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Review

My Way: Noncanonical Biosynthesis Pathways for Plant Volatiles

Pulu Sun,^{1,2} Robert C. Schuurink,² Jean-Claude Caissard,¹ Philippe Hugueney,³ and Sylvie Baudino^{1,*}

Plant volatiles are crucial for various interactions with other organisms and their surrounding environment. A large number of these volatiles belong to the terpenoid and benzenoid/phenylpropanoid classes, which have long been considered to be exclusively synthesized from a few canonical pathways. However, several alternative pathways producing these plant volatiles have been discovered recently. This review summarizes the current knowledge about new pathways for these two major groups of plant volatiles, which open new perspectives for applications in metabolic engineering.

Noncanonical Metabolic Pathways

Plants produce various volatile organic compounds for different purposes like plant growth and development (e.g., ethylene, nitric oxide). Other volatiles can play a major role in interactions with their surrounding environment, including **direct/indirect defense** (see [Glossary](#)) against herbivores and pathogens, attraction of pollinators, and plant–plant interactions [1–6]. Up to 5–10% of assimilated carbon may be converted into plant volatiles and even more when plants are under stress [7,8]; in floral scents alone, more than 1700 different volatiles have been identified [9]. According to their biosynthetic origin and chemical structure, plant volatiles can be grouped into several classes, including: terpenoids, benzenoids/phenylpropanoids, fatty acid derivatives, amino acid derivatives, and carbohydrate derivatives such as furanones [2,3,9,10]. For decades it was believed that nearly all of these compounds were exclusively manufactured through a limited number of pathways shared by all plants. However, recently several publications indicate that different plants have evolved alternative pathways to produce the same compounds. These alternative pathways often occur through convergent evolution, which has been extensively discussed in [11].

In this review we focus on the biosynthesis pathways of two major groups of plant volatiles: low-molecular-weight terpenoids (including monoterpenes, sesquiterpenes, and homoterpenes) and benzenoids/phenylpropanoids. The review complements the latest reviews on volatile terpenoid [3,4,12] and benzenoid/phenylpropanoid [2,3,13] biosynthesis and specifically deals with the implications of recently discovered alternative metabolic pathways.

New Players in Plant Volatile Biosynthesis

Here we describe the recent and unexpected characterization of proteins belonging to families that had never been associated with volatiles before.

Involvement of Isopentenyl Kinase in Terpene Biosynthesis

Terpenes are one of the largest and most structurally diverse groups of natural products, including more than 40 000 compounds [4]. All terpenes are constructed from two types of five-carbon

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Isopentenyl phosphate kinase (IPK) recycles the isopentenyl phosphate/dimethylallyl phosphate (IP/DMAP) pool that is likely to be formed by the action of phosphatases on IPP/DMAPP and contributes to the formation of both mevalonic acid (MVA) and methylerythritol (MEP) pathway-derived terpenes.

Cytosolic monoterpene synthases (LdGES, FaNES1) and a diphosphohydrolase (RhNUDX1) have been linked to the biosynthesis of acyclic monoterpenes.

The complete iridoid biosynthesis pathway has been identified recently, indicating a complex biosynthesis network of monoterpenes in the cytosol.

Z-prenyl diphosphates (NPP and Z,Z-FPP) can also act as substrates for mono- and sesquiterpene synthases, respectively.

There are three different routes for the biosynthesis of phenylacetaldehyde from phenylalanine.

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molecules: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) [14]. Sequential condensation of IPP and DMAPP leads to the formation of prenyl diphosphates, the precursors of most terpenes; that is, geranyl diphosphate (GPP, C₁₀), farnesyl diphosphate (FPP, C₁₅), geranylgeranyl diphosphate (GGPP, C₂₀), and geranylgeranyl farnesyl diphosphate (GFPP, C₂₅). Generally, GPP and FPP are the precursors of monoterpenes (C₁₀) and sesquiterpenes (C₁₅), respectively, while GGPP is the precursor of diterpenes (C₂₀). GFPP is the newly discovered precursor of sesterterpenes (C₂₅) [15]. Sequential condensation of FPP and GGPP results in precursors of triterpenes (C₃₀) and tetraterpenes (C₄₀), respectively. Further catalytic reactions (e.g., cyclization of an acyclic precursor followed or not followed by Wagner–Meerwein rearrangements, hydroxylation, dehydroxylation, reduction, oxidation, or glycosylation) on the parental skeletons give rise to a great diversity of terpenoid-derived products.

There are two well-established pathways generating IPP and DMAPP: the cytosolic mevalonic acid (MVA) pathway and the plastidic methylerythritol phosphate (MEP) pathway (reviewed in [16]). In the MVA pathway, IPP is generated by decarboxylation from mevalonate-5-diphosphate by mevalonate diphosphate decarboxylase (MVD or MDD) [17]. In the MEP pathway, IPP is generated from 4-hydroxy-3-methylbut-2-enyl-diphosphate (HMBPP) by HMBPP reductase [18]. In the MEP pathway, both IPP and DMAPP can be produced by HMBPP reductase [19]. In the MVA pathway, IPP is converted into DMAPP by IPP isomerase (IDI) [20], which also operates in plastids and mitochondria [21].

However, recent studies have shown that the genes encoding the final two enzymes of the MVA pathway, namely phosphomevalonate kinase and MDD, are absent in the **Archaea**. These organisms use an alternative route via isopentenyl phosphate kinase (IPK) for the formation of IPP [22]. Dellas and colleagues found that IPK also exists in all green plants whose genomes have been sequenced. Fully functional IPKs are distributed across all three domains of life; thus, this does not seem to be a case of convergent evolution. In *Arabidopsis thaliana*, it was shown that IPK is a new member of the plant terpenoid metabolic network [23]. This cytosolic IPK is coexpressed with various genes involved in the MVA pathway and downstream terpene biosynthesis pathways, including MDD, farnesyl diphosphate synthase, squalene synthase and C-8,7 sterol isomerase [23]. Using *A. thaliana ipk* and *ipk/mdd1* double mutants, it was found that IPK functions in parallel to the MVA pathway. In addition, overexpression of the cytosolic *AtIPK* in tobacco increased the production of both FPP- and GPP-derived terpenes in the cytosol and plastids, suggesting communication between these two compartments. The proposed function of IPK in *A. thaliana* is then to recycle the isopentenyl phosphate/dimethylallyl phosphate (IP/DMAP) pool that is likely to be formed by the action of phosphatases on IPP/DMAPP (Figure 1).

Involvement of a Nudix Hydrolase in Monoterpene Biosynthesis

Monoterpenes are well known as components of floral scent [24] and also as constituents of essential oils of aromatics plants that are widely used in the cosmetic, perfume, food [25], and pharmaceutical industries [26]. Because of their high economic relevance, monoterpene biosynthesis has been extensively studied. The biosynthesis of monoterpenes usually requires a catalytic reaction triggered by monoterpene synthases, converting C₁₀ prenyl diphosphates to various cyclic and acyclic products [27]. No monoterpene synthase has so far been functionally characterized in rose (*Rosa* sp.), although sesquiterpene synthases have been associated with the biosynthesis of sesquiterpenes in this genus [28]. Recently, a terpene synthase-independent pathway to monoterpenes has been reported in rose. By comparing the volatile profiles and differential gene expression of scented and unscented rose cultivars, an unexpected enzyme – a Nudix hydrolase, RhNUDX1 – was found to be responsible for the formation of geraniol [29]. The function of *RhNUDX1* was elucidated by using recombinant protein on several potential substrates, by co-localizing the gene with a major QTL for geraniol production, and by analyzing

Glossary

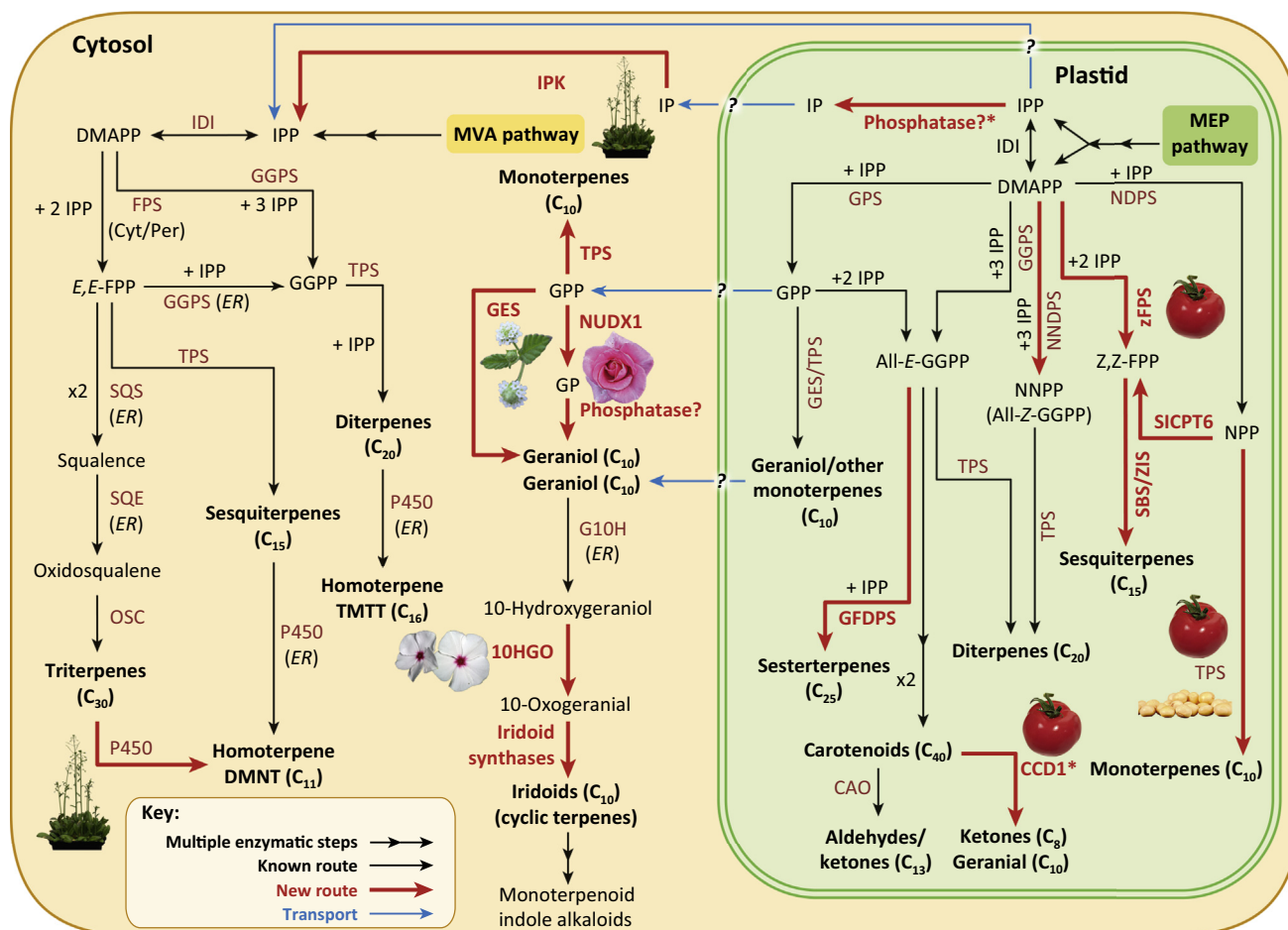
Archaea: microbes that have no cell nucleus or any other membrane-bound organelles (prokaryotes) constituting a domain or kingdom of single-celled microorganisms, like bacteria. Archaea have isoprenoids as side chains in their membrane lipids instead of fatty acids as in bacteria.

Glandular trichomes: specialized 'hairs' on the surface of plants that can produce and secrete metabolites such as terpenes.

(Plant) direct defense: characteristics of a plant that negatively affect the physiology or behavior of herbivores. Direct defense can be classified as mechanical defense, like thorns or thick cuticle, or (induced) defense like toxic specialized metabolites.

(Plant) indirect defense: herbivore-induced characteristics of a plant that can enhance the performance of natural enemies of herbivores using additional resources such as alternative food (extrafloral nectar, pollen) or herbivore-induced plant volatiles.

Polyphyletic group: characterized by one or more similar phenotypes due to convergence or reversion from different ancestors.



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Figure 1. Known and Novel Terpene Biosynthesis Pathways in Plant Cytosol (Cyt) and Plastids. Terpene precursors are synthesized by the mevalonic acid (MVA) pathway (cytosolic) and the methylerythritol phosphate (MEP) pathway (plastidic). Abbreviations: ER, endoplasmic reticulum; Per, peroxisome; CAO, carotenoid oxygenase; CCD1, carotenoid cleavage dioxygenase 1; DMAPP, dimethylallyl diphosphate; DMNT, 4,8-dimethylnona-1,3,7-triene; *E,E/Z*-FPP, all *E/Z* farnesyl diphosphate; FPS, *E,E*-FPP synthase; zFPS, *Z,Z*-FPP synthase; G10H, geraniol 10-hydroxylase; 10HGO, 10-hydroxygeraniol dehydrogenase; GES, geraniol synthase; GFDPS, geranyl farnesyl diphosphate synthase; GP, geranyl monophosphate; GPP, geranyl diphosphate; GPS, GPP synthase; (All-*E*)-GGPP, (all *E*) geranylgeranyl diphosphate; GGPS, GGPP synthase; IDI, isopentenyl diphosphate isomerase; IP, isopentenyl phosphate; IPK, IP kinase; IPP, isopentenyl diphosphate; NDPS, neryl diphosphate synthase; NNPP, neryl neryl diphosphate; NNDPS, neryl neryl diphosphate synthase; NPP, neryl diphosphate; NUDX1, Nudix hydrolase 1; OSC, oxidosqualene cyclase; P450, cytochrome P450 monooxygenase; SBS, santalene and bergamotene synthase; SICPT6, *Z*-prenyltransferase 6 of *Solanum lycopersicum*; SQE, squalene epoxidase; SQS, squalene synthase; TMTT, 4,8,12-trimethyltrideca-1,3,7,11-tetraene; TPS, terpene synthase; ZIS, α -zingiberene synthase. *The location of these proteins is unknown. Soybean image obtained from CSIRO (<https://commons.wikimedia.org/w/index.php?curid=35476410>). *Lippia dulcis* image obtained from https://commons.wikimedia.org/wiki/File:Lippia_dulcis.jpg.

RNAi RhNUDX1 transgenic plants. The results indicated that RhNUDX1 used GPP as the substrate, as classical terpene synthases, but hydrolyzed one phosphate resulting in geranyl monophosphate (Figure 1). Thus, a further catalytic reaction with the assistance of an unidentified phosphatase to form geraniol is required. This mode of action differs from all known monoterpene synthases, which remove two phosphates in one reaction [29]. In addition, the RhNUDX1 enzyme is located in the cytosol, indicating that its substrate, GPP, was obtained either by transport from plastids or via cytosolic generation, but this remains to be solved. The discovery of such a unique pathway has provided a potential molecular marker for scented-rose breeding. Meanwhile, it also has raised many of questions on, for example, how this pathway evolved and how is it distributed in the plant kingdom [30].

The Complex Pathway to Iridoids

Iridoids represent a broad class of monoterpenoids derived from 10-oxogeranial that have interesting pharmaceutical and antibacterial properties [31]. Madagascar periwinkle (*Catharanthus roseus*) has been used as a model plant for extensive studies of the iridoid pathway. The monoterpene branch of this pathway starts with the formation of geraniol by a classical plastidial geraniol synthase [32]. Geraniol 10-hydroxylase (G10H), a cytochrome P450 enzyme that is anchored to the endoplasmic reticulum (ER), converts geraniol into 10-hydroxygeraniol [33]. Since the downstream protein G10H is located in the cytosol [34], this indicates that in, *C. roseus*, geraniol is transported from plastids to cytosol perhaps through stromules, as they are closely associated with the ER [35]. The product from the first reaction, 10-hydroxygeraniol, is then converted by a cytosolic 10-hydroxygeraniol dehydrogenase (Cr10HGO) (also called 8HGO) into 10-oxogeranial (also 8-oxogeranial) [31,36]. Subsequently, 10-oxogeranial serves as substrate for the newly discovered iridoid synthases, which show high similarity to progesterone-5 β -reductases [37]. This unusual cyclization step leads to the formation of cyclic monoterpenoids (e.g., *Z*-*E*-nepetalactol), which are in turn converted to iridoids [31,37–40]. Thus this is another example of a new noncanonical player involved in terpene biosynthesis.

Biosynthesis of Vanillin

The most important phenylpropanoid volatile as seen from an economic perspective is certainly vanillin. It was recently shown that a single hydratase/lyase-type enzyme designated vanillin synthase (VpVAN) catalyses direct conversion of ferulic acid or its glucoside into vanillin or vanillin glucoside, respectively [41]. The conversion of ferulic acid to vanillin catalyzed by VpVAN is thought to occur via an initial hydration addition reaction followed by a retro-aldol elimination reaction. The VpVAN protein shows high sequence similarity with cysteine proteinases, including some important conserved residues. The cysteine protease family encompasses a large group of enzymes with versatile physiological functions. The involvement of such proteins in volatile biosynthesis is unexpected and their characterization offers new opportunities for the vanilla-pod-based industries.

New Substrates for Old Enzymes

As already mentioned, classical substrates for monoterpene synthases and sesquiterpene synthases are GPP and *E,E*-FPP, respectively. However, it has been shown that some enzymes use alternative substrates.

First, some plastidial monoterpene synthases use the *Z* isomer form of GPP, neryl diphosphate (NPP), to form monoterpenes. A plastidial *Z*-prenyltransferase, neryl diphosphate synthase 1 (NDPS1/SICPT1), was recently identified in the **glandular trichomes** of cultivated tomato (*Solanum lycopersicum*) [42,43]. Recombinant NDPS1/SICPT1 protein was able to use both IPP and DMAPP to generate NPP [42,43]. By analyzing transgenic tomato plants with reduced *NDPS1/SICPT1* transcript levels, a decrease in the production of the monoterpene β -phellandrene was observed [43]. Both results indicate that NDPS1/SICPT1 provides NPP substrate for the biosynthesis of monoterpenes.

Alternative substrates such as NPP are not routinely tested in functional characterization of plant terpene synthases. So far, few identified plastidial monoterpene synthases have shown preference for NPP rather than GPP as a substrate. Well-described examples are: (i) a phellandrene synthase (SIPHS1) [42]; (ii) a limonene synthase (ShLMS) [44]; (iii) a pinene synthase (ShPIS) [44]; and (iv) a nerol synthase (GmNES) [45]. Recombinant SIPHS1 protein, which was highly expressed in the glandular trichomes of *S. lycopersicum*, converted NPP mainly to β -phellandrene and, to a lesser extent, to four other monoterpenes: δ -2-carene, α -phellandrene, limonene, and γ -terpinene [42]. A similar result was found in another *in vitro* study of the recombinant PHS1 protein of wild tomato (*Solanum habrochaites*) (ShPHS1), which exhibited high specificity

to NPP and generated β -phellandrene and δ -2-carene [44]. The NPP preference characteristic of PHS1 could be further confirmed by the overexpression of NDPS1 and PHS1 in *S. lycopersicum* fruits, which resulted in a monoterpene blend [46]. Another two monoterpene synthases, ShLMS and ShPIS found in the glandular trichomes of *S. habrochaites*, solely used NPP as the substrate and mainly generated limonene and α -pinene, respectively [44]. In soybean (*Glycine max*), a nerol synthase (GmNES) exclusively converted NPP to nerol [45].

Sesquiterpenes are a group of C_{15} terpenes that play important roles in various physiological and ecological processes of plants. Besides being constituents of floral scents, sesquiterpenes are also known as repellents of insects (e.g., 7-epizingiberene, *R*-curcumene) [47] and as antimicrobial compounds (e.g., *E*- β -caryophyllene) [48]. It is generally agreed that *E,E*-FPP is the precursor for the biosynthesis of most sesquiterpenes and their corresponding sesquiterpene synthases are located in the cytosol. However, in glandular trichomes of wild tomatoes sesquiterpenes can also be synthesized from *Z,Z*-FPP. Sallaud *et al.* [49] discovered a *Z,Z*-farnesyl diphosphate synthase (zFPS) in *S. habrochaites* that catalyzed the formation of *Z,Z*-FPP from IPP and DMAPP and a sesquiterpene synthase, santalene and bergamotene synthase (SBS), that used *Z,Z*-FPP as the substrate to generate a mixture of these two sesquiterpenes. In a later study, additional *Z*-prenyltransferases were found in *S. lycopersicum*, with SICPT6 being able to convert NPP into *Z,Z*-FPP in roots and red fruits [43]. More sesquiterpene synthases that use *Z,Z*-FPP – for example, zingiberene synthase (ShZIS) – to generate 7-epizingiberene in glandular trichomes of *S. habrochaites* have also been identified [44,50].

The Puzzling Localization of Enzymes

Almost all of the identified monoterpene synthases have been located inside plastids and, with a few exceptions, inside mitochondria [51,52] based on either predicted plastid transit peptides or by visualization using fusions with GFP. However, monoterpene biosynthesis may also be present in the cytosol (Figure 1). The idea that monoterpenes can be made in the cytosol goes back to the synthesis of shikonin [53]. The first-discovered cytosolic monoterpene synthase is actually a dual-function terpene synthase, nerolidol synthase 1 in *Fragaria* $\times$ *ananassa* (FaNES1), that can react with both GPP and FPP to produce linalool or nerolidol, respectively. Its localization in the cytosol was demonstrated by GFP fusion of the FaNES1 protein [51]. In the same study, FvPINS, a genuine monoterpene synthase, was also localized in the cytosol. Moreover, (S)-linalool and (–)- α -pinene in the fruit of *F. x ananassa* and *Fragaria vesca*, respectively, were found, using deuterium (2H) labeling, to be generated exclusively via the cytosolic MVA pathway [54]. In raspberry fruits, feeding experiments also demonstrated that (S)-linalool and (–)- α -pinene were exclusively synthesized via the cytosolic MVA pathway [55]. These studies in Rosaceae revealed that monoterpenes can be also synthesized in the cytosol rather than only in plastids and that precursors probably come from the MVA pathway. Cytosolic monoterpene synthases were also discovered in other species. In *Lippia dulcis*, a cytosolic geraniol synthase (*LdGES*) has been characterized [56]. Both transient and stable transformation of tobacco confirmed monoterpene synthase activity of *LdGES* resulting in the production of geraniol-derived products. Studies with GFP fusions of *LdGES* by transient transformation assays in *Nicotiana benthamiana* indicated its cytosolic localization [56]. *Ocimum basilicum* α -zingiberene synthase (ZIS) is a cytosolic bifunctional terpene synthase that uses not only FPP but also GPP as substrates to produce both sesqui- and monoterpenes [57].

The presence of these cytosolic monoterpene synthases or bifunctional enzymes suggests that there is a complex biosynthetic network of monoterpenes in cytosol for some species. This also raises the question of the origin of their substrates. Are they exported from plastids or are they synthesized in the cytosol? Several lines of evidence indicate that molecules such as GPP can be exported from plastids to cytosol. Coexpression in tomato fruits of the snapdragon small catalytic unit of GPP synthases (GPPS-SSU) (plastidial) and the cytosolic bifunctional ZIS from

O. basilicum [57] resulted in increased accumulation of ZIS-derived monoterpenes, demonstrating the transport of GPP from plastid to cytosol [58]. The hypothesis of GPP export from plastids is also supported by a kinetic study of the export of prenyl diphosphates in isolated chloroplast envelope membrane vesicles of spinach (*Spinacea oleracea*) [59]. It showed that the export rates of IPP and GPP are relatively higher than those of DMAPP and FPP. Finally, ectopic expression of GES from *Valeriana officinalis* in three different cell compartments (plastids, cytosol, and mitochondria) showed that there is considerable (bi)directional GPP exchange between these compartments [60]. In addition, no cytosolic GPP synthase (GPPS) has been found so far, with the possible exception of one from *Lithospermum erythrorhizon*, which has not been characterized [61]. Although these experimental results indicate that plastid-generated GPP could support cytosolic monoterpene biosynthesis, no transporter of IPP or GPP has been characterized to date. Another possibility, albeit less probable, is that GPP could be generated in the cytosol as a byproduct of FPPS activity. As GPP is an intermediate in the synthesis of FPP, it could be that part of it is released and used for the biosynthesis of monoterpenes in the cytosol. However, the amount of GPP available might not be sufficient to support the biosynthesis of a large quantity of monoterpenes.

Sesquiterpenes were first thought to be synthesized exclusively in the cytosol, using precursors generated via the MVA pathway. However, there seem to be some exceptions to this rule. Labeling experiments in snapdragon flowers have shown that IPP and DMAPP, used for the biosynthesis of sesquiterpenes, were produced in plastids via the MEP pathway [62]. Since snapdragon sesquiterpene synthases are localized in the cytosol [63], this indicates transport of IPP/DMAPP from plastids to cytosol. More recently it has become clear that all identified Z-prenyltransferases, related to mono- and sesquiterpene synthases, are located inside plastids [43,45,49], suggesting that the major pool of Z-prenyl diphosphates may be mainly produced in plastids. Concomitantly, several sesquiterpene synthases able to use these Z-prenyl diphosphate substrates in the plastids have been identified in wild tomato [50]. The MVA and MEP pathways contribute to the biosynthesis of sesquiterpenes at equal rates in the glandular trichomes of *Stevia rebaudiana* [64] and both routes are also utilized in grape berries [65]. Thus, there are multiple examples of 'metabolic crosstalk' in the biosynthesis of sesquiterpenes, as summarized in [66].

Some diterpenes are also made in the cytosol. While diterpene synthases are primarily located in plastids, geranylinalool synthase from *A. thaliana*, which is involved in the biosynthesis of (*E,E*)-geranylinalool, the precursor of 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), resides in the cytosol or the ER [67]. It is thus likely that its substrate GGPP is present in these compartments.

Different Pathways for the Same Compounds

Biosynthesis of Homoterpenes

Homoterpenes (more precisely, tetra-nor-polyterpenes, but in this field commonly referred to as homoterpenes) belong to a special class of terpenes that are generated through degradation of regular terpenes. They are known to be floral constituents of night-scented flowers like Orchidaceae and Liliaceae as well as damage-induced leaf volatiles that may attract natural enemies of herbivorous insects [68–72]. The two most-studied homoterpenes are the C₁₁ homoterpene 4,8-dimethylnona-1,3,7-triene (DMNT) and the C₁₆ homoterpene TMTT, which were thought to be derived from sesquiterpenoids (e.g., nerolidol) and diterpenoids (e.g., geranylinalool) by oxidative degradation, respectively [68]. In *A. thaliana* it was shown that one P450 enzyme (CYP82G1) was able to catalyze the formation of both compounds [70]. However, a recent study showed that in *A. thaliana* the precursor for biosynthesis of DMNT is not limited to nerolidol [73]. Interestingly, DMNT can be generated by degradation of a C₃₀ triterpenoid, arabidiol, in *A. thaliana* roots. This degradation reaction was catalyzed by another cytochrome P450 monooxygenase, CYP705A1 (At4g15330), probably located in the ER [74]. The reaction is induced by

Biosynthesis of Geranial

Plastid

- Shikimate pathway
- Arogenate pathway
- L-Phenylalanine

Cytosol

- L-Phenylalanine (via pCAT from plastid)
- Enzymes: PAL, C4H, 4CL, CCoAOMT, CFAT
- Pathway: L-Phenylalanine → t-Cinnamic acid → p-Coumaric acid → p-Coumaroyl-CoA → Caffeoyl-CoA → Feruloyl-CoA → Coniferyl alcohol
- Coniferyl alcohol → Coniferyl acetate (via CFAT)

Peroxisome

- Enzymes: PXA1, CHD, KAT, BPBT*, IGS1, EGS1
- Pathway: Coniferyl-CoA → 3-Hydroxy-3-phenylpropanoyl-CoA → 3-Oxo-3-phenylpropanoyl-CoA → Benzoyl-CoA
- Benzoyl-CoA → Benzoic acid (via Thioesterase?) → Benzoic acid
- Benzoic acid → Methylbenzoate (via BSMT)
- Benzoyl-CoA → Benzylbenzoate (via BPBT*)
- Benzoyl-CoA → Phenylethybenzoate (via BPBT*)

Other Pathways and Enzymes

- Phenethylamine → 2-Phenylacetaldehyde (via PAAS)
- 2-Phenylacetaldehyde → 2-Phenylethanol (via PAR)
- 2-Phenylethanol → 2-Phenylpyruvate (via PPDC)
- 2-Phenylpyruvate → Phenylpyruvate (via AAAT/ArAT)
- Phenylpyruvate → Benzaldehyde (via AOs)
- Benzaldehyde → Benzoin (via AOs)
- Benzoin → Benzoin glycoside (via AOs)
- Benzoin glycoside → Benzoin glucoside (via AOs)
- Benzoin glucoside → Benzoin glucuronide (via AOs)
- Benzoin glucuronide → Benzoin glucuronide (via AOs)
- Benzoin glucuronide → Benzoin glucuronide (via AOs)

Key:

- Multiple enzymatic steps →
- Known route →
- Unproven route - - - - -
- New route →
- Transport →

Abbreviations: 4CL, 4-coumarate CoA-ligase; AAAT, amino acid aminotransferase; AADC, aromatic amino acid decarboxylase; AOs, aldehyde oxidases; ArAT, amino acid transaminase; BPBT, benzoyl-CoA:benzylalcohol/2-phenylethanol benzoyltransferase; BSMT, benzoic acid/salicylic acid carboxyl methyltransferase; BZO1, homolog gene of cinnamoyl CoA ligase in *A. thaliana*; C4H, cinnamic acid 4-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; CFAT, coniferylalcohol acetyltransferase; CHD, cinnamoyl-CoA hydratase/dehydrogenase; CNL, cinnamoyl-CoA ligase; EGS1, eugenol synthase 1; IGS1, isoeugenol synthase 1; KAT, 3-ketoacyl-CoA thiolase; PAL, phenylalanine ammonia lyase; PAAS, phenylacetaldehyde synthase; PAR, phenylacetaldehyde reductase; pCAT, plastidial cationic amino acid transporter; PEA, phenethylamine; PPDC, phenylpyruvate decarboxylase; PXA1, peroxisomal ATP-binding cassette (ABC) transporter 1.

Biosynthesis of 2-Phenylethanol (2-PE)

In terms of diversity, benzenoids and phenylpropanoids are considered the second largest class of plant volatiles after terpenoids [2]. They are also involved in various interactions with the surrounding environment [2,3]. This class of compounds is mainly derived from L-phenylalanine (Phe), one of the three aromatic amino acids synthesized via the shikimate pathway [80]. Comprehensive reviews on the biosynthesis of benzenoids [13] and phenylpropanoids [81] are available. Here we only briefly emphasize the alternative routes that plants have developed to make the same compounds.

Briefly, benzenoids are C_6-C_1 compounds, having an aromatic six-carbon ring with one carbon attached. Their formation is initialized by L-phenylalanine ammonia lyase (PAL), which deaminates Phe to *t*-cinnamic acid (CA) (Figure 2). The subsequent route from CA to benzenoid-related products proceeds via the β -oxidative pathway (in peroxisomes), the non- β -oxidative pathway (in the cytosol), or a combination of the two, as recently reviewed by Widhalm and Dudareva [13].

Other volatiles derived from Phe – for example, phenylacetaldehyde (PAld) and 2-PE – are important scent compounds in numerous flowers such as rose and petunia. The genes and enzymes responsible for the biosynthesis of these phenylpropanoid-related compounds (C_6-C_2 , an aromatic six-carbon ring with two carbons attached) have been identified and characterized. Interestingly, plants have developed different pathways to convert Phe to PAld, all located in the cytosol (Figure 2). In petunia (*Petunia hybrida* cv. Mitchell), a bifunctional phenylacetaldehyde synthase (PAAS) can achieve both decarboxylation and oxidation reactions and convert Phe to PAld [82]. By contrast, in tomato (*S. lycopersicum* cv. M82) fruits, Phe is first converted to phenethylamine (PEA) by an aromatic L-amino acid decarboxylase (AADC) and then subjected to oxidation by an as yet to be identified oxidase [83]. The conversion from PAld to 2-PE in tomato is catalyzed by a phenylacetaldehyde reductase (PAR) [84]. In rose two enzymes have been identified, RhPAAS of *Rosa* $\times$ *hybrida* cv. Fragrant Cloud [82,85] and Rose-PAR of *Rosa* $\times$ *damascena* Mill. [86], which possess functions similar to PAAS in petunia and PAR in tomato, respectively. Surprisingly, another pathway leading to PAld biosynthesis from Phe also exists in rose. In this pathway, PAld is first converted into phenylpyruvate (PPA) by an aromatic amino acid aminotransferase (RyAAAT3), which then undergoes decarboxylation to form PAld by a phenylpyruvic acid decarboxylase [87,88]. This is another example, as in homoterpene biosynthesis in *A. thaliana*, of two different routes used in one species to produce the same compound. The difference with *A. thaliana* here is that the two alternative pathways are supposed to function in parallel in the same petal tissue. Finally, an additional route was discovered recently in the fruit of melon (*Cucumis melo*) in which Phe is converted by an amino acid transaminase (CmArAT1) to its corresponding α -keto acids, which are efficient precursors for subsequent modifications to synthesize various aromatic compounds, including PAld [89].

Concluding Remarks and Future Perspectives

In recent years, numerous studies have revealed that plants have evolved complex routes for terpene biosynthesis. For example, monoterpenes, which can be synthesized in both plastids and the cytosol, can be generated from GPP, NPP, and even long-chain terpenoids and can be synthesized by canonical monoterpene synthases or by a terpene synthase-independent pathway (RhNUDX1 in roses). Similar plasticity for the biosynthesis of benzenoids and phenylpropanoids is also observed. The diversity of these volatile biosynthesis pathways is often an example of convergent evolution, with multiple pathways giving rise to similar or identical products evolving independently across various taxa, perhaps as the result of different adaptive responses to similar environments or ecological niches [11]. In rose, the alternative pathway identified for the production of 2-PE could be activated in response to seasonal changes in temperature [88]. Examples of convergent evolution occur also in the biosynthesis of other, non-volatile, specialized metabolites.

Outstanding Questions

How are the prenyl diphosphates, precursors of terpenes, transported between subcellular compartments? What are the proteins responsible for such transport?

Could geranyl diphosphate be synthesized in the cytosol? If yes, what are the enzymes responsible for such biosynthesis?

Are there other distinct enzymes (besides NUDX1 and terpene synthases) involved in the biosynthesis of terpenes?

When two pathways operate in the same species for the production of one compound, what are the molecular mechanisms that regulate the two pathways?

What are the environmental parameters that favor the evolution of alternative pathways for the production of a given compound?

For example, the *N*-methyltransferase (NMT) genes, which are involved in caffeine formation in *Coffea canephora*, nest within a gene clade distinct from those of cacao (*Theobroma cacao*) and tea (*Camellia sinensis*), which suggests a **polyphyletic** origin of NMT activity [90]. Thus, due to the biodiversity in land plants, it seems probable that there exist several yet to be discovered alternative pathways for the biosynthesis of identified plant volatile compounds. As for Nudix hydrolase in rose, previously characterized enzymes may possess completely different functions in other plants. It is possible that the capacity of Nudix hydrolases has been recruited for other functions in different plants. For example, another Nudix hydrolase might be able to use IPP as substrate to produce IP, which seems to limit the biosynthesis of terpenes in the cytosol [23]. Moreover, other novel enzymes could be discovered over time during extensive studies of plant volatile biosynthesis (see Outstanding Questions). The use of RNA sequencing as well as available plant and microbial genomes will facilitate the future identification of these proteins [91–93].

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