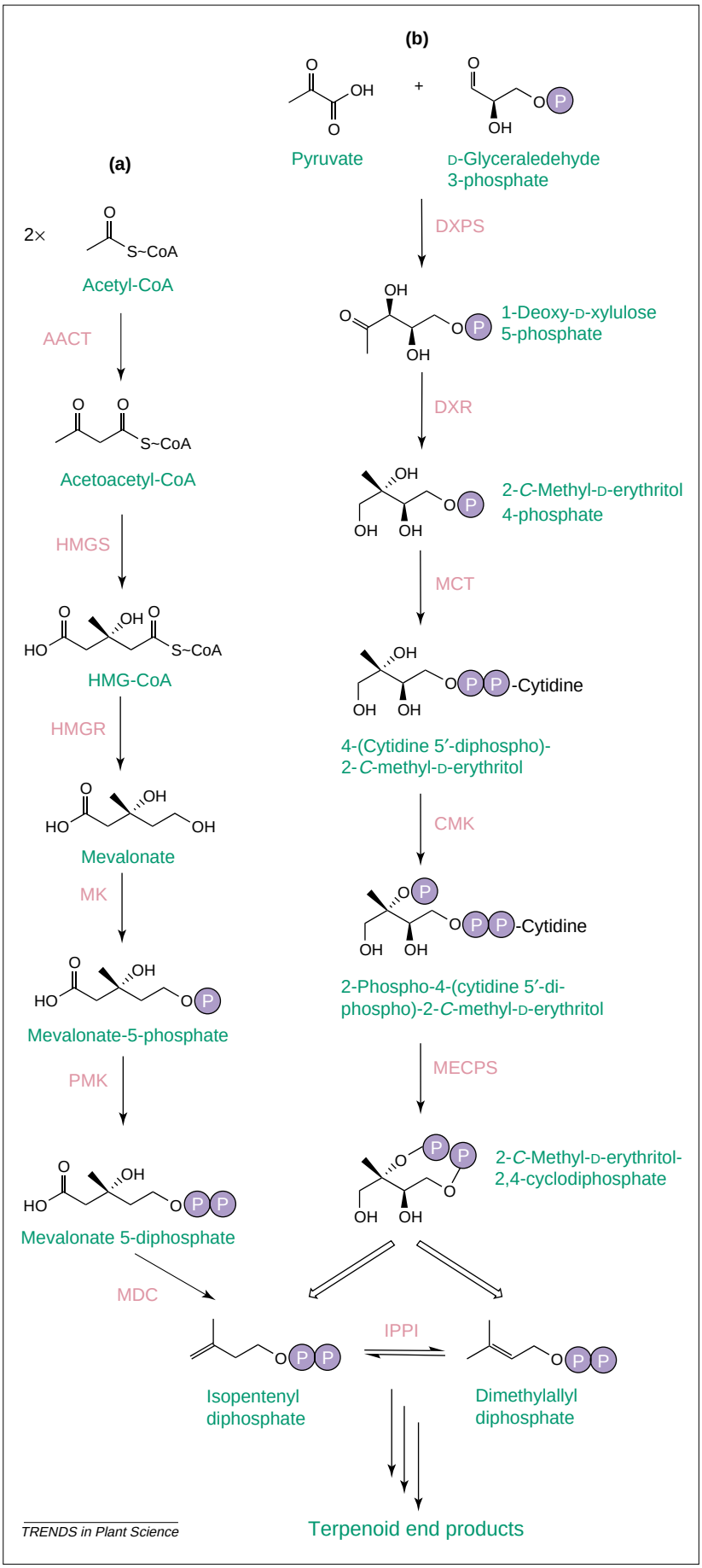

Fig. 4. The mevalonate pathway (a) and the mevalonate-independent (deoxyxylulose phosphate) pathway (b) for the biosynthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The phosphate moiety is highlighted in purple and the large open arrows indicate unidentified steps. Abbreviations: AACT, acetyl-CoA:acetyl-CoA *C*-acetyltransferase; CMK, 4-(cytidine-5′-diphospho)-2-*C*-methylerythritol kinase; DXPS, 1-deoxyxylulose-5-phosphate synthase; DXR, 1-deoxyxylulose-5-phosphate reductoisomerase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; IPPI, isopentenyl diphosphate isomerase; MCT, 2-*C*-methylerythritol-4-phosphate cytidyltransferase; MDC, mevalonate-5-diphosphate decarboxylase; MECPS, 2-*C*-methylerythritol-2,4-cyclodiphosphate synthase; MK, mevalonate kinase; PMK, phosphomevalonate kinase.

fragrance-free flower) to impart scent to this plant [61]. Transgenic petunia plants expressing the heterologous gene produced linalool but the entire product was conjugated as the non-volatile linaloyl β-D-glucoside and thus had no effect on the olfactory properties of the transformants.

In a separate study, *Lis* was expressed in carnation (*Dianthus caryophyllus*, another important ornamental) under the CaMV 35S promoter [62]. In this case, transformed plants produced and emitted linalool and linalool oxides, although production levels were too low to affect the scent of the transgenic plants. *Lis* was also expressed in transgenic tomato (*Lycopersicon esculentum*) in an attempt to improve the flavor of the fruits [63]. In this case, expression of the transgene driven by the tomato late-ripening-specific E8 promoter led to the production of (*S*)-linalool and 8-hydroxylinalool in ripening fruits without altering the biosynthesis of other terpenoids such as tocopherols, lycopene, β-carotene and lutein. The concentrations of linalool and 8-hydroxylinalool in the ripe fruit of transgenic plants were sufficient for olfactory detection [63]. These pioneering experiments show that monoterpenes can be synthesized in heterologous systems through metabolic engineering. However, the fate of these products in transgenic organisms (metabolism, including conjugation, storage or emission) cannot be predicted. Further effort is needed to improve the production levels and emission efficiency of the monoterpenes.

Plants can also be used as factories for the production of commercially useful monoterpenes. Good targets in this regard are the pyrethrins. These irregular monoterpenoid esters are produced in the secretory ducts of the floral tissue of certain *Asteraceae* and are among the most economically important natural insecticides [64–66]. The production of pyrethrins depends upon their extraction from *Chrysanthemum cinerariaefolim*, and this source of supply consistently falls short of demand [66]. The pathway for the biosynthesis of pyrethrins involves relatively few steps [64] and it could be feasibly engineered into other plants. This type of pathway engineering has been successfully accomplished by introducing the entire (three-step) biosynthetic pathway for the production of β-carotene (provitamin A) into transgenic rice endosperm [67].

The feasibility of producing novel terpenoids via metabolic engineering has also been demonstrated in peppermint, in which suppression of the gene encoding menthofuran synthase led to the accumulation of relatively high levels (5–10% total oil) of two monoterpenes [(–)-*trans*-piperitone oxide, and (+)-piperitenone oxide] that are not normally produced in this species (S.S. Mahmoud *et al.*, unpublished). Pathway engineering of monoterpene metabolism in monoterpene-producing species such as peppermint, which have specialized secretory structures, is relatively straightforward. Transplanting monoterpene biosynthetic pathways into plants that do not normally accumulate these metabolites and hence lack specialized secretory structures is likely to be much more challenging because the ability of these species to transport and store large quantities of volatile, hydrophobic products is presently unknown.

Use of transcription factors

An attractive strategy for engineering monoterpene production is to manipulate the expression of transcription factors that coordinately regulate the structural genes for all steps of an extended pathway. Examples in which a single transcription factor coordinately regulates the expression of multiple genes are known in animals [68,69] and plants [70–73], including the Myb- and Myc-related transcription factors for the global regulation of anthocyanin biosynthesis in maize [70,71]. Transcription factors that regulate multiple monoterpene biosynthetic genes have not yet been described. However, most genes directly involved in the biosynthesis of *p*-menthane monoterpenes in peppermint are transcriptionally regulated in a coordinated fashion [55] and it seems likely that the expression of these genes is controlled by a common transcription factor. Overexpression of this factor (or factors) would be expected to enhance pathway flux globally and to lead to increased production yield of the essential oil.

A conceptually attractive alternative approach to increasing monoterpene production is to manipulate transcription factors to increase the density of the specialized structures (such as glandular trichomes and resin ducts) that are the specific sites of monoterpene production and storage. Trichome differentiation has been studied most extensively in *Arabidopsis*, which produces only simple unicellular (non-secretory) hair-like structures on leaf surfaces. Trichome formation and development are complex, involving interactions between several positive and negative regulators [74–77]. Positive transcriptional regulators include GL1, AtMYB23, TTG1 and GL3 [74,77]. Interestingly, AtMYB23 and GL1 can also inhibit leaf trichome formation when overproduced in transgenic plants, although ectopic production of these transcription factors results in formation of trichomes on tissues that normally do not produce them [75,78]. Overproduction of GL1 or AtMYB23 alone is insufficient to induce substantial trichome formation [75]. However, co-production of GL1 with the R protein of maize (a homolog of TTG1) significantly increases trichome number in *Arabidopsis* [77]. Two negative regulators of trichome formation in *Arabidopsis* have also been identified [79,80].

It can be assumed that control of the development of glandular trichomes involves similar transcription factors. However, the process is likely to be much more complex, involving the formation of three distinct cell types and a subcuticular storage receptacle as well as the production of considerable cellular machinery for monoterpene biosynthesis, metabolite trafficking and secretion of product to the storage cavity. Nevertheless, both positive and negative transcriptional regulators of the process can be anticipated, the respective enhancement and silencing of which should lead to an increase in trichome number and thus essential oil production.

Metabolic engineering of monoterpene biosynthesis

Metabolic engineering of monoterpene biosynthesis in plants is currently an active area of research. The feasibility of increasing monoterpene yield and altering metabolite composition in essential oil plants has been demonstrated, and the transgenic production of monoterpenes to enhance scent and flavor in flowers [61,62] and fruits [63] has been described. Similar approaches to engineer these pathways for the purpose of importing terpenoid-based plant defenses or to exploit allelopathic interactions can be readily envisioned. However, limitations might arise in the transplantation of monoterpene biosynthetic pathways into normally nonproducing hosts because of the absence of secretory structures and their associated transport and storage machinery.

There are clear indications that precursor (IPP and DMAPP) supply is a limiting factor in the biosynthesis of monoterpenes from the plastidial DXP pathway. To improve flux to these basic isoprenoid precursors requires the identification of the slow steps of this recently elucidated pathway [42], and the overexpression of the responsible genes. The definition of regulatory mechanisms that control monoterpene metabolism and the identification of transcription factors that coordinately regulate gene expression for these extended pathways can be expected to improve production yields markedly, as will the identification and characterization of proteins involved in the intracellular trafficking and secretion of monoterpenes. Finally, the characterization of the regulatory elements that control cellular differentiation leading to the formation of specialized secretory structures (such as glandular trichomes and resin ducts) can be expected to have a major effect on the transgenic production of monoterpenes and related natural products.

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