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Review

Plant volatile terpenoid metabolism: Biosynthetic genes, transcriptional regulation and subcellular compartmentation

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ABSTRACT

Volatile terpenoids released from different plant parts play crucial roles in pollinator attraction, plant defense, and interaction with the surrounding environment. Two distinct pathways localized in different subcellular compartments are responsible for the biosynthesis of these compounds. Recent advances in the characterization of genes and enzymes responsible for substrate and end product biosynthesis as well as efforts in metabolic engineering have revealed new aspects of volatile terpenoid biosynthesis. This review summarizes recent progress in the characterization of volatile terpenoid biosynthetic genes, their spatio-temporal expression patterns and subcellular localization of corresponding proteins. In addition, recent information obtained from metabolic engineering is discussed.

1. Introduction

Volatile terpenoids represented by mainly isoprene (C_5), monoterpenes (C_{10}) and sesquiterpenes (C_{15}) constitute the largest class of plant volatile compounds. They play important roles in direct and indirect plant defense against herbivores and pathogens, in reproduction by attraction of pollinators and seed disseminators, and in plant thermotolerance [1]. Apart from their importance in plant physiology and ecology, volatile terpenoids are also used as natural flavor and aroma compounds and have beneficial impact on humans as health promoting compounds [2]. All terpenoids are synthesized from the universal five carbon precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which are derived from two alternate biosynthetic pathways localized in different subcellular compartments (Fig. 1). While DMAPP formed in plastids is used by isoprene synthase (ISPS) to form isoprene in some plants, IPP and DMAPP precursors are further condensed by prenyl diphosphate synthases in the respective compartments to form prenyl diphosphate intermediates serving as substrates for a large group of terpene synthase (TPS) enzymes, resulting in the final terpenoid compounds [1]. In the past several years there has been a significant progress in identification and characterization of volatile terpenoid biosynthetic genes and enzymes, determination of their spatio-temporal expression and compartmentalization, and metabolic engineering. This has led to several new insights resulting in better understanding of volatile terpenoid biosynthesis.

2. Formation of IPP and DMAPP

In plants two biosynthetic pathways are responsible for the synthesis of IPP and DMAPP, the universal precursors of all terpenoids. The classical cytosolic mevalonic-acid (MVA) pathway gives rise to IPP from acetyl-CoA [3], whereas the plastidial 2-*C*-methyl-erythritol 4-phosphate (MEP) pathway [4] described during the 1990s leads to the formation of IPP and DMAPP from pyruvate and glyceraldehyde-3-phosphate (Fig. 1). Although the subcellular compartmentalization of two pathways allows them to operate independently, metabolic cross-talk between the two pathways has been reported [5,6].

The MVA pathway starts with the condensation of two molecules of acetyl-CoA into acetoacetyl (AcAc)-CoA by the enzyme AcAc-CoA thiolase (AACT) (Fig. 1). The first plant AACT was cloned from radish (*Raphanus sativus*) by functional complementation of a yeast mutant defective for AACT [7]. Recent characterization of *Arabidopsis* genes encoding AACT1 and AACT2 and analysis of T-DNA insertion mutants for both genes indicated that only AACT2 is involved in the MVA pathway, whereas AACT1 could possibly be associated in the last step of fatty acid degradation [8,9]. In the second step, HMG-CoA is formed by the condensation of one molecule of acetyl-CoA with one molecule of AcAc-CoA. It was

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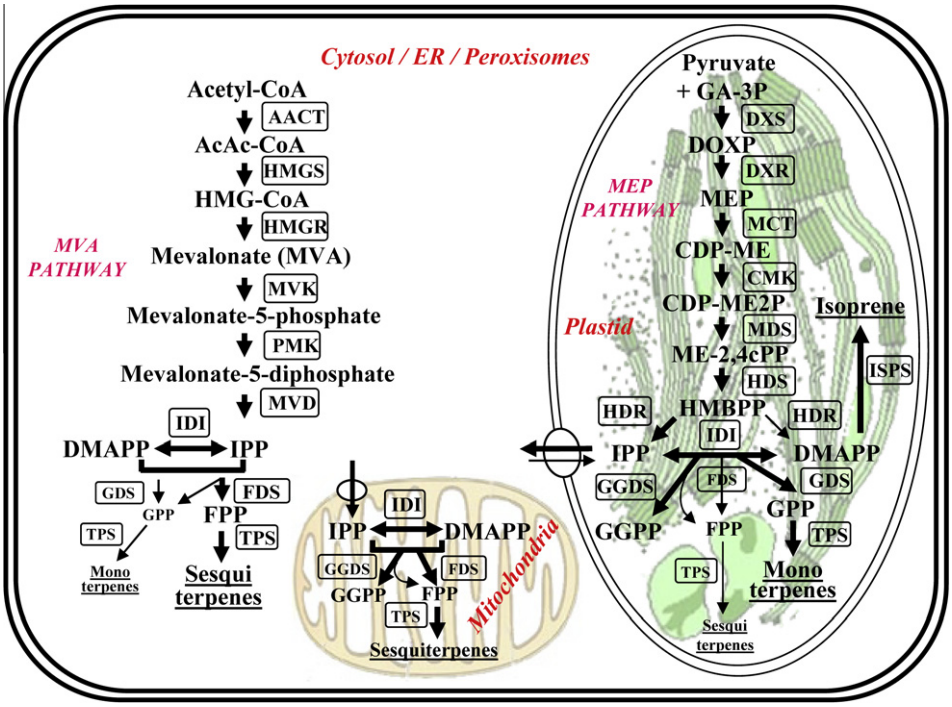

Fig. 1. Biosynthetic pathways and their compartmentalization leading to volatile terpenoids in plants. AACT, acetoacetyl-CoA thiolase; AcAc-CoA, acetoacetyl-CoA; CDP-ME, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol; CDP-ME2P, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol phosphate; CMK, CDP-ME kinase; DMAPP, dimethylallyl diphosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; DXR, DOXP reductoisomerase; DXS, DOXP synthase; FDS, farnesyl diphosphate synthase; FPP, farnesyl diphosphate; GA-3P, glyceraldehyde-3-phosphate; GDS, geranyl diphosphate synthase; GGDS, geranyl geranyl diphosphate synthase; GGPP, geranyl geranyl diphosphate; GPP, geranyl diphosphate; HDR, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; HDS, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase; HMBPP, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthase; IDI, isopentenyl diphosphate isomerase; IPP, isopentenyl diphosphate; ISPS, isoprene synthase; MCT, 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase; MDS, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; ME-2,4cPP, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; MVD, mevalonate diphosphate decarboxylase; MVK, mevalonate kinase; PMK, phosphomevalonate kinase; TPS, terpene synthase. Names of the enzymes are boxed and volatile terpenoids are underlined.

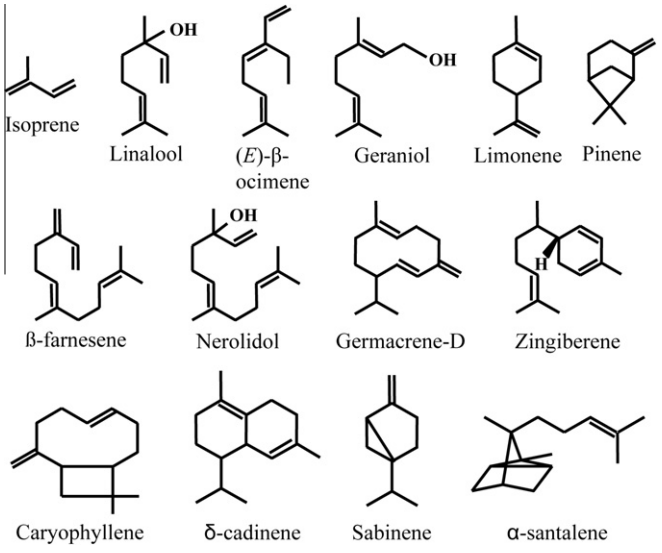

Fig. 2. Structures of representative volatile terpenoid compounds.

earlier proposed that the conversion from acetyl-CoA to HMG-CoA involves two enzymatic steps carried out by a single enzyme [10]. However, HMG-CoA synthase (HMGS) from *Arabidopsis* was reported to functionally complement yeast mutants defective for HMGS [11]. Further biochemical characterization of HMGSs from *Brassica juncea* [12] and *Hevea brasiliensis* [13] that catalyze the conversion of acetyl-CoA and AcAc-CoA to HMG-CoA indicated that the conversion from acetyl-CoA to HMG-CoA involves two independent enzymes; AACT and HMGS. In the next step, HMG-CoA reductase (HMGR), a NADPH-dependent enzyme that catalyzes a double reduction reaction involving four electron transfers, catalyzes the biosynthesis of mevalonate from HMG-CoA [14]. HMGR has been extensively studied in some plant species. For example, *Arabidopsis* contains two differentially expressed HMGR genes which encode three isoforms [15], whereas in snapdragon (*Antirrhinum majus*) three genes encoding HMGR have been identified [16]. The downstream steps from mevalonate to IPP involve two phosphorylations and a decarboxylation event carried out by mevalonate kinase (MVK), phosphomevalonate kinase (PMK), and mevalonate diphosphate decarboxylase (MVD) enzymes, respectively. Although the enzymes involved in these steps are well studied in yeast and mammalian systems, there is surprisingly little information available in terms of their biochemical characterization in plants. IPP derived from the MVA pathway in the cytosol, is further acted upon by isopentenyl diphosphate isomerase (IDI), a divalent, metal ion-requiring enzyme, to form dimethylallyl diphosphate (DMAPP) [17].

The mevalonate-independent pathway, named the MEP pathway was first elucidated in *Escherichia coli* and subsequently plant homologues have been characterized using a combination of biochemical and genomic approaches [18]. The MEP pathway consists of seven enzymatic steps involved in the formation of IPP and DMAPP from pyruvate and D-glyceraldehyde 3-phosphate (GAP) (Fig. 1). The first step in this pathway is the condensation of

pyruvate and glyceraldehyde 3-phosphate to form 1-deoxy-D-xylulose-5-phosphate (DOXP) by DOXP synthase (DXS). DOXP is then transformed into MEP by DOXP reductoisomerase (DXR), also called MEP synthase. MEP is further converted to 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (HMBPP) by the consecutive enzymatic action of 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase (MCT), 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase (CMK), 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS) and (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS). The last step is the branching of HMBPP to IPP and DMAPP catalyzed by the simultaneous enzymatic action of a single enzyme, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) [19]. Although the HDR in the MEP pathway produces both IPP and DMAPP, albeit at 85:15 ratio [20], the plastid localized IDI is involved in substrate optimization by catalyzing IPP isomerization, (Fig. 1). Based on [2-^{13}C,4-^{2}H]deoxyxylulose double-labeling studies in tobacco BY-2 (TBY-2) and *Catharanthus roseus* cells, it was suggested that the regulation of DMAPP/IPP balance by IDI may significantly differ from one plant to another plant species [20]. Although cDNAs encoding IDIs have been isolated from different plants, only *Arabidopsis* IDIs have been biochemically characterized [21].

After the formation of DMAPP, isoprene synthase (ISPS) utilizes DMAPP to produce isoprene (Fig. 1), a 5-carbon compound (Fig. 2) generally emitted from the leaves of many deciduous broad-leaved tree species. ISPSs have been sequenced from different plant species and the sequences and gene structures indicate that they all belong to TPS-b family that also includes other monoterpene and sesquiterpene synthases [22]. However, ISPSs differ from monoterpene and sesquiterpene synthases in two properties; requirement of high temperature for their activity (45–50 °C) and very high K_m for DMAPP [23].

3. Formation of prenyl diphosphate precursors

Following the formation of IPP and DMAPP, prenyltransferases located in both compartments utilize IPP and DMAPP to produce prenyl diphosphates (Fig. 1). Condensation of one DMAPP and two IPP molecules catalyzed by farnesyl diphosphate synthase (FDS) leads to the formation of FPP (C_{15}), the natural precursor of sesquiterpenes [1]. In plants, FDSs have been cloned and characterized from several species and like all prenyltransferases, they contain two aspartate-rich DD(X)$_{2-4}$D motifs (where "X" represents any amino acid) that are essential for prenyl–substrate binding [24]. Until recently, all plant FDSs involved in sesquiterpene biosynthesis were thought to produce FPP in the trans (*E*) configuration, however, recently a FDS capable of catalyzing the condensation of IPP and DMAPP in the cis (*Z*) configuration forming *Z,Z*-FPP, the substrate for biosynthesis of type II sesquiterpenes, has been reported in *Solanum habrochaites* [25].

Geranyl diphosphate synthase (GDS) catalyzes the condensation of one molecule each of IPP and DMAPP to produce GPP (C_{10}), the universal precursor of monoterpenes [1]. GDSs have been isolated and functionally characterized in a limited number of plant species and are known to exist as homodimeric and heterodimeric structures composed of two identical sub-units or one small sub-unit (SSU) with one large sub-unit (LSU), respectively. All homodimeric GDSs and the LSU of heterodimeric GDSs (GDS·LSU) contain two aspartate-rich DD(X)$_{2-4}$D, with the exception of a recently identified homodimeric GDS from orchid *Phalaenopsis bellina* [26]. All GDSs characterized to date produce GPP as the main product, however, it was recently demonstrated that a homodimeric bifunctional geranyl and geranylgeranyl diphosphate synthase (PaIDS1), capable of forming both GPP and GGPP in substantial amounts is involved in monoterpene and diterpene formation in *Picea abies* [27]. The SSU of heterodimeric GDS by itself is catalytically inactive whereas the LSU alone could be either inactive [28], or function as geranylgeranyl diphosphate synthase (GGDS) on its own [29,30], and only the interaction between the SSU and LSU leading to the formation of a heterodimer results in an active GDS. However, the SSU lacks a DD(X)$_{2-4}$D motif and is catalytically inactive alone, but its interaction with LSU modifies the chain length of the product formed by LSU from GGPP to GPP. Recently it was shown that the two conserved CxxxC motifs (where "x" can be alanine, leucine, isoleucine, valine, glycine, serine or methionine) present in all SSUs and one conserved CxxxC motif in all LSU and GGDS are crucial for the interaction between SSU and LSU/GGDS [30,31]. Further a new clade of proteins, designated as SSU-II subfamily present in both angiosperms and gymnosperms were identified, which like SSU-I subfamily, not only modify the chain length of the product, but also accelerate the GDS activity of the LSU [30]. In addition to the formation of FPP and GPP by FDS and GDS, respectively, it has been proposed that FPP and GPP could potentially be derived from promiscuous side reactions of GGDS and FDS, respectively [32].

4. Formation of monoterpenes and sesquiterpenes

Following the formation of the prenyl diphosphate precursors GPP and FPP, an array of structurally diverse cyclic and acyclic monoterpenes and sesquiterpenes is generated by the action of a large family of enzymes known as terpene synthases/cyclases (TPSs) [1] (Fig. 1). The reaction mechanisms of TPSs involve a divalent-cation-dependent ionization of the prenyl diphosphate substrate and the formation of reactive carbocation intermediates [33]. All TPSs have similar properties with respect to their native molecular mass (either monomers or homodimers) and requirement of a divalent metal ion (usually Mg^{2+} or Mn^{2+}) for activity [34]. Many TPSs catalyze the formation of a single product; however several multiproduct mono/sesqui TPSs have also been recently isolated and characterized [35]. The biosynthesis of monoterpene compounds (C_{10}) is catalyzed by specialized monoterpene synthases, which utilize GPP to form various skeletal types (Fig. 2). In the last several years, a number of monoterpene synthases has been isolated and characterized from various plant species of both angiosperm and gymnosperm group [35]. Generally, monoterpene synthases are between 600 and 650 aa in length and are larger than sesquiterpene synthases by 50–70 aa, due to the presence of a N-terminal transit peptide required for plastid targeting [34]. Sesquiterpenes (C_{15}) are synthesized from FPP by sesquiterpene synthases and are structurally more diverse than monoterpenes, due to the increased number of different cyclizations possible with five additional carbon atoms (Fig. 2). To date, several sesquiterpene synthases have been cloned and biochemically characterized from various plant species [35].

5. Spatial and temporal expression of volatile terpenoid biosynthetic genes

Volatile terpenoids are often biosynthesized and emitted from specific plant tissues at a particular time. There is significant number of reports describing the spatio-temporal expression of TPSs correlating with volatile terpenoid biosynthesis and emission, indicating that volatile terpenoid biosynthesis is mainly regulated at the level of transcription. In *P. alba* leaves, *ISPS* mRNA expression was strongly induced by heat stress and light irradiation, but was substantially decreased in the dark, suggesting that isoprene emission was regulated at the transcriptional level [36]. Also, studies of the regulation of *ISPS* gene in poplar (*Populus canescens*) and kudzu (*Pueraria montana*) have indicated that, during leaf development,

the onset of isoprene emission is controlled by *ISP*S transcription or mRNA turnover [37,38]. Also, it was recently reported that in *Populus trichocarpa*, isoprene emission and accumulation of *ISPS* mRNA begin at the same developmental stage [22].

In *Clarkia breweri*, linalool synthase (*LIS*) transcripts are found at high levels in the stigma, styles and petals. In situ hybridization on cross-sections of flower buds indicated that *LIS* mRNA transcripts are present mainly in the secretory zone of the four-lobed stigma and also in epidermal layers of petals, the major source of emitted linalool [39]. In snapdragon flowers, TPS genes responsible for the formation of myrcene, ocimene, linaool and nerolidol are highly expressed in upper and lower petal lobes, the parts of the flower involved in the production and emission of these compounds [40,41]. Analysis of mRNA expression over flower development showed that myrcene and ocimene synthase mRNAs were detected first in mature flower buds and its level increased until it peaked on day 4 after anthesis and declining thereafter. Moreover, the steady state mRNA levels of myrcene and ocimene synthase genes exhibited rhythmic expression, having a strong correlation with corresponding monoterpene emission [40]. RT-PCR as well as RNA blotting analysis of *Cstps1*, the gene encoding valencene synthase in 'Valencia' oranges, showed that *Cstps1* transcript accumulation continued progressively towards fruit maturation, thus corresponding well with the timing of valencene accumulation. Further, upon ethylene treatment, *Cstps1* exhibited enhanced mRNA expression, which corresponded with increased accumulation of valencene [42]. Expression of *TPS* genes from grapevine, valencene synthase (*VvVal*), germacrene-D-synthase (*VvGerD*) and monoterpenol synthase (*VvTer*) displayed distinctive correlation with grapevine flowering and fruit ripening. All three genes exhibited strongest mRNA expression in flower buds shortly before opening at bloom when the transcripts could be involved in the formation of floral terpenoids. During berry development, only the transcripts of *VvVal* were detected during late ripening of the berries [43]. The expression patterns of the transcripts of four cDNA clones encoding monoterpene synthases in citrus (*Citrus unshiu*) fruits exhibited variation among tissues and development stages, but the expression of all four clones occurred mainly in peel at an early stage of fruit development and disappeared or decreased in fruit at later stages [44]. Analysis of mRNA expression levels of specific TPSs in the glandular trichomes of basil cultivars showed that *LIS* expression was highest in the cultivar producing mostly (*R*)-linalool, whereas in a cultivar that synthesizes mostly geraniol, transcripts of geraniol synthase were the most abundant. Similarly, sesquiterpene synthases such as δ-cadinene synthase, selinene synthase and germacrene synthase were highly expressed in the cultivars with the highest levels of their respective products [45]. *FaNES1* responsible for the formation of the major volatiles of strawberry fruit, nerolidol and linalool, is predominantly expressed in ripe cultivated strawberry fruit, and expression levels correlated with the presence or absence of nerolidol and linalool in the different cultivated and wild strawberry lines tested [46]. In hop (*Humulus lupulus*) inflorescence, transcript levels of genes encoding the monoterpene synthases HlMTS1 and HlMTS2 followed the same developmental pattern as the monoterpene metabolites. Similarly, expression of *HlSTS1*, a gene encoding sesquiterpene synthase catalyzing the formation of caryophyllene and humulene, was abundant in those tissues with high levels of humulene and caryophyllene, paralleling the levels of these compounds in hop trichomes during development [47]. In *Arabidopsis* flowers, monoterpene and sesquiterpene synthases are not expressed in flower petals; instead, their expression is limited to the stigma, anthers, nectaries and sepals, suggesting that the volatile terpenoids synthesized in the *Arabidopsis* flower might not only function as short range attractants of pollinating insects, but might be of equal importance for the defense of floral tissues by inhibiting the growth of microbial pathogens or repelling herbivores from particularly vulnerable sites of the flower [48]. Expression of kiwi fruit (*Actinidia deliciosa*) sesquiterpene synthases, farnesene (*AdAFS1*) and germacrene synthases (*AdGDS1*) was significantly higher in flowers than in leaf tissue. Within floral tissues, expression of both genes was highest in petals and stamens [49]. *ZSS1* and *ZSS2*, which encode *Zingiber* sesquiterpene synthases, α-humulene synthase and β-eudesmol synthase, respectively, showed strongest expression in rhizomes correlating with a higher content of α-humulene and eudesmaol in rhizome oil [50,51]. In *Lavandula angustifolia* the expression of two TPS genes, *LaLIMS* and *LaLINS* displayed a good correlation with emission of respective compounds during inflorescence development [52].

In contrast to the correlation between TPS gene expression and corresponding terpenoid accumulation/emission, only fragmented data exists for expression of upstream genes in relation to volatile biosynthesis. In *Arabidopsis*, the high levels of *DXS* and *DXR* gene expression were also found in the inflorescences, consistent with the relatively high emission of terpenoids from this part of the plant [53]. In snapdragon, *DXS* and *GDS·SSU*, similar to the *TPSs*, are highly expressed in upper and lower petals and display rhythmic expression that coincides with monoterpene emissions [29]. Similarly, *GDS·SSU* and *LSU* in hop were highly expressed in trichomes where myrcene is synthesized [30]. In the orchid *P. bellina*, expression analysis of *PbGDS* gene encoding a homodimeric GDS showed that its expression was flower specific, and that maximal expression was concomitant with maximal emission of monoterpenes on day 5 post-anthesis [26]. In *P. abies*, expression of *PaIDS1* encoding a bifunctional GDS and GGDS was highest in wood where oleoresin comprising of monoterpenes and diterpenes is accumulated [27].

6. Subcellular localization of volatile terpenoid biosynthetic enzymes

In plants two biosynthetic pathways located in different subcellular compartments are involved in volatile terpenoid biosynthesis. In general, the MVA pathway is considered to operate in the cytosol, however current knowledge regarding the subcellular compartmentalization of enzymes involved in this pathway is limited. Transient expression of *Arabidopsis* homologues of AACT fused to a green fluorescent protein (GFP) showed that AACT2, the enzyme shown to be involved in the MVA pathway [8] is localized in the cytosol, whereas AACT1, suggested to be involved in fatty acid degradation, is located in peroxisomes [9,54]. Cytosolic localization of HMGS catalyzing the second step of the MVA pathway has been demonstrated for *B. juncea* HMGS [55]. Although all four *B. juncea* HMGS isoforms were found to contain a PTS-2-like sequence, in vitro localization studies using GFP-fusion constructs and subcellular fractionation of overexpressed fusion proteins confirmed that at least BjHMGS1 is a cytosolic enzyme [55]. In the case of HMGR, it was earlier reported that two radish genes encoding HMGR, when expressed in yeast yielded membrane-bound active enzyme indicating the enzyme is targeted to the ER [56], which is consistent with the insertion of tomato HMGR into microsomal membranes [57]. In *Arabidopsis*, a multipronged microscopy and cell fractionation approach revealed that HMGR1S, the housekeeping form of the enzyme, is localized within ER and also within spherical, vesicular structures located in the cytoplasm and within the central vacuole in differentiated cotyledon cells [58]. A more recent localization study using trans-membrane domain (TMD)-GFP fusion in TBY-2 cells indicates an (reversible) association of at least one isoform of HMGR with the cytoskeleton, whereby the housekeeping and the stress-induced enzymes behave quite differently [59]. It was demonstrated that HMGR1 is ER-localized, and a

second stress-induced HMGR2 forms globular structures connected by tubules, which is due to the presence of a serine upstream of the ER retention motif [59]. Although the subcellular localization of the downstream MVA pathway enzymes, MK, PMK and MVD, has been shown to be cytosolic in humans [60], localization of plant counterparts has not yet been reported.

In contrast to limited reports on subcellular localization of MVA pathway enzymes, it has been shown that all enzymes of the plant MEP pathway are localized to plastids. The plastidic localization of DXS was reported initially by immunohistochemistry in *Arabidopsis* leaf [61] and *Marchantia polymorpha* gemmae [62] using an anti-DXS antibodies. Further GFP-fusion experiments for DXS from *Arabidopsis* [63] and tomato [64] confirmed its plastidial localization. *Arabidopsis* DXR has been localized in plastids by transient expression of a DXR–GFP fusion protein in *Arabidopsis* cells [53,63] and by immunolocalization studies using anti-DXR antibody followed by electron microscopy [53]. Experimental evidence for chloroplast localization of MCT was recently demonstrated by transient expression of transit peptide-GFP fusion in *Arabidopsis* protoplasts [63]. The plastidial localization of CMK was reported for *Arabidopsis* [63,65] and *Ginkgo biloba* [65] CMKs by transient expression of GFP-fusion protein in *Arabidopsis* protoplasts. MDS, the next enzyme in the pathway was shown to be localized to plastids by transient expression of *Arabidopsis* MDS fused to N-terminus of GFP in *Arabidopsis* protoplasts [63]. The plastidial localization of HDS has been independently demonstrated by transient expression of GFP-fusion proteins in *Arabidopsis* leaves [66] and protoplasts [63]. HDR, the final enzyme involved in plastidial IPP and DMAPP formation was shown to be localized to the *Arabidopsis* chloroplast using chloroplast fractionation and immunoblot analysis [67] and by GFP-fusion and transient expression experiments with HDRs from *Arabidopsis* [63].

IPP isomerase (IDI) in *Arabidopsis* is encoded by *IDI1* and *IDI2* [68]. IDI1, when fused to GFP, displayed localization in plastids, and IDI2 was targeted to the mitochondria [21,69], suggesting that these IDIs provide DMAPP for plastidial- and mitochondrial-derived isoprenoids. Additional IDI activity encoded by both genes was present in the cytoplasm [21]. Further, shorter transcripts for both *IDI* genes encoding IDI proteins lacking the N-terminal extensions [21] were localized exclusively to peroxisomes [69]. However, this peroxisomal localization of IDIs and the suggestion that, part of the MVA pathway in plants is also located in peroxisomes needs to be further validated, as it was earlier claimed that in humans MVA pathway enzymes were localized to peroxisomes, but were later clearly demonstrated to be exclusively localized in the cytosol by a detailed multipronged approach [60].

With regard to isoprene synthases, all investigated ISPSs are located in chloroplasts. Initial biochemical studies in willow (*Salix discolor*) leaves revealed that ISPS is located in chloroplasts [70]. Recently, by immunogold-labeling with polyclonal antibodies generated against recombinant ISPS protein, it was demonstrated that poplar (*Populus canescens*) ISPS is localized in the chloroplast stroma as well as attached to the stromal side of the thylakoid membrane [23]. In transient expression studies, when the N-terminal 55 amino acids of *P. alba* ISPS were fused to GFP, the fluorescence was localized in chloroplasts of onion peel cells and tobacco leaf guard cells, indicating that the N-terminal sequence of PaISPS is a transit peptide for plastidial sorting [36].

Although all GDSs are known to contain a transit peptide for their plastid targeting, there are some ambiguous reports on actual GDS localization. Evidence for plastid localization of GDS was shown by biochemical localization studies in *Vitis vinifera* [71]. However, cell fractionation, marker enzyme assays, and immunodetection studies in *Lithospermum erythrorhizon* cell cultures indicated that GDS is cytosolic [72]. In *Arabidopsis* it was reported that, similar to FDS, two forms of GDS are present, depending on which methionine is used for initiating translation, and immunoblot analysis of the chloroplast proteome revealed that GDS is localized in chloroplasts [61]. Further, in situ localization studies using anti-GDS antibodies revealed that GDS is localized to chloroplasts of *Arabidopsis* leaf [61], *M. polymorpha* gemmae [62], leucoplasts and chloroplasts of *Citrofortunella mitis* fruit epicarp and chromoplasts of *Pinus* leaves [61]. However the presence of GDS in the cytosol could not be unequivocally determined [61]. Recently, immunogold-labeling using antibodies made against the *A. majus* GDS.SSU (involved in monoterpenoid biosynthesis in snapdragon) followed by electron microscopy demonstrated that GDS·SSU is found exclusively within the leucoplasts of the conical epidermal cells [29]. In the case of hop (*H. lupulus*) GDS, the LSU–GFP fusion protein accumulated exclusively in chloroplasts of tobacco leaf epidermal cells, whereas the SSU–GFP fusion protein aggregated around chloroplasts, suggesting that SSU could be targeted to non-pigmented leucoplasts in hop trichomes [30].

Like most of MVA pathway enzymes, the information on localization of FDS in plants is limited. In *Arabidopsis*, there are two genes *FDS1* and *FDS2*, encoding isoforms FDS1S, FDS1L and FDS2 [73,74]. It has been reported that *FDS1S* and *FDS1L* are both transcribed by the *FDS1* gene from alternative transcription start site and the corresponding FDS1S and FDS1L proteins differ only at their N-terminus. With respect to FDS1S, FDS1L contains an additional 41 amino acids at its N-terminus, which targets the enzyme into mitochondria, where it could be involved in providing FPP for the production of mitochondrial isoprenoid compounds such as ubiquinone, heme A, tRNA species, and prenylated proteins [74]. In contrast, it is believed that FDS1S and FDS2 could be located in the cytosol/ER compartment [75] with FDS1S functioning as a housekeeping gene to provide FPP for isoprenoids to general plant cell functions, while FDS2 would be involved in the synthesis of isoprenoids for more specialized functions [76]. In rice, immunolocalization and protease protection assays indicated that FDS was localized in chloroplasts. Further, based on immunoblot analysis of subcellular fractions using antibodies against rice FDS, it was concluded that wheat and tobacco FDS are also localized to chloroplasts. However, the enzyme activity of FDS reported in this study was not carried out, leaving its actual function unanswered [77]. Recently, a *z*FDS (catalyzing the formation of FPP in cis (*Z*) configuration) from wild tomato containing a 45 aa N-terminal transit peptide was shown to be localized to chloroplasts, suggesting that *z*FDS uses IPP and DMAPP provided by the plastidic MEP pathway [25].

All cloned monoterpene synthases are 50–70 amino acids longer than sesquiterpene synthases because of the presence of a typical transit peptide in their N-terminus responsible for chloroplast targeting [34]. Although a large number of monoterpene synthases has been isolated, subcellular localization has been reported only for a few of them. The plastidic localization of (4*S*)-limonene synthase (LIS) from different plants has been reported using various techniques [46,61]. In snapdragon, AmNES/LIS-2 involved in linalool biosynthesis was recently shown to be localized to plastids by transient expression assays of GFP-fusion proteins [41]. In addition, the plastid localization of AmNES/LIS-2 was confirmed by analysis of AmNES/LIS-2 enzyme activity in purified leucoplasts and by chloroplast import experiments [41]. Contrary to the general notion that all monoterpenes are localized to the plastids, some recent reports indicate that monoterpene synthases can deviate from this pattern and be localized to the cytosol as was demonstrated in the case of FaNES1 and FvPIN of strawberry [46], or can have a dual plastid and mitochondrial localization, as was reported for FaNES2 from strawberry and α-terpineol synthase from *Magnolia* [46,78]. Two putative monoterpene synthases, MtTps3 and MtTps4 from *Medicago trancutula* were reported to be localized to chloroplast by in vitro import experiments [79].

Sesquiterpene synthases are responsible for the biosynthesis of C_{15} sesquiterpenoid compounds from FPP and have been reported to be located in the cytosol, consistent with the conventional notion that sesquiterpenes are synthesized in cytosol [17]. A bifunctional nerolidol/linalool synthase from strawberry (FaNES1) and snapdragon (AmNES/LIS-1) has been localized in cytosol by GFP localization studies [41,46] and chloroplast import assay [41]. Two sesquiterpene synthases, (+)-germacrene D synthase and (*E*,*E*)-α-farnesene synthase responsible for the volatile profile of kiwifruit (*A. deliciosa*) flowers, when fused to the N-terminus of GFP, were localized in the cytoplasm [49]. Chloroplast import experiment with two putative sesquiterpene synthases, MtTps1 and MtTps2 from *Medicago truncatula* showed that these two proteins are not imported into isolated chloroplasts, indicating their cytosolic localization [79]. Although it is not expected of sesquiterpene synthases, some of them are reported to contain a putative plastid targeting sequence at their N-terminus similar to monoterpene synthases. *Pinus sylvestris* PsTPS2, a sesquiterpene synthase responsible for products with a germacrene skeleton, contains an N-terminal 37 amino acid putative signal peptide [80]. Interestingly, a 210-amino acid N-terminal signal peptide, a feature of conifer diterpene synthases, is also found in the sesquiterpene synthases of Norway spruce (*P. abies*) [81], and grand fir [34]. However, none of these putative signal peptides have been experimentally validated. Recently, a sesquiterpene synthase, Santalene and Bergamotene Synthase (SBS) isolated from the wild tomato *S. habrochaites* that synthesizes type II sesquiterpenes from *Z*,*Z*-FPP was shown to be localized to the plastids using GFP-fusion experiment [25].

7. Volatile terpenoid engineering

Metabolic engineering of volatile terpenoids is important not only for improving a considerable number of traits in crops, but also for understanding fundamental aspects of terpenoid biosynthesis in plants. Volatile terpenoid engineering studies in some plants has revealed the presence of GPP and FPP pools in non-native compartments (cytosol and plastid/mitochondria, respectively), which could be due to the promiscuous side reactions of FDS or GGDS in the respective compartments or perhaps due to a cytosol localized GDS or plastid/mitochondria localized FDS, respectively (Fig. 1). Transgenic *Arabidopsis* and potato plants expressing the plastid targeted strawberry FaNES1 capable of forming both linalool and nerolidol from GPP and FPP, respectively, emitted small amounts of nerolidol in addition to expected high levels of linalool, indicating that a small pool of FPP is present in plastids [82]. Similarly, patchoulol synthase (PTS), a sesquiterpene synthase from *Pogostemon cablin*, when targeted to the plastids in tobacco led to the accumulation of small amount of patchoulol and several other sesquiterpenes, indicating the presence of a small FPP pool in tobacco plastids [32], which is consistent with the localization of FDS in tobacco chloroplasts [77]. In another study when FaNES1 was targeted to mitochondria in *Arabidopsis* there was about 30-fold increase in nerolidol production [83] compared to transgenic plants with a plastid targeted FaNES1 [82], suggesting that mitochondria in *Arabidopsis* have a readily available FPP pool, which is formed by a FDS isoform localized to mitochondria [76] (Fig. 1). However, the possibility of cytosolic FPP uptake by mitochondria to a certain extent may not be ruled out, as it was shown that the exogenously supplied farnesol is absorbed and integrated into the side-chain of ubiquinone, a typical mitochondrial compound [84]. Transgenic tobacco plants overexpressing a cytosolic limonene synthase (truncated at the N-terminus) produced low levels of limonene indicating the presence of a small GPP pool in the cytosol [85]. Furthermore, α-zingiberene synthase (ZIS), a sesquiterpene synthase from basil, when overexpressed in tomato fruits under the control of a fruit ripening-specific promoter resulted in the accumulation of a number of monoterpenes including α-thujene, α-pinene, β-phellandrene and γ-terpinene in addition to high levels of α-zingiberene and several other sesquiterpenes [86], indicating the availability of a GPP pool in the cytosol of tomato fruits.

Metabolic engineering of volatile terpenoids has also revealed new aspects on IPP flux in the cytosol and plastids. It was reported that redirection of a sesquiterpene biosynthetic pathway from its natural cytosolic location to chloroplasts by simultaneous overexpression of plastid targeted FDS and PTS led to a dramatic increase in patchoulol accumulation 100–10 000 greater than when targeted to the native locale suggesting that IPP flux through the MEP pathway exceeds that of the MVA pathway [32]. In a recent study in tobacco, overexpression of a snapdragon GDS·SSU (an upstream gene in plastdial volatile terpenoid biosynthesis) under the control of flower specific *C. breweri LIS* promoter resulted in a large increase in emitted monoterpenes in both leaves and flowers, suggesting that the endogenous level of GPP available to monoterpene synthases is limiting [31]. The transgenic plants emitted reduced levels of sesquiterpenes, suggesting a metabolic cross-talk between the plastidic MEP pathway and cytosolic MVA pathway in tobacco and that the introduced GDS·SSU in transgenic plants increased flux towards GPP formation in plastids, thereby decreasing the IPP pool and reducing its transport to the cytosol, resulting in reduced sesquiterpene formation. Also, the transgenic plants exhibited strong chlorosis and a reduction in stature due to the reduced levels of GGPP-derived metabolites including chlorophyll, carotenoids and gibberillic acids (GAs), which suggested that the limitation of IPP due to flux redirection toward GPP formation likely plays a major role in the observed effects on GGPP-derived metabolites [31]. Similarly, transgenic tomato expressing basil geraniol synthase accumulated high amounts of monoterpenes but at the expense of a GGPP-derived metabolite, lycopene [87]. Thus, to achieve an overproduction of a compound of interest without negatively interfering with the synthesis of vital entities by metabolic engineering will be a true challenge.

Volatile terpenoid engineering studies in plants have further demonstrated the existence of 'silent metabolism', a term recently coined for the presence of occult enzymes without any apparent endogenous substrate or function [88]. For example, when *Clarkia LIS* was overexpressed in petunia, tomato and carnation, in addition to the over-accumulation of linalool, these plants also produced linalyl-glucoside [89], 8-hydroxylinalool [90] and linalool oxides [91], respectively, according to the silent metabolism present in that particular plant/tissue. Similarly, when basil geraniol synthase (GES) was overexpressed in tomato fruits in addition to geraniol, at least eleven novel metabolites accumulated in fruits, which are absent in control fruits of untransformed plants, indicating the presence of silent array of enzymes, which are likely triggered when a novel substrate is present [88]. Tobacco plants transgenic for snapdragon GDS·SSU displayed a marked increase in (*E*)-β-ocimene emission from leaves relative to controls and began to emit a new monoterpene myrcene, which was absent in control plants suggesting the presence of a silent myrcene synthase in leaves. Also, flowers of transgenic tobacco plants produced significant amount of (*E*)-β-ocimene, which was not detected in control plants, indicating that flowers may contain a silent ocimene synthase [31].

8. Conclusion

Research over the last decade has resulted in a significant improvement in our understanding of the volatile terpenoid

biosynthesis. Although characterization of most volatile terpenoid pathway genes and enzymes is carried out, still some gaps need to be filled with respect to some of the MVA pathway enzymes. Spatio-temporal expression of volatile terpenoid biosynthetic genes suggests that volatile terpenoid biosynthesis is mainly regulated at the level of transcription. It was generally accepted that GPP and monoterpenes are synthesized in plastids, whereas FPP and sesquiterpenes are produced in the cytosol, but subcellular localization and metabolic engineering studies have demonstrated that this cannot be a general rule, at least in some particular species and tissues. As an increasing number of TPSs and upstream genes are characterized from different species, many more novel aspects of the complex nature of volatile terpenoid biosynthesis will be revealed. The information thus far obtained has given new insights into volatile terpenoid metabolism and will be of great use in future efforts towards rational metabolic engineering of plant volatile terpenoids to improve traits such as plant defense, improved pollinator attraction, flavor and aroma quality and increasing the production of valuable volatile terpenoids.

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