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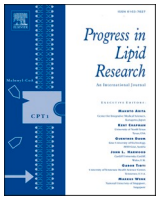

Review Article

Plant terpenoid biosynthetic network and its multiple layers of regulation

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A B S T R A C T

Terpenoids constitute one of the largest and most chemically diverse classes of primary and secondary metabolites in nature with an exceptional breadth of functional roles in plants. Biosynthesis of all terpenoids begins with the universal five-carbon building blocks, isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP), which in plants are derived from two compartmentally separated but metabolically crosstalking routes, the mevalonic acid (MVA) and methylerythritol phosphate (MEP) pathways. Here, we review the current knowledge on the terpenoid precursor pathways and highlight the critical hidden constraints as well as multiple regulatory mechanisms that coordinate and homeostatically govern carbon flux through the terpenoid biosynthetic network in plants.

1. Introduction

Terpenoids are a group of compounds that are found in all branches of life and represent one of the largest classes of metabolites in nature. In the plant kingdom they occur with exceptional diversity serving a broad range of physiological functions in key processes such as photosynthesis, respiration, growth, and development. These primary metabolic activities require terpenoids including chlorophylls, carotenoids, quinones, dolichols, sterols, and the hormones gibberellins, strigolactones, brassinosteroids, and abscisic acid. Moreover, plant terpenoids are essential for ecological processes [1–3] where mono-, sesqui-, di-, and triterpenes, as well as various terpenoid derivatives such as iridoid glycosides and terpene indole alkaloids play important roles in the beneficial and antagonistic interactions of plants with their surrounding environment. Despite the enormous structural and functional diversity of plant terpenoids, they are all derived from the universal five-carbon building blocks isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). In general, there are two pathways known for the biosynthesis of IPP and DMAPP, the mevalonic acid (MVA) pathway and the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway (Fig. 1). In most organisms only one of these pathways is present, with the MVA pathway being found in archaea and all eukaryotes except Chlorophyta [4], and the MEP pathway operating within eubacteria and plastids of protists and algae [5]. Exceptionally, both the MVA and MEP pathways are active in plants and contribute to the formation of the IPP and DMAPP precursors for terpenoids [6–8] leading to a complex regulatory network that coordinates these two pathways and the biosynthesis of downstream terpenoid products. Currently, all the enzymes and corresponding genes of the MEP and MVA pathways have been elucidated [9–11]. Furthermore, numerous enzymes involved in the metabolism of IPP and DMAPP have been described, with an emphasis on prenyltransferases [12–15] and terpene synthases [16,17]. Likewise, a large set of distinct downstream enzymes has been identified and characterized that further modify the basic terpenoid backbones contributing to the diversity of plant terpenoids. These terpenoid modifying enzymes include cytochrome P450 monooxygenases [18,19], alcohol dehydrogenases [20], double-bond reductases [21], dehydrogenases [22–24], BAHD (named after benzyl alcohol *O*-acetyltransferase, anthocyanin *O*-hydroxycinnamoyltransferase, anthranilate N-hydroxycinnamoyl/benzoyltransferase, deacetylvindoline 4-*O*-acetyltransferase) acyltransferases [25–28], serine carboxy peptidase-like acyltransferases [29], oxidoreductases [30], and glycosyltransferases [31], among others.

To date, despite the extensive knowledge on individual enzymatic steps, we are still far from a complete and comprehensive understanding of the plant terpenoid biosynthetic network. In particular little is known about the multiple regulatory mechanisms acting at the genomic, transcriptional, metabolite, and enzymatic levels. In addition, a detailed understanding of compartmentalization, transport processes, and formation of protein complexes remains elusive. Therefore, the main focus

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of this review is to outline the current knowledge on these various aspects of the plant terpenoid biosynthetic network as well as to define respective open questions that will need to be addressed by future research efforts. The urgent need for a better understanding of the underlying regulatory mechanisms has been highlighted by the outcomes of many metabolic engineering efforts to alter or improve plant terpenoid metabolism which are frequently hampered by low product yields, unexpected alternative products, or undesired phenotypes. Since plant terpenoids contribute to important agronomic traits (e.g. crop protection and fruit aromas) and are also used by humans as flavors, fragrances, preservatives, pharmaceuticals and biofuels, the emerging knowledge about the architecture and regulation of the plant terpenoid biosynthetic network will be necessary and highly valuable for improving the outcome of metabolic engineering towards producing desired terpenoids in plants.

2. The MVA and MEP pathways creating IPP/DMAPP pools

2.1. Short overview of pathways

In plants, the IPP/DMAPP-producing MVA and MEP pathways generally act independently and are compartmentally separated. The MVA pathway consists of six enzymatic reactions distributed between the cytosol and peroxisomes (Fig. 1). This pathway is initiated by a stepwise condensation of three molecules of acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which undergoes reduction to MVA followed by two subsequent phosphorylations and a decarboxylation/elimination step with formation of IPP as the final product which is then reversibly isomerized into DMAPP [32,33]. IPP and DMAPP units from the MVA pathway act as precursors for various terpenoids including sesquiterpenes, triterpenes, and sterols [32,34], dolichols [35] and, in photosynthetic tissues, ubiquinone [36,37]. It should be mentioned that out of four enzymes within the early steps of the MVA pathway, HMG-CoA reductase (HMGR), responsible for the formation of MVA, is bound to the endoplasmic reticulum (ER), which likely contributes to the functional and regulatory properties of this key enzyme [10,38,39]. Previously HMGR was considered to catalyze the rate-limiting step of the MVA pathway [9]. However, multiple metabolic engineering studies revealed that overexpression of *HMGR* alone is not always sufficient to achieve high-yields of target terpenoids in plants and thus pointed towards the presence of an additional step(s) limiting the flux in the MVA pathway [40,41]. Recently, phosphomevalonate (MVAP) kinase (PMK) catalyzing phosphorylation of the phosphomevalonate intermediate was identified as a previously unknown regulatory hub in the plant MVA pathway. It imposes a constraint on flux, and together with HMGR plays a major role in controlling flux through the MVA pathway [42]. Simultaneous transient overexpression of Arabidopsis *PMK* and *HMGR* in tobacco leaves not only increased emission of sesquiterpenes, but also that of acyclic monoterpenes suggesting that, when sufficient, IPP produced in the cytoplasm via the MVA pathway can drive plastidial terpenoid biosynthesis as well [42].

In contrast to the MVA pathway, the MEP pathway operates exclusively in plastids with seven enzymatic steps beginning with the condensation of D-glyceraldehyde 3-phosphate (GAP) and pyruvate (Pyr) to produce 1-deoxy-D-xylulose 5-phosphate (DXP) by DXP synthase (DXS) (Fig. 1). Early research identified DXS as the rate controlling enzyme for the MEP pathway in Arabidopsis chloroplasts [43]. DXP is subjected to isomerization/reduction with formation of the pathway's characteristic intermediate, MEP. Five consecutive steps are required to convert MEP to IPP and DMAPP with the final formation of IPP and DMAPP catalyzed by hydroxymethylbutenyl diphosphate (HMBPP) reductase (HDR) [44]. The MEP pathway was initially found to yield a 5:1 ratio of IPP and DMAPP [45], which was generally accepted to be conserved across different plant species. However, recent analysis of simultaneous IPP and DMAPP formation by phylogenetically distinct plant HDRs revealed that the product ratio is species-specific and can vary by up to 40-fold [46]. By changing the ratio of IPP and DMAPP substrates through HDR and IPP isomerase (IDI), plants can regulate the prenyl diphosphate product outcome by making more FPP/GGPP in the presence of an excess of IPP and favor GPP or even isoprene formation when DMAPP is in excess [46–48]. The MEP pathway derived IPP and DMAPP are most often used to produce a suite of primary metabolites such as chlorophyll, carotenoids and plastoquinone involved in photosynthesis [37,49–51] and phytohormones including gibberellins, abscisic acid, and strigolactones [52–54]. This pathway also supplies precursors for the formation of secondary metabolites such as mono- and diterpenoids commonly involved in defense [55] and communication [56,57]. Additionally, the MEP pathway has been the focus of biotechnological efforts to increase flux towards commercially valuable metabolites including either endogenous compounds such as carotenoids [58] and bioactive diterpenoids [59] or newly introduced compounds like, for example, patchoulol [40].

2.2. Classical rate limiting steps of MVA and MEP pathways

Many efforts to understand the regulation of terpenoid production have focused on the classical rate-limiting steps, catalyzed by DXS and HMGR of the MEP and MVA pathways, respectively (Fig. 1). In general, plants contain multiple copies of differentially regulated isoforms of these key enzymes. For DXS, three distinct classes have been identified that are preferentially involved in different cellular and developmental processes and exhibit distinct expression profiles [60–62]. Class 1 DXS is almost ubiquitous across the plant kingdom and primarily active in photosynthetic tissue where it typically performs a 'housekeeping' role for the biosynthesis of primary metabolites, such as photosynthetic pigments including carotenoids and chlorophylls in the chloroplast [43,63–65]. However, at least two species, *Arabidopsis halleri* and *Triticum aestivum*, apparently lack this class of DXS that might suggest the existence of distinct adaptations to maintain essential flux through the MEP pathway [66]. *A. halleri* has been proposed to use an ancestral bacterial type DXS while *T. aestivum* likely utilizes a class 2 DXS to fulfill the housekeeping role of class 1 DXSs present in other species [66].

Class 2 DXS is often associated with the production of secondary metabolites either in specialized tissues, such as glandular trichomes, flowers, and some roots, or in response to infection, herbivory and extreme temperature conditions [60,67–77]. However, this division between class 1 and 2 *DXS* expression and functions is not rigid, and varies drastically between tissue, organ, cultivar, species, and throughout growth and development [60,61,71,74,76,77]. Anomalously, the *Arabidopsis thaliana* genome appears not to contain a class 2 DXS despite the retention of this gene in both angiosperms and gymnosperms. However, there is an additional DXS-like protein with high homology to DXS1, but without DXS activity [78]. As such, class 1 DXS fulfills the roles of both class 1 and 2 DXSs in this model plant that has a characteristically low production of secondary terpenoid metabolites. Interestingly, overexpression of *Morus notabilis* class 2 *DXS* under a 35S promoter in Arabidopsis, led to an increase in chlorophyll and carotenoid levels whereas the corresponding class 1 DXS was instead involved in gibberellic acid production [79]. This partial switch between functional roles of class 1 and 2 DXS highlights the species-specific and post-transcriptional regulation of DXS enzymes towards terpenoid end products.

Class 3 of DXS is not universal across the plant kingdom and may have emerged much later than class 1 and 2 as it is so far found exclusively in dicots [60,66,80,81]. The functional role of these class 3 DXSs remains enigmatic with a proposed involvement in post-embryonic development and biosynthesis of developmentally related phytohormones [60,66,80,81]. Some members of this class lack DXS activity primarily due to the loss of the C-terminal transketolase domain which further complicates their functional characterization [61,66,74]. Tomato represents one of the species where a single copy of all three classes of DXS have clear separation in function and expression profiles. Here,

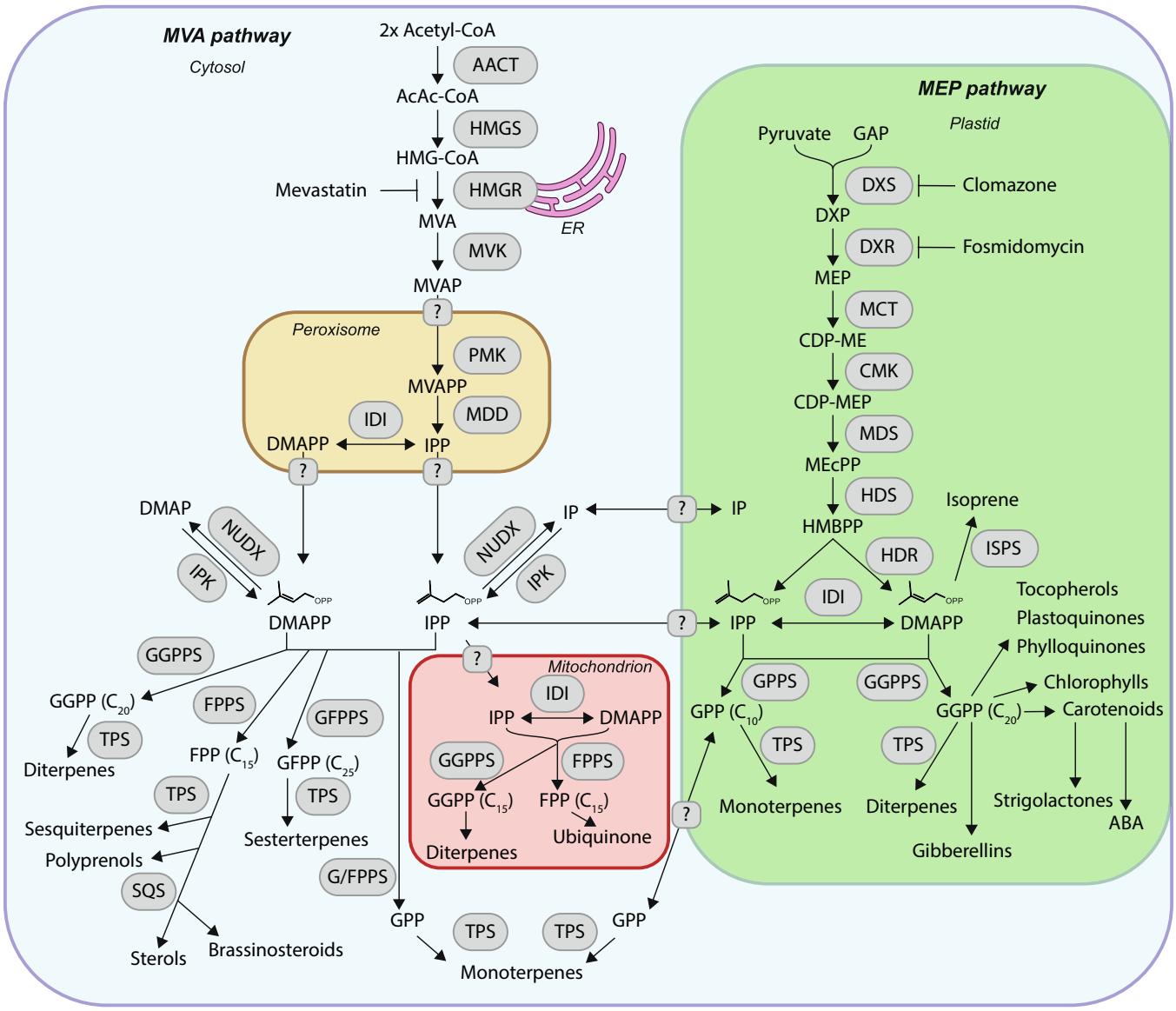

Fig. 1. Overview of isoprenoid biosynthesis in plants.
The 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway enzymes are located in the plastid and those of the mevalonic acid (MVA) pathway are distributed between the cytosol, endoplasmic reticulum (ER) and peroxisomes. Compound abbreviations are as follows: abscisic acid (ABA); acetyl Coenzyme A (Acetyl-CoA); acetoacetyl-CoA (AcAc-CoA); 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (CDP-ME), 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol phosphate (CDP-MEP); dimethylallyl phosphate (DMAP); dimethylallyl diphosphate (DMAPP); 1-deoxy-D-xylulose-5-phosphate (DXP); farnesyl diphosphate (FPP); glyceraldehyde 3-phosphate (GAP); geranylfarnesyl diphosphate (GFPP); geranylgeranyl diphosphate (GGPP); geranyl diphosphate (GPP); (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP); 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA); isopentenyl phosphate (IP); isopentenyl diphosphate (IPP); 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP); mevalonate 5-phosphate (MVAP); mevalonate 5-diphosphate (MVAPP). Enzyme abbreviations are as follows: AcAc-CoA thiolase (AACT); CDP-ME kinase (CMK); DXP reductoisomerase (DXR); DXP synthase (DXS); FPP synthase (FPPS); bifunctional geranyl/farnesyl diphosphate synthase (G/FPPS); GFPP synthase (GFPPS); GGPP synthase (GGPPS); GPP synthase (GPPS); HMBPP reductase (HDR); HMBPP synthase (HDS); HMG-CoA reductase (HMGR); HMG-CoA synthase (HMGS); IPP isomerase (IDI); IP kinase (IPK); isoprene synthase (ISPS); MEP cytidylyltransferase (MCT); mevalonate 5-diphosphate decarboxylase (MDD); MEcPP synthase (MDS); mevalonate kinase (MVK); nudix hydrolase (NUDX); phosphomevalonate kinase (PMK); squalene synthase (SQS); terpene synthase (TPS).

class 1 *DXS* shows the highest expression in leaves and ripe fruits, class 2 is expressed in flowers, and class 3 has typically low expression but the highest of the three in developing embryos [61].

Generally, overexpression of *DXS*, encoding the classical rate limiting step of the MEP pathway, increases MEP-derived terpenoid production [82–88], however, the overall effect often depends on the class of *DXS* and surrounding metabolic context. In the specialized environments of leucoplasts and terpenoid rich compartments, class 2 DXS may not be the rate-limiting enzyme catalyst, but rather contributes to a more complex regulatory network involving DXP reductoisomerase (DXR). For example, overexpression of *DXR*, but not class 2 *DXS* alone, increased essential oil yields in peppermint [89,90]. Also, *DXR* or 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP) synthase (*MDS/MECS*) overexpression increased flux towards terpenoid indole alkaloids in *Catharanthus roseus* hairy root culture [91] and a positive correlation was observed in *Amomum villosum* between volatile terpene production and expression of *DXR* as opposed to *DXS* [92]. Interestingly, in *Cassia tora*, *DXR* overexpression led to increased growth, primarily through chlorophyll production, that was not observed upon overexpression of class 1 *DXS*, indicating that DXR may fulfill the typical regulatory role of DXS in primary metabolism in some contexts [93].

Within the MVA pathway, *HMGR* exists as a multigene family which in plants encodes exclusively class 1 HMGR protein. This class, found in eukaryotes and some archaea [94,95], differs from the class 2 enzymes of eubacteria and other archaea based on the differences in the catalytic domain structure, the presence of N-terminal transmembrane domains and the NAD(P)H cofactor preference. Analysis of 56 *HMGR*s from 14 representative plant species showed that gene structure and the corresponding protein architecture are highly conserved throughout land plants [94]. Indeed, most plant HMGRs contain the two N-terminal transmembrane domains that are linked to the catalytic cytoplasmic domain. Phylogenetic analysis further revealed that higher plant *HMGR*s are derived from one ancestral gene and form four distinct groups with two encompassing all monocot *HMGR*s and two containing the dicot ones [94]. Analysis of available sequenced genomes also uncovered great variations in the number of *HMGR* genes depending on plant species. The number ranges from two copies in *Arabidopsis thaliana* and eight in soybean (*Glycine* max) [96] up to 30 copies in chicory (*Cichorium intybus* L.), 20 of which form an extraordinary cluster in a 0.6-Mb genomic region on chromosome 2 [97]. Chicory is known for its high accumulation of sesquiterpenes which occurs in roots and stems where most genes (17 of 30) are highly expressed. In many plant species, multiple *HMGR* paralogs exhibit differential expression based on tissue type and developmental stage [83,98–101]. Interestingly, in Arabidopsis which contains the simplest known HMGR family in plants, the number of differentially expressed transcripts is extended by using an alternative transcription start site that leads to an HMGR1 isoform, HMGR1L, with an extra 50 amino acids at the N-terminus [102]. The typical shorter transcripts of *HMGR1* were detected at high levels in all tissues implicating the housekeeping role of the encoded enzyme, while *HMGR1L* had a restricted distribution and likely performs a specialized role in the synthesis of specific isoprenoids in particular cell types or at specific developmental stages.

The presence of multiple *HMGR* paralogs in plant genomes greatly challenged and slowed the identification of their functional diversification. Nevertheless, it has been shown that they often exhibit different expression patterns in response to methyl jasmonate (MeJA) and methyl salicylate treatments as well as stress conditions including wounding, extensive salt, high and low temperatures, drought, and light exposure

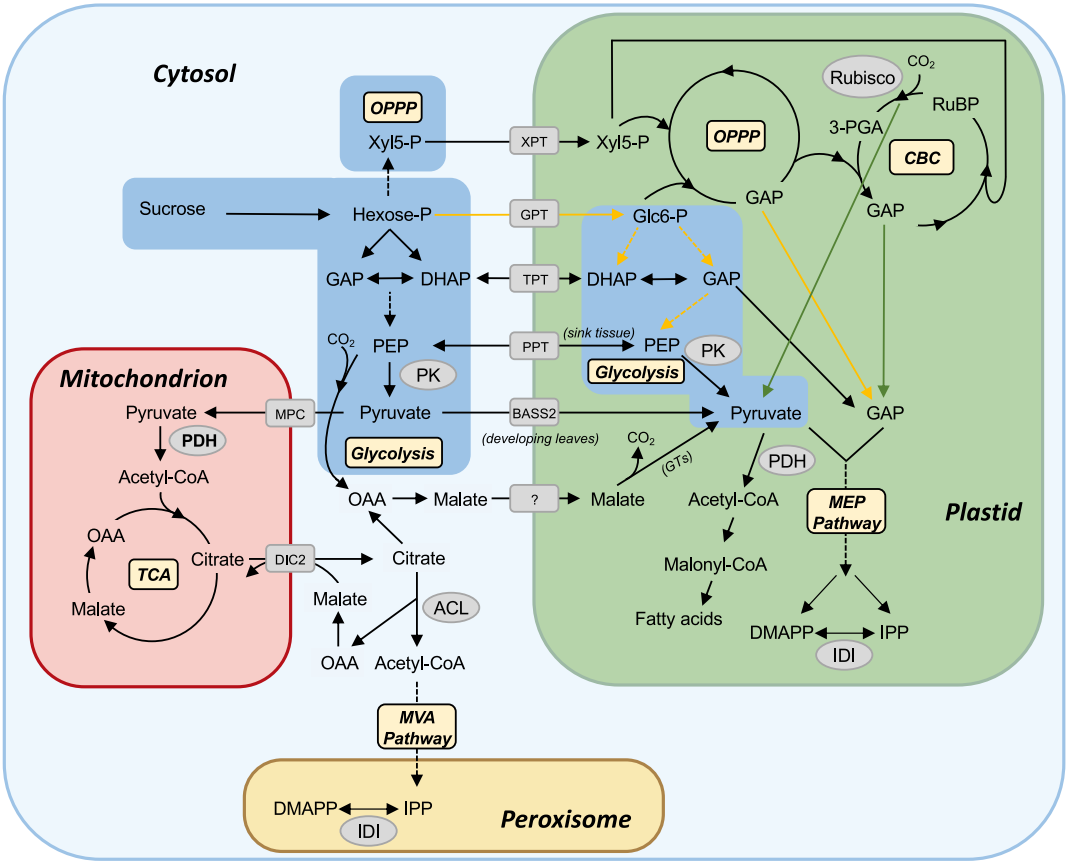

Fig. 2. Carbon supply for terpenoid production in plants.
The MVA pathway relies on cytosolic acetyl-CoA and the plastidic MEP pathway on pyruvate and D-glyceraldehyde 3-phosphate (GAP), which are provided by multiple different primary metabolic pathways depending on tissue and developmental stage. Pathways for photoautotrophic tissues are shown by green arrows, while those for non-photosynthetic tissues by yellow arrows. Cytosolic acetyl-CoA is formed by ATP citrate lyase (ACL) and precursors are provided by cytosolic glycolysis via the mitochondrial tricarboxylic acid (TCA) cycle. GAP for the MEP pathway can be supplied from the Calvin-Benson cycle (CBC), through glycolysis and the oxidative pentose phosphate pathway (OPPP) in plastids, and by import of xylulose 5-phosphate (Xyl5-P) derived from the cytosolic OPPP replenishing the CBC. Likewise, pyruvate can be supplied to the MEP pathway through a number of different routes including i) plastidic glycolysis, ii) cytosolic glycolysis combined with phosphoenolpyruvate (PEP) or pyruvate import, iii) β-elimination reaction catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), iv) import and decarboxylation of cytosolic malate. Additional enzyme, transporter and metabolite abbreviations are as follows: acetyl Coenzyme A (acetyl-CoA); bile acid:sodium symporter family protein 2 (BASS2), dihydroxyacetone phosphate (DHAP), dicarboxylate carrier 2 (DIC2); dimethylallyl diphosphate (DMAPP); glucose 6-phosphate (Glc6-P), glucose 6-phosphate/phosphate translocator (GPT); hexose phosphate (Hexose-P); isopentenyl diphosphate isomerase (IDI); isopentenyl diphosphate (IPP); mitochondrial pyruvate carrier (MPC); oxaloacetate (OAA); pyruvate dehydrogenase (PDH); 3-phosphoglycerate (3-PGA); pyruvate kinase (PK); phosphoenolpyruvate/phosphate translocator (PPT); ribulose bisphosphate (RuBP); triose phosphate/phosphate translocator (TPT); Xyl5-P/phosphate translocator (XPT). Solid arrows indicate individual enzymatic or transport steps, dashed arrows indicate pathways including multiple enzymatic steps. GTs; tomato type VI glandular trichomes.

[83,99,103] mirroring the scenario seen for *DXS*s. Direct genetic evidence for functional divergence of two Arabidopsis HMGR isoforms was obtained using T-DNA mutants. Mature *hmg-1* mutant plants displayed dwarfism, early senescence, and male sterility, whereas *hmg-2* mutant plants showed no visible morphological changes [104]. In addition, functional characterization of HMGR isoforms was performed by heterologous overexpression of their encoding genes in Arabidopsis. Overexpression of soybean *GmHMGR4* and *GmHMGR6* both increased Arabidopsis root length and total sterol and squalene levels [96]. Similarly, overexpression of the ginseng (*Panax ginseng* 'Meyer') *PgHMGR1*, but not *PgHMGR2*, resulted in Arabidopsis plants with enhanced production of sterols and triterpenes [105]. However, overexpression of apple *MdHMGR5* enhanced Arabidopsis tolerance to oxidative stress by scavenging reactive oxygen species [106]. These transgenic plants, under oxidative stress conditions, had not only enhanced activities of antioxidant enzymes, but also higher germination rate, longer primary root length and high levels of chlorophyll and proline [106]. Overall, these results uncover some specific functional roles of HMGR isoforms and show that despite catalyzing the same reaction they promote different effects depending on their origin and physiological conditions. Future systematic analysis of all HMGR isoforms in different plant species will unveil further details into their functional diversification and distinct regulatory properties in controlling flux through the MVA pathway.

3. Carbon supply for terpenoid production

While it has long been known that the MVA and MEP pathways utilize acetyl-CoA, and Pyr and GAP, respectively (Fig. 2), the details of how these pathways are supplied with primary metabolite precursors for terpenoid formation have been studied only in recent years. Acetyl-CoA is formed independently in the cytosol and several organelles including plastids, mitochondria, and peroxisomes, and currently there is no evidence of its transport across organellar membranes [107]. In plastids and mitochondria acetyl-CoA is synthesized from pyruvate by pyruvate dehydrogenase (PDH) complexes. While in plastids acetyl-CoA is primarily used for fatty acid biosynthesis, in mitochondria it is converted to citrate which enters the tricarboxylic acid (TCA) cycle. In contrast to these two organelles, cytosolic acetyl-CoA, utilized for the formation of MVA pathway derived terpenoids, is synthesized by ATP citrate lyase (ACL) from citrate (Fig. 2) [108]. This citrate originates from the mitochondrial TCA cycle that uses pyruvate imported from the cytosol by the mitochondrial pyruvate carrier (MPC) [109] followed by subsequent oxidation by the mitochondrial PDH. Citrate is also exported from mitochondria by the dicarboxylate carrier 2 (DIC2) [110] in exchange for malate derived from oxaloacetate in the cytosol, the second product of the reaction catalyzed by ACL. Antisense repression of *ACL* in Arabidopsis resulted in pleiotropic effects including a severe dwarf phenotype [111], unfortunately the effect of reduced ACL activity and cytosolic acetyl-CoA levels on terpenoid metabolism was not analyzed. In contrast, the overexpression of *ACL* in Arabidopsis led to only a weak but significant increase in sterol levels. However, tissue-specific *ACL* overexpression in laticifers of dandelion resulted in a significant increase in the rubber and triterpene content [112,113] further demonstrating that improved cytosolic acetyl-CoA supply affects flux through the MVA pathway. Recently, it has been shown that metabolism in one compartment can indirectly affect acetyl-CoA biosynthesis in another compartment despite the fact that there is no evidence of its direct exchange between subcellular organelles. Indeed, a significant increase of sesquiterpene formation was observed when an inactive form of biotin carboxyl carrier protein was expressed in tobacco, partially inhibiting the conversion of acetyl-CoA to malonyl-CoA by the acetyl-CoA carboxylase complex involved in plastidic fatty acid biosynthesis [114]. This inhibition likely leads to a build up of pyruvate in plastids due to inhibition of PDH by acetyl-CoA. Pyruvate then accumulates in the cytosol, and ultimately enters the mitochondria where it is metabolized via the TCA cycle to citrate that finally supports acetyl-CoA formation by ACL in the cytosol (Fig. 2).

In contrast to the MVA pathway, there are, depending on the plant tissue and plastid type, multiple different metabolic processes that can contribute to supplying GAP and Pyr to the MEP pathway (Fig. 2). While in chloroplasts GAP can be supplied directly from the Calvin-Benson cycle (CBC), in non-green tissues GAP is derived through glycolysis and the pentose phosphate pathway (PPP). However, both the latter pathways can also replenish intermediates of the CBC in tomato glandular trichomes [115]. Recent isotopic labeling studies [116] imply that the cytosolic oxidative pentose phosphate pathway can further contribute to this process by converting hexose phosphates to ribulose 5-phosphate and its isomer xylulose 5-phosphate, both of which can be imported into chloroplasts by the xylulose 5-phosphate/phosphate translocator (XPT) [117,118].

Likewise, the origin of the Pyr required for the MEP pathway differs depending on the developmental stage and plant tissue, and until very recently appeared so complex that, based on labeling studies, it was considered a paradox [119]. In developing embryos and various non-photosynthetic tissues, fully active glycolytic pathways operate in both the cytosol and plastids to supply pyruvate to the MEP pathway. In contrast, mature photosynthetic tissues do not have an operational plastidic glycolysis pathway due to the drastic reduction of phosphoglyceromutase (PGM) and enolase (ENO) activities, which are required for the conversion of 3-phosphoglycerate (3-PGA) to phosphoenolpyruvate (PEP), and thus another source is required to provide Pyr for the MEP pathway [120,121]. A potential alternative route is the import of PEP derived from cytosolic glycolysis into plastids, via the phosphoenolpyruvate/phosphate translocator (PPT) [122], and its subsequent conversion to Pyr by plastidic pyruvate kinase (PK) (Fig. 2). The import of PEP into plastids is considered an important source of Pyr for plastid localized metabolism such as fatty acid biosynthesis in embryos and seedlings [123–126] as well as potentially for isoprene formation [127]. However, the Arabidopsis *cue1* mutant lacking a functional PPT has normal levels of fatty acids in leaves [128] and was found to have the same Pyr concentrations as wild-type plants and unchanged DXP levels when normalized to GAP concentration [121]. As such, PPT likely provides PEP to plastid metabolism in sink tissues but does not supply precursors for the MEP pathway in photosynthetic tissue such as Arabidopsis rosette leaves. The direct import of Pyr into plastids via the pyruvate/sodium symporter BASS2 (BILE ACID:SODIUM SYMPORTER FAMILY PROTEIN 2) could represent another way of providing this MEP pathway precursor, in particular in developing leaves where its expression is significantly higher compared to mature leaves [129]. Indeed, a more severe growth retardation was observed in Arabidopsis *bass2* mutants compared to wild type upon inhibition of the MVA pathway with mevastatin, indicating that BASS2 contributes to the supply of Pyr in the chloroplasts of young developing tissues. However, further analysis of adult *bass2* mutant Arabidopsis plants revealed wild type levels of Pyr, DXP and MEcPP [121] suggesting that the import of Pyr into chloroplasts via BASS2 does not contribute to supplying this precursor to the MEP pathway in this photoautotrophic tissue. Remarkably, it has long been known that in chloroplasts ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) forms pyruvate as a minor product through β-elimination of the *aci*-carbanion intermediate [130]. It was shown that Rubisco derived Pyr accounts for ~2% of the carbon exiting the CBC [121,130]. Moreover, recent whole-plant feeding with $^{13}CO_2$ under low O_2 conditions resulted in an increase in Pyr concentration and labeling as well as enhanced flux through the MEP pathway [121]. Subsequent isotopically nonstationary metabolic flux analysis accounting for Pyr supplied by Rubisco resolved the Pyr paradox and suggested that Rubisco provides a major source of Pyr in chloroplasts, thus connecting the MEP pathway directly to photosynthetic carbon assimilation. Yet another scenario has been recently discovered in the glandular trichomes of tomato which possess chloroplasts, however, only genes of photosystems I and II were highly

expressed but not genes encoding CBC enzymes [115]. In this tissue, invertases and sucrose synthases, involved in sucrose degradation, as well as genes of the cytosolic and plastidic glycolysis pathways were also highly expressed, suggesting that these pathways supply precursors for terpenoid metabolism while photosynthesis only provides energy and reducing power. Remarkably, this study revealed that in glandular trichomes of tomato, Pyr for the MEP pathway appears to originate from the plastidic glycolysis or the plastidic NADP-malic enzyme that produces Pyr by decarboxylation of malate which is imported via a yet unknown transporter. In contrast, the monoterpene producing secretory cells of peltate glandular trichomes (PGTs) found in many species of the Lamiaceae such as spearmint, peppermint, and basil contain leucoplasts. Hence, they are not photosynthetic and must rely on a carbon source imported from the underlying tissue for all metabolic needs, including monoterpene biosynthesis. A comparative transcriptomic analysis of spearmint (*Mentha spicata*) PGTs, and leaves with and without PGTs removed [131] showed that not only the expression of terpene metabolic genes was enriched in PGTs, but also the abundance of transcripts of genes involved in the glycolysis, tricarboxylic acid cycle, and oxidative pentose phosphate pathway. While photosynthetic and chlorophyll biosynthetic gene transcripts were lacking in PGTs, transcripts for sucrose synthase, invertases, glucose 6-phosphate/phosphate translocator, and phosphoenolpyruvate/phosphate translocator were enriched in PGTs suggesting that sucrose from neighboring leaf tissue supplies carbon for metabolism in PGTs. Previous analysis of expressed sequence tags from PGTs of peppermint (*Mentha* × *piperita*) [132] and basil (*Ocimum basilicum*) [133] also had revealed a high expression of genes involved in sucrose catabolism, glycolysis, and pentose phosphate pathway.

4. Prenyltransferases and downstream branching terpenoid metabolism

IPP and DMAPP produced by the MEP and MVA pathways are used by short-chain *trans*-prenyltransferases which catalyze the head-to-tail condensation of prenyl diphosphates to form the terpenoid precursors GPP (geranyl diphosphate, C10), FPP (farnesyl diphosphate, C15), GGPP (geranylgeranyl diphosphate, C20) and GFPP (geranylfarnesyl diphosphate, C25). As IPP and DMAPP are present in multiple compartments, subcellular localization of the short-chain prenyltransferases and their corresponding products determines the specificity and in many cases initial organellar location of downstream terpenoid product biosynthesis, unless specific exchange of prenyl diphosphates between compartments occurs. For a long time, it was accepted that GPP and GGPP are synthesized in plastids from MEP-derived IPP/DMAPP by GPP synthases (GPPSs) and GGPP synthases (GGPPSs), respectively, whereas FPP is produced in the cytosol and mitochondria from MVA-derived precursors by FPP synthases (FPPSs). This "classical" view has changed in recent years with the identification of multiple subcellular localizations of GGPPSs, which reside in mitochondria and the endoplasmic reticulum (ER) in addition to their conventional localization in plastids [134,135], as well as with numerous studies suggesting the existence of a GPP pool in the cytosol. The formation of differentially targeted FPPS and GGPPS isoforms was shown to result from the use of alternative transcription start sites which generate two in-frame proteins differing only in their N-termini. The longer transcripts encode proteins that are transported into mitochondria and/or plastids, while the shorter transcripts produce cytosolically located proteins lacking the respective targeting signals [134,136,137]. Dual subcellular localization, in mitochondria and plastids, was also shown for recently identified Arabidopsis GFPPSs [138], whereas GFPPS found in glandular trichomes of the woody plant *Leucosceptrum canum* only localized to the plastid [139]. While the fate of mitochondrial GFPP in Arabidopsis remains unknown, plastidial GFPPS localization is consistent with the biochemical properties of this prenyltransferase which uses GGPP as a preferred allylic substrate and also suggests that sesterterpenoid biosynthesis relies on MEP pathway-derived precursors [139].

The existence of a cytosolic GPP pool was known for a long time from numerous metabolic engineering studies targeting normally plastid localized monoterpene synthases to the cytosol and resulting in monoterpene production [40,140–145]. Additionally, monoterpene synthases naturally targeted to the cytosol [17,140,141], as well as bifunctional cytosolic mono−/sesquiterpene synthases capable of synthesizing monoterpenes [140,143,146,147] supported the existence of a cytosolic GPP pool. However, the origin of GPP in the cytosol and how widespread this phenomenon is in the plant kingdom remained unknown. The cytosolic GPP could originate via metabolic crosstalk from the MEP pathway, as was demonstrated by increased cytosolic monoterpene production upon the overexpression of the small subunit of a heteromeric plastidic GPPS in the fruits of transgenic tomato lines expressing a bifunctional cytosolic mono−/sesquiterpene synthase [143,145]. However, early feeding experiments with pathway-specific labeled precursors in strawberry and raspberry fruits, and rose petals also revealed that the MVA pathway is the major contributor to the biosynthesis of monoterpenes and GPP-derived compounds in these plant species [148–150]. Moreover, it was shown that shikonin production in *Lithospermum erythrorhizon* roots relies on cytosolic GPPS activity [151] and the cytosolic Nudix hydrolases, which dephosphorylate prenyldiphosphates (GPP and bornyl diphosphate), are involved in geraniol formation in roses [152], pelargonium [153], and tea [154], as well as borneol and camphor biosynthesis in *Wurfbainia villosa* [155]. GPPSs capable of producing GPP in the cytosol were only recently isolated and characterized from *L. erythrorhizon* [156,157], roses [47], and pelargonium [158]. In contrast to plastidial GPPSs which evolved from GGPPSs [159], the newly discovered cytosolic GPPSs were found to be evolutionarily related to FPPSs (Fig. 3). These recent studies also revealed that the emergence of cytosolic GPPS activities from bona fide FPPSs occurred independently in phylogenetically distant members of the Rosaceae and Boraginaceae resulting in two types of enzymes, bifunctional geranyl/farnesyl diphosphate synthases (G/FPPSs), like those in roses, producing both GPP and FPP, and monofunctional enzymes with strict product specificity like the *L. erythrorhizon* GPPS [47,156,158]. Previous biochemical characterization of FPPSs from various plant species showed that many of them can produce small or trace amounts of GPP in addition to FPP in vitro [47], however, the relevance of this activity for cytosolic monoterpene formation *in planta* remains to be determined.

All plant short-chain prenyltransferases are homomeric enzymes except for GPPSs [163], which have both homomeric and heteromeric architectures depending on the plant species (Fig. 4). Homomeric GPPSs have been found in gymnosperm and some angiosperm species [164–168], while the heteromeric GPPSs have been reported only in angiosperms including *Arabidopsis thaliana*, *Mentha* × *piperita*, *Solanum lycopersicum*, *Antirrhinum majus*, *Clarkia breweri*, *Catharanthus roseus*, *Cucumis sativus*, and *Humulus lupulus* so far (Fig. 3) [17,166,169–173]. Homomeric GPPSs of a GGPPS-like group predominantly produce GPP, although bifunctional variants with combined GPPS/FPPS activities were reported in the orchid *Phalaenopsis bellina* [167] and pedunculate oak *Quercus robur* [164]. Moreover, a GPPS with high similarity to the *Q. robur* GPPS, but producing substantial amounts of FPP and GGPP in addition to GPP, was discovered in Norway spruce *Picea abies* [164].

The heteromeric GPPSs consist of a large subunit (GPPS-LSU) that is structurally related or identical to GGPPSs (Fig. 3) [171,172,174] and serves as a catalytic unit, and a small subunit (GPPS-SSU), which shares ~20% similarity with plant GGPPSs or GPPSs and acts as a regulatory unit [163]. Large subunits usually possess GGPPS activity on their own (Fig. 4) [171]. However, depending on plant species they either could be inactive, like in mint and lavender [169,175], or produce, in addition to GGPP, GPP at different ratios: ~ 1:2 (GGPP:GPP) in *C. roseus* [166] and ~ 2:1 in *S. lycopersicum* [170], along with small amounts of

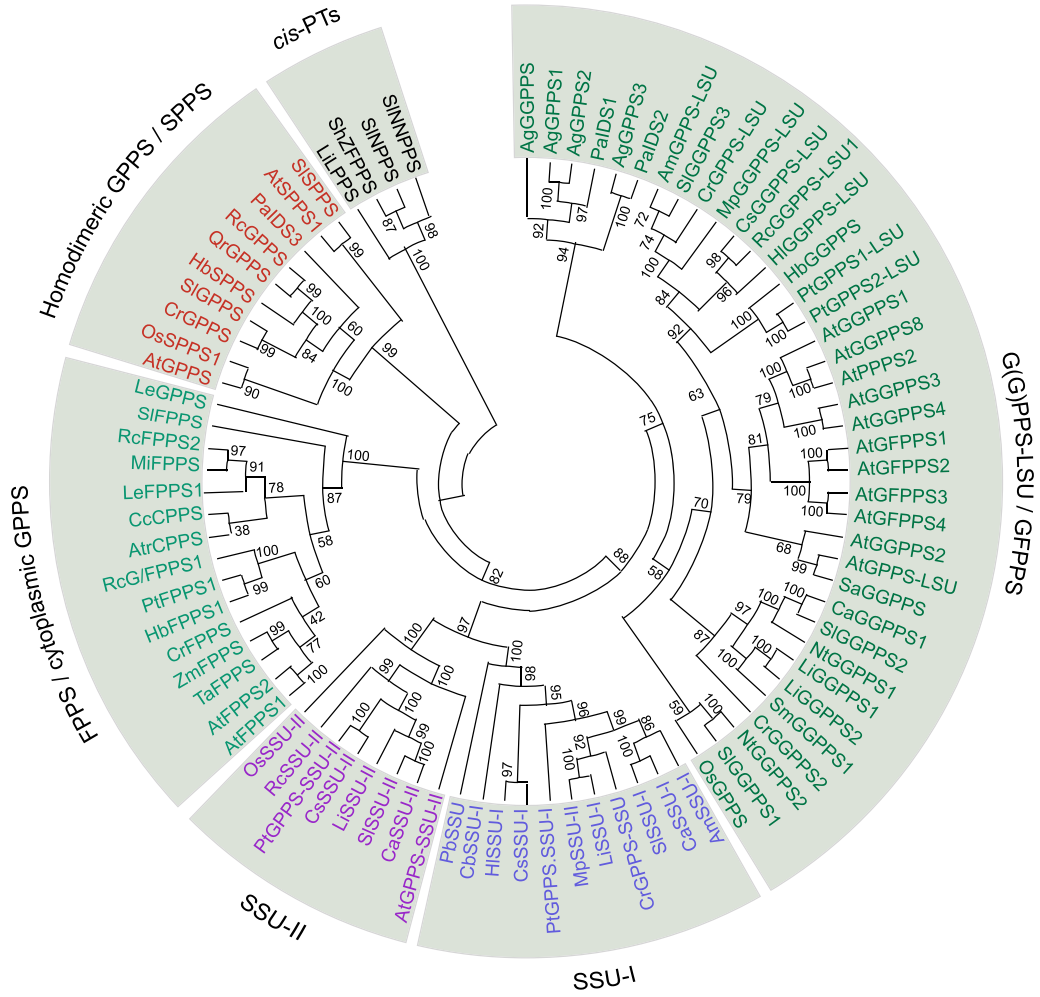

Fig. 3. Phylogenetic relationships of prenyltransferases.
Maximum likelihood tree constructed from protein sequences of biochemically characterized short-chain prenyltransferases. Numbers at tree branches correspond to bootstrap values. Protein sequences were aligned using the MUSCLE (codon) algorithm in MEGA-X [160] with the following parameters: gap open: −2.9, gap extend: 0, hydrophobicity multiplier: 1.2, clustering method: UPGMA. Next, maximum likelihood trees were reconstructed using IQ-Tree [161] implementing the Jones-Taylor-Thornton (JTT) matrix-based substitution model [162] using a discrete gamma distribution with 4 rate categories and ultrafast bootstrapping (1000 replicates). Proteins are color-coded according to their group of clustering; *cis*-prenyl transferases (*cis*-PT); farnesyl diphosphate synthases (FPPS) and cytoplasmic geranyl diphosphate synthases (GPPS); homodimeric GPPS and solanesyl diphosphate synthases (SPPS); large subunits (LSU) of hetero/homo-dimeric geranylgeranyl diphosphate synthases (GGPPS) and heterodimeric GPPS and geranylfarnesyl diphosphate synthases (GFPPS); small subunits I (SSU-I) and II (SSU-II). The first letters of each enzyme refers to the species: *Abies grandis* (Ag); *Antirrhinum majus* (Am); *Arabidopsis thaliana* (At); *Artemisia tridentata* (Atr); *Capsicum annuum* (Ca); *Clarkia breweri* (Cb); *Chrysanthemum cinerariaefolium* (Cc); *Catharanthus roseus* (Cr); *Cucumis sativus* (Cs); *Hevea brasiliensis* (Hb); *Humulus lupulus* (Hl); *Lithospermum erythrorhizon* (Le); *Lavandula* × *intermedia* (Li); *Mangifera indica* (Mi); *Mentha* × *piperita* (Mp); *Nicotiana tabacum* (Nt); *Oryza sativa* (Os); *Picea abies* (Pa); *Phalaenopsis bellina* (Pb); *Populus trichocarpa* (Pt); *Quercus robur* (Qr); *Rosa chinensis* (Rc); *Sinapis alba* (Sa); *Solanum habrochaites* (Sh); *Solanum lycopersicum* (Sl); *Salvia miltiorrhiza* (Sm); *Triticum aestivum* (Ta); *Zea mays* (Zm).

FPP [17,172]. In contrast, small subunits are generally catalytically inactive as they lack either the first aspartate rich motif (FARM), or both the FARM and second aspartate rich motif (SARM) in SSU-I and SSU-II, respectively. These motifs are essential for catalytic function and binding of prenyl diphosphate substrates by *trans*-prenyltransferases [12,176,177]. Despite the lack of catalytic activity, the small subunits possess two conserved CxxxC motifs (where x is any hydrophobic amino acid) shown to play a critical role in physical interactions between large and small subunits [172].

To date, only two types of SSUs, SSU-I and SSU-II, have been identified (Figs. 3 and 4). It appears that SSU-Is, upon interaction with their corresponding large subunits, change the product specificity of the latter towards GPP formation [17,166,169,171–174,178], whereas SSU-IIs enhance the activity and product specificity of GGPPSs [17,172], or facilitate GGPP channeling to specific downstream metabolic branches, such as the biosynthesis of chlorophylls and carotenoids [179,180]. Analysis of the *SSU-I* and *SSU-II* expression in different plant species revealed that *SSU-I* expression is restricted to specific tissues rich in monoterpenes [171–173], while *SSU-IIs* are largely constitutively expressed [17,179]. Unlike the angiosperm-specific SSU-Is, SSU-IIs are widely spread across seed plants [159,177], suggesting an essential function(s) of SSU-II in fundamental processes including, and likely not limited to, promoting and channeling GGPPSs for the biosynthesis of primary metabolites [179,180].

Until recently, *trans*-prenyl diphosphates were considered as the exclusive precursors for the formation of plant terpenoids except for some long-chain polyisoprenoids like rubber and dolichols. However, several short-chain *cis*-prenyltransferases have been characterized in plants additionally increasing the diversity of secondary terpenoid products (Fig. 3). These enzymes catalyze the head-to-tail condensation

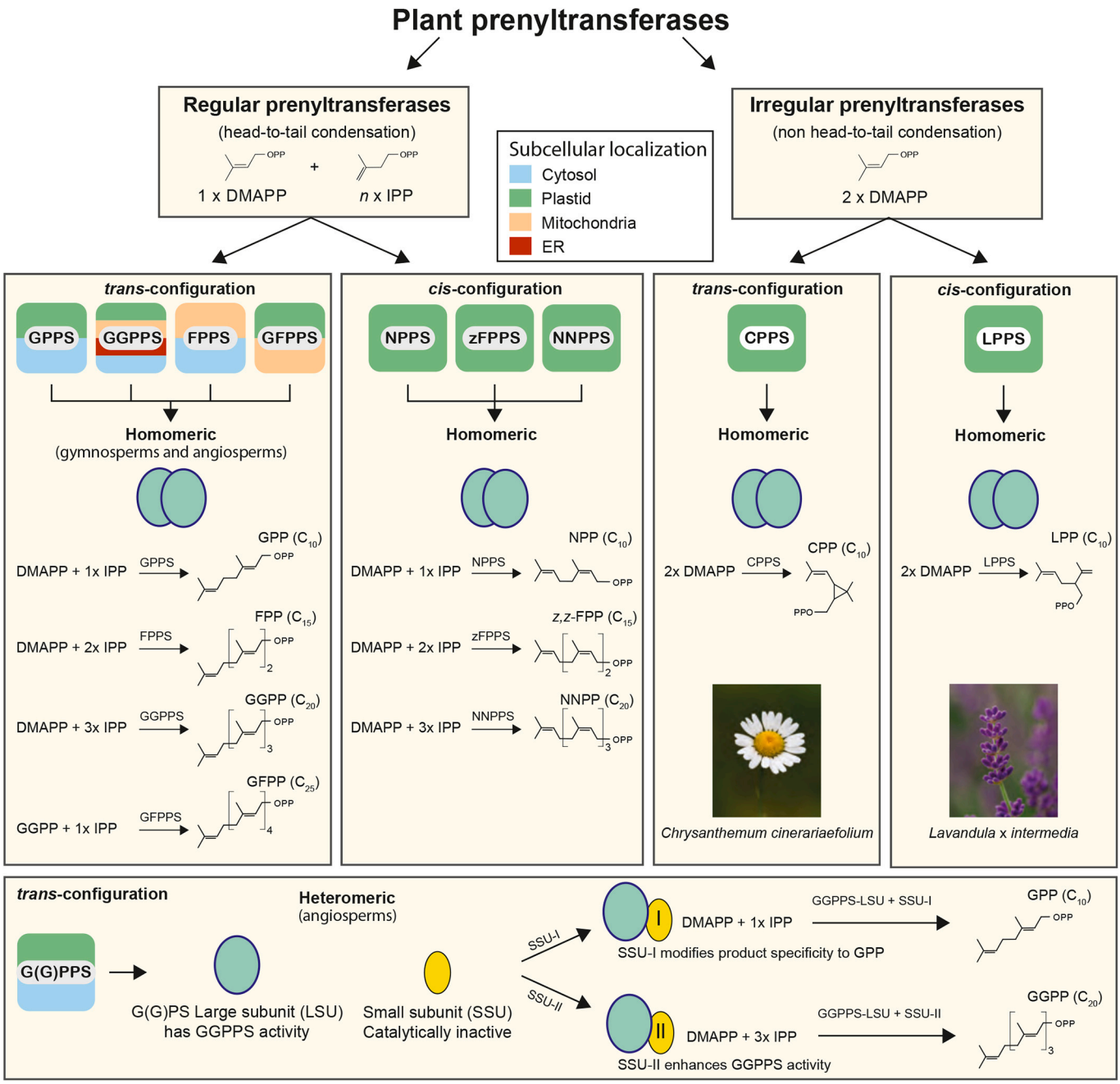

Fig. 4. Overview of subcellular localization and architecture of prenyl transferases in plants and stereochemistry of their products.
Short-chain prenyltransferases are subdivided by their mechanism of IPP/DMAPP condensation (i.e. head-to-tail and non head-to-tail) and stereochemical configuration of their product(s) (i.e. *trans* and *cis*). The colored boxes indicate the reported subcellular localization of prenyltransferases. Abbreviations: isopentenyl diphosphate (IPP); dimethylallyl diphosphate (DMAPP); geranyl diphosphate (GPP); farnesyl diphosphate (FPP); geranylgeranyl diphosphate (GGPP); geranylfarnesyl diphosphate (GFPP); neryl diphosphate (NPP); nerylneryl diphosphate (NNPP); *cis*,*cis*-farnesyl diphosphate (*z*,*z*-FPP); chrysanthemyl diphosphate (CPP); lavandulyl diphosphate (LPP); GPP synthases (GPPS); GGPP synthase (GGPPS); FPP synthase (FPPS); GFPP synthase (GFPPS); NPP synthase (NPPS); NNPP synthase (NNPPS); *z*,*z*-FPP synthase (zFPPS); LPP synthase (LPPS); CPP synthase (CPPS); G(G)PPS large subunit (LSU); G(G)PPS small subunit (SSU).

of IPP and DMAPP in the *cis* conformation to form neryl diphosphate (NPP, the *cis* isomer of GPP), *cis*,*cis*-FPP (*z*,*z*-FPP) and nerylneryl diphosphate (NNPP, the all-*cis* isomer of GGPP) (Fig. 4) [15,181]. Although *cis*- and *trans*-prenyltransferases use similar allylic and homoallylic substrates, the former appeared to be evolutionarily unrelated to *trans*-prenyltransferases and, independent of the size of product formed, are all localized in the plastids. Consistent with the discovery of *cis*-prenyltransferases, a limited number of terpene synthases, which use almost exclusively these *cisoid* substrates, were reported in plants [182–185]. While these terpene synthases were found predominantly in *Solanum* species, where they are mainly expressed in leaf glandular trichomes, there is increasing data to suggest that they also exist in other plant species including soybean leaves [186] and glandular trichomes of the *Eremophila* species endemic to Australia [187]. To date, terpene synthases have been characterized systematically from only a small number of plant species relative to the predicted abundance of these enzymes. However, in the limited cases where both *transoid* and *cisoid* isomers of prenyl diphosphates were tested in enzyme assays, it was found that some terpene synthases could exhibit substrate promiscuity and use both substrates [17,188–190]. These findings raised several

questions about i) how broadly *cisoid* substrates are distributed across the plant kingdom as well as various organs and tissues, ii) how often terpene synthases utilize these substrates, and iii) if a correlation exists between the presence of *cisoid* substrates and substrate stereo-selectivity of expressed terpene synthases in specific cells.

Another interesting aspect of terpenoid metabolism is the inhibitory effect of NPP on GGPPS activity which was uncovered by overexpressing *NPP synthase* in tomato fruits which naturally lack NPP [191]. Further biochemical analysis showed that NPP competitively inhibited GGPPS with respect to DMAPP and non-competitively with respect to IPP. With this discovery the question arises if and how plants accommodate this and potentially other *cisoid* compounds in the plastids with high GGPPS activity, which is essential for chlorophyll and carotenoid biosynthesis. These data also shed light on why NPP synthases were predominantly found in the glandular trichomes of tomato leaves and stems [15,182], as such localization helps protect photosynthetic source tissue from accumulated NPP. In soybean leaves, the only currently known case where both NPP- and GGPP-derived terpenoids are produced at high levels in a single organ, it is possible that NPP is channeled to the corresponding monoterpene synthase which was shown to have a strict substrate specificity towards NPP [186]. This would allow the utilization of a *cisoid* substrate without its release to the plastids thus negating potential inhibition of GGPPS and the associated deleterious effects on primary metabolism. Alternatively, soybean GGPPS(s) could be potentially insensitive to NPP inhibition which remains to be analyzed.

In addition to short-chain prenyltransferases catalyzing the head-to-tail condensation of IPP and DMAPP, irregular prenyltransferases responsible for the non-head-to-tail condensation of five-carbon building blocks were also isolated from a few plant species (Figs. 3 and 4). This list includes *Lavandula* × *intermedia* lavandulyl diphosphate synthase (LPPS) which catalyzes the head-to-middle condensation of two DMAPP molecules resulting in the formation of lavandulyl diphosphate, the precursor of the monoterpene alcohol lavandulol [192], and *Chrysanthemum cinerariaefolium* chrysanthemyl diphosphate synthase (named CPPS or CDS), which catalyzes irregular c1′-2–3 linkage of two DMAPP molecules producing *trans*-chrysanthemyl diphosphate, the precursor of pyrethrins, the most widely used plant-derived pesticide [193]. Both irregular C10 prenyltransferases are highly expressed in flowers and are targeted to plastids to provide substrates for the biosynthesis of irregular monoterpenes. While LPPS is closely related to *cis*-prenyltransferases, CPPS/CDS groups with the *trans*-prenyltransferase family and is structurally and evolutionarily related to FPPSs [194] (Fig. 3). Evolvement of CPPS from FPPSs required not only a shift in size of the product, but also a change in enzyme activity from chain elongation to cyclopropanation. Interestingly, a CPPS/CDS (FDS-5) with unusual activity has been isolated from *Artemisia tridentata* yielding the two irregular monoterpenoid precursors CPP and LPP from DMAPP, in addition to GPP from IPP and DMAPP [195]. This CPPS/CDS is an example of a promiscuous and relatively inefficient enzyme that recently evolved from a highly specialized FPPS parent likely by gene duplication and random mutagenesis [194]. Stepwise domain swapping between the authentic FPPS and CPPS from *A. tridentata* revealed that this new irregular prenyltransferase activity required numerous mutations throughout the protein beyond the active site [194]. While plant genomes in general contain multiple FPPS encoding genes, only comparatively few FPPSs were characterized in detail. Currently it is difficult to predict how many FPPSs are in a transition state towards developing irregular activities which could render them beneficial for plant defense and thus promote retention of multiple FPPS isoforms with different product specificities.

5. The roles of compartmentalization and transport in the regulation of terpenoid biosynthesis

Compared to other organisms, plants are distinctive because they possess both the MVA and MEP pathways. Despite the compartmental separation of these two terpenoid precursor pathways, some metabolic crosstalk between them has been discovered, most notably through the IPP/DMAPP pools and some downstream prenyl diphosphate products. While the entirety of the MEP pathway takes place in the plastid [196–200], the localization of MVA pathway enzymes is more complex (Fig. 1). The first step of the MVA pathway, the condensation of two molecules of acetyl-CoA, is catalyzed by acetoacetyl-CoA thiolase (AACT). While the Arabidopsis genome encodes two AACTs, AACT1 (At5g47720) and AACT2 (At5g48230), further functional characterization revealed that only AACT2 is involved in the MVA pathway [201,202]. Subcellular localization studies utilizing green fluorescent protein (GFP) fusion proteins demonstrated that AACT1 is located in peroxisomes while AACT2 is localized in the cytosol [201,203]. Moreover, a proteomics analysis of Arabidopsis leaf peroxisomes also identified AACT2 suggesting a potential dual localization of this MVA pathway enzyme [204,205]. Two AACT homologs were also identified in tobacco, and analysis of their subcellular localization demonstrated that one, NtAACT1, is targeted towards peroxisomes by its C-terminal PTS-1 signal, while the second, NtAACT2, is cytosolic and involved in the MVA pathway [206]. In addition, a recent immunogold electron microscopy study of terpenoid biosynthesis in laticifer cells of *Euphorbia helioscopia* found that its AACT is mainly localized in the ER, vacuoles originating from the ER, and the cytosol around these vacuoles [207]. These results suggest that, in some plants, AACT may operate in multiple subcellular compartments, though its functional role in these compartments remains unclear. The subcellular localization of the second enzyme of the MVA pathway, HMG-CoA synthase (HMGS), has only been studied in a very limited number of plant species. While putative peroxisome targeting PTS-2 sequences were identified in all *Brassica juncacea* HMGS isoforms, GFP localization studies demonstrated that *B. juncacea* HMGS1 and *Cornus officinalis* HMGS are cytosolic enzymes [208,209]. Remarkably, in red algae, HMGS activity was demonstrated by cytochemical and electron microscopy analyses to be localized within vacuolar-type organelles named mevalonosomes [210].

HMGR, the next enzyme of the MVA pathway, has a catalytic domain that is exposed to the cytosol and two N-terminal hydrophobic transmembrane regions that were initially found to integrate into ER membranes [211,212]. However, further detailed analysis revealed that the enzyme is also found in spherical vesicular structures within the cytosol and the central vacuole [213]. Additionally, GFP-fusion constructs carrying the membrane domains of two tobacco HMGRs also showed differential subcellular localization with one HMGR isoform being targeted to the ER, while the second isoform was found in globular structures that appear to be connected to actin filaments [214]. Likewise, ginseng (*Panax ginseng*) HMGR, fused with cyan fluorescent protein (CFP) or red fluorescent protein (RFP) at its C-terminus, was found in spherical vesicles upon expression in Arabidopsis and co-localized with ER, plastid, and peroxisome markers [105]. Remarkably, recent studies have demonstrated the capacity of the membrane domain of plant HMGRs to induce the proliferation of ER membranes and formation of organized smooth ER (OSER) structures [215,216] which were found to be highly dynamic in their formation and disassembly and contain high levels of endogenous HMGR. These OSER structures consist of symmetrically stacked ER membrane arrays [217] that appear to be directly or indirectly involved in biosynthetic and secretory functions, and develop naturally in specific tissues.

In contrast to HMGR very little is known about the subcellular localization of the three last enzymes of the MVA pathway in plants, mevalonate kinase (MVK), PMK, and mevalonate diphosphate (MVAPP) decarboxylase (MDD). Remarkably, putative peroxisome targeting PTS-2 sequences, usually located internally or near the N-terminus, were found in all these enzymes from Arabidopsis, *Catharanthus roseus*, and *Salvia miltiorrhiza* [218–220]. Since the presence of PTS-2 signals is not always sufficient for peroxisomal targeting, fusion constructs with yellow fluorescent protein (YFP) were utilized to verify the subcellular localization of these MVA pathway enzymes from Arabidopsis and

C. roseus. While the MVK-YFP fusion protein was found in the cytosol, PMK-YFP and MDD-YFP primarily localized in peroxisomes and only a small quantity remained in the cytosol [219]. However, immunogold electron microscopy studies with anti-MDD antibodies revealed that MDD in laticifers of *E. helioscopia* is localized in the ER, small ER derived vacuoles, and cytoplasm [221].

Interconversion of IPP and DMAPP, catalyzed by IDI, favors the formation of different chain length prenyl diphosphates by prenyltransferases based on the ratio of these precursors. Two genes, *IDI1* and *IDI2*, are present in the Arabidopsis genome each encoding an IDI precursor protein with an N-terminal extension that targets the proteins to plastids and mitochondria respectively [218,222,223]. In addition, shorter transcripts have been identified for both Arabidopsis *IDI* genes encoding IDI proteins that lack these N-terminal extensions [222,223]. Both short versions of these IDIs carry a C-terminal PTS-1 peroxisomal-targeting motif and the respective GFP fusion proteins were indeed shown to be localized in peroxisomes [218]. Two *IDI* genes have also been identified in tobacco and tomato [224,225]. The IDI1 homologs in these species both carry N-terminal extensions that appear to function in plastid targeting based on the plastidial localization of a tobacco IDI1-GFP fusion construct [224] and the reduction of plastidial carotenoid accumulation in the tomato *fcd1* mutants lacking a functional IDI1 [225]. Both IDIs in tobacco and tomato also carry a C-terminal HKL amino acid sequence like the Arabidopsis IDIs, but it remains to be determined if these putative PTS-1 motifs result in peroxisomal targeting. The rice genome was found to encode two IDI isoforms (OsIDI1 and OsIDI2) as well, and bioinformatic analysis predicted the presence of N-terminal organellar targeting sequences and peroxisomal-targeting motifs in both. Further subcellular localization studies for the rice IDIs utilizing GFP fusion proteins and immuno-electron microscopy revealed that both OsIDIs are localized in mitochondria, peroxisomes, and remarkably also the ER, while only OsIDI2 was found in chloroplasts [226]. In contrast to other plant species, only a single *IDI* gene is present in the *Catharanthus roseus* genome that produces long and short transcripts giving rise to corresponding proteins with and without an N-terminal transit peptide, respectively [227]. Utilizing C-terminal GFP fusion constructs the long IDI isoform was shown to be targeted to both plastids and mitochondria, while internal YFP fusion constructs revealed that the short isoform is targeted to peroxisomes in agreement with the presence of a PTS-1 motif at its C-terminus. Unless the C-terminal peroxisomal targeting sequence was blocked, IDI isoforms appear to be located in peroxisomes, mitochondria, and plastids, but not the cytosol in plants (Fig. 1). [218,222–224]. Further systematic analysis of IDIs throughout the plant kingdom will reveal if this protein exists in the cytosol. The recently discovered isopentenyl phosphate kinase (IPK) however, localizes to the cytosol and contributes to the formation of IPP and DMAPP [228], and together with peroxisomal IDI and IPP/DMAPP transporter(s), as well as crosstalk with the plastidial MEP-derived pools, regulates the availability of these building blocks for multiple downstream terpenoid biosynthetic pathways (Fig. 1).

As mentioned earlier (see section 4), the "classical" model that GPP and GGPP are exclusively synthesized by respective prenyltransferases in plastids had to be revised based on the recent discovery of GPPSs that are located in the cytosol, and the identification of GGPPSs in multiple additional subcellular locations including the cytosol, endoplasmic reticulum and mitochondria [134,135,137]. Likewise, FPPS homologs have been identified and characterized from numerous different plant species and it has become obvious that many plants contain at least two isoforms of this prenyltransferase involved in different functions ([229–231]; and summarized in [232]). In some plants, all isoforms are localized in the cytosol [195,233], while in other plants FPPS isoforms were found to be targeted to multiple different subcellular compartments. In the Arabidopsis genome two *FPPS* genes, *FPS1* and *FPS2*, were identified [136,234]. Remarkably, two different transcripts are derived from *FPS1* through the use of alternative transcription start sites that subsequently result in the formation of two different isoforms, FPPS1S and FPPS1L. The FPPS1L protein contains an N-terminal extension that was found to target this isoform into mitochondria [136,235] where it provides precursors for the formation of terpenoid compounds such as ubiquinone and heme A. In contrast, the Arabidopsis FPPS1S and FPPS2 isoforms both lack an N-terminal extension and were shown to be localized in the cytosol (Fig. 1) [236]. A similar situation was recently observed in cultivated and wild *Solanum* species that contain three transcribed *FPPS* genes [237]. While two of these tomato isoforms, FPPS1 and FPPS3, do not carry an N-terminal extension and a cytosolic localization was demonstrated for the FPPS1 protein [17], the FPPS2 protein has an N-terminal extension that is predicted to be a mitochondrial targeting sequence [237]. In contrast, the short FPPS in *C. roseus* was found to primarily localize to the peroxisome, where it co-localizes with the final steps of the MVA pathway, while being only partially retained in the cytosol [219,227,238].

Although the synthesis of IPP and DMAPP occurs in separated cellular compartments, the metabolic crosstalk between the MVA and MEP pathways has been shown in multiple plant species (Fig. 1) [239–242]. Such connectivity of the isoprenoid biosynthetic pathways allows the MEP pathway, often with a higher carbon flux than the MVA route, to support biosynthesis of terpenoids in the cytosol [243–245]. The directional contribution and the magnitude of crosstalk between these pathways in terpenoid biosynthesis is species- and/or organ-specific [145,246,247] and depends on their developmental stage [40,47,243,248]. Indeed, in snapdragon, the MEP pathway provides precursors for cytosolic sesquiterpene formation [244] whereas in rose flowers, substantial import of MVA pathway-derived isoprenoid intermediates occurs from the cytosol to plastids thus contributing, along with the MEP pathway, to β-ionone biosynthesis [47]. Additionally, in cotton seedlings, both pathways make substantial contributions to isoprenoid formation with crosstalk between the pathways occurring at different rates in various organs [249]. Both pathways contributed to squalene biosynthesis in leaves and cotyledons, as determined by feeding isotopically labeled MEP and MVA pathway precursors, while exclusively the MVA pathway supported squalene biosynthesis in roots.

Currently, no transporter of isoprenoid precursors across the inner envelope membrane of plastids has been identified. However, two independent studies have provided biochemical evidence for the existence of IPP, DMAPP, GPP and FPP, transport over the (inner) plastidial membrane [250,251], although the gene encoding such a transporter remains unknown. To date there is no agreement on the mechanism of translocation. Bick and Lange (2003) [250] proposed a Ca^{2+} gated IPP/proton symporter while Flügge and Gao (2005) [251] suggested that IPP is transported by a uniporter that depends on the presence of other phosphorylated compounds or inorganic phosphate. In addition to the exchange between the cytosol and plastids, movement of IPP and DMAPP out of peroxisomes, as well as their import into mitochondria will undoubtedly require transporters, the natures of which remain unknown. Moreover, how MVAP is imported into peroxisomes is also elusive, further highlighting the need to identify the transporters involved in terpenoid biosynthesis and intermediate exchange.

6. Regulation of MEP and MVA pathway fluxes towards terpenoid production

The MEP and MVA pathways are known to be subjected to a "multi-layered" form of regulation allowing plants to (1) balance the allocation of carbon resources, energy and reducing power, (2) fine-tune the synthesis of specific spectra of isoprenoids, and (3) maintain appropriate levels of key intermediates and end products. Overaccumulation of terpenes often leads to auto-toxic effects arising from membrane disruption and impairment of cellular functions. In general, the metabolic flux through the MEP and MVA pathways varies substantially depending on plant species, organ, tissue, and developmental stage, as well as biotic and abiotic environmental conditions, demanding the proper allocation of carbon and multiple levels of regulation.

6.1. Transcriptional regulation and coordination of the MVA and MEP pathways

The MEP and MVA pathways are compartmentally separated, enabling efficient production of terpenoid end products that are essential for development and adaptation to various types of stresses. As a result, the pathways are particularly active at different developmental stages with the MVA pathway operating early in plant and organ development when sterol biosynthesis is required for cell division and expansion [10]. Conversely, the MEP pathway is most active in photosynthetic tissue and some ripening fruits where pigments derived from GGPP accumulate [10,252]. Interestingly, both pathways are induced by MeJA and herbivory to favor production of secondary terpenoid products for defense, often mediated by transcription factors (TFs) [72,83,92,253–256]. The distinct contributions of the MEP and MVA pathways to terpenoid biosynthesis requires efficient coordination and orchestrated cooperation, the regulatory mechanisms of which remain largely unknown.

Light is an essential environmental signal which inversely affects both the MEP and MVA pathways. It transcriptionally upregulates expression of most MEP pathway genes, and also affects the level of GAP availability for carbon flux through the pathway [257,258]. In contrast, light has an opposite effect on the transcription of MVA pathway genes which are highly expressed in the dark through a complex regulatory network involving multiple TFs [258]. In Arabidopsis aerial tissues, transcription of MEP pathway genes generally correlates with the 'morning loop' core circadian clock genes and exhibits negative correlation with genes of the MVA pathway and the 'evening loop' [259]. The two pathways retain this inverse expression profile also during deetiolation of rice leaves. Indeed, the expression of MVA pathway genes remains highest in the dark and starts to decrease 4 h post illumination when a simultaneous increase in expression of MEP pathway genes occurs, demonstrating a balanced regulation of these two compartmentally separated pathways [260]. Interestingly, consistent with the different functions of DXS proteins (see section 2.2 above), class 2 and 3 *DXS*s were co-expressed with the MVA pathway genes, while class 1 *DXS* was co-expressed with the MEP pathway genes. It has been shown that light induces expression of MEP pathway genes via the light-dependent degradation of transcriptional repressors, namely Phytochrome Interacting Factors (PIFs), sometimes in conjunction with the transcription factor long hypocotyl 5 (*HY5*) which activates expression of class 1 *DXS* and *DXR* [261]. Also, light-dependent degradation of PIF3 induces the synthesis of the MEP pathway derived constituent of α-tocopherol (vitamin E) in tomato fruit [262]. Despite, the fixed negative correlation between the expression of the MEP and MVA pathway genes, their diel separation is flexible, and examples exist wherein the MEP pathway is upregulated in the dark, and MVA pathway in the light [83,259,260,263]. Moreover, analysis of the 5′ regions of the MEP and MVA pathway genes revealed that *MDD* and *DXS* contain putative *cis*-acting elements responsive to light, hormones, and various stresses [76,264].

In addition to light, numerous other factors were found to transcriptionally regulate the MEP and MVA pathway genes and increase production of corresponding terpenoids in response to UV [265,266], temperature [267], salt stress [268], drought [72], and abscisic acid and salicylic acid [253]. The presence of multiple gene copies that are subject to regulation by different factors allows the plant to adapt to various environmental conditions as exemplified by the *DXS* gene family. Class 2 *DXS* is upregulated in response to MeJA and darkness, and downregulated by drought, while class 1 *DXS* is upregulated in the light, consistent with their roles in secondary (linalool and (*E*)-β-ocimene) and primary (chlorophyll and carotenoids) metabolism, respectively [72]. Such a distinction may be important for the plant response to biotic stresses as well, enabling a shift in carbon budget towards secondary defense compounds when facing herbivory, while maximizing growth in the absence of biotic stress thus allowing for efficient plant growth-defense trade-offs. In addition to direct coordination of most genes within either the MEP or MVA pathway, the biosynthesis of downstream terpenoids is also often linked with these precursor pathways through transcriptional regulation [269,270]. Indeed, in Arabidopsis, GGPPS11, which is the only highly expressed GGPPS in green tissues, is co-expressed with both the upstream MEP pathway, and the downstream photosynthesis-related isoprenoid biosynthetic pathway [271], thus allowing high flux specifically towards essential photosynthetic pigments. Moreover, further direction of carbon flux from the MEP and MVA pathways towards final products occurs through transcriptional regulation of prenyltransferases and terpene synthases by a suite of TFs including members of NAC (no apical meristem (NAM)/Arabidopsis transcription activation factor (ATAF)1,2/cup-shaped cotyledon (CUC2)), AP2/ERF (APETALA2/Ethylene Responsive Factor), bHLH (basic-helix-loop-helix), bZIP (basic leucine zipper), MYB (v-myb avian myeloblastosis viral oncogene homolog), and WRKY families [272–278].

6.2. Regulation at the genetic level: Co-expression and clustering of pathway genes

Several genes of the MEP and MVA pathway were found to be co-expressed and form independent gene modules. This is achieved through the presence of common *cis*-regulatory elements in genes of the individual pathways without overlapping elements between them. The presence of common *cis*-acting elements allows a single TF or set of regulatory proteins to modulate and coordinate expression of the entire module in response to various cues [10]. Beyond the terpenoid precursor pathways, genes encoding downstream steps of terpenoid metabolism such as various prenyltransferases and carotenoid biosynthetic genes [269,279] are often found to be included in these co-expression gene networks. By analyzing transcriptome data, co-expression networks can be used to infer potential membership of a gene of interest within a biosynthetic pathway, as exemplified by the co-expression of Arabidopsis *IPK* within a gene module containing MVA pathway, *FPPS*, and sterol biosynthetic genes [228]. Similarly, co-expression analysis can be used to predict new modules of genes belonging to a common biosynthetic network as shown for the gene module containing (−)- and (+)-linalool synthases along with respective CYP450s involved in the hydroxylation and epoxidation of linalool in Arabidopsis flowers [280]. The ever increasing availability of genome and transcriptome data from various plants and conditions, along with advanced gene-expression databases and analysis tools [281] provides a foundation for accelerated prediction and deciphering of the remaining pieces of the terpenoid network throughout the plant kingdom.

Metabolic gene clusters typically contain genes of the same pathways in close proximity on a chromosome, and in plant genomes they can serve to stabilize pathways over generations while also providing a mechanism for coordinating transcriptional regulation [282]. Such gene clusters have been identified for specialized metabolic processes and have also been identified across long chromosomal distances or even on distinct chromosomes in 'loose' or 'split' gene clusters, but in close physical proximity through three-dimensional chromosomal remodeling [283–287]. Due to the typical random dispersion of MEP and MVA pathway, as well as prenyltransferase genes throughout plant genomes, such chromosome-level topological changes could enable the co-regulation of these spatially distal pathway genes in response to different conditions but this still requires experimental validation [10,288–291]. Since a steadily growing number of biosynthetic gene clusters has also been identified for terpenoid metabolism [287] here we will only focus on a select number of examples to highlight some of their features. Several terpenoid biosynthetic gene clusters have been identified in the tomato (*S. lycopersicum*) genome [17] including one on chromosome 8 that includes two *cis*-prenyltransferase genes (*CPT1*, *CPT2*), five TPS genes (*TPS18*, *TPS19*, *TPS20*, *TPS21*, *TPS41*) and two cytochrome P450 oxidoreductase genes. Since TPS19 and TPS20 were

found to utilize the CPT1 product NPP and likewise TPS18 and TPS21 utilize the CPT2 product NNPP, the co-expression of these clustered genes potentially avoids inhibitory effects of these *cis*-prenyl diphosphates on other parts of terpenoid metabolism. Similar to this tomato gene cluster encoding enzymes that produce monoterpenes for defense against insect herbivores [292,293], genes encoding enzymes involved in the production of diterpene phytoalexins in response to pathogen infections have been found in biosynthetic gene clusters [287,294,295]. Additional clusters have been implicated in triterpene biosynthesis in multiple plants species [287], most interestingly in the form of split gene clusters that control the biosynthesis of specialized triterpenes called cucurbitacins in cucumber [296]. These split clusters are found across three distinct chromosomes regulated by the same two transcription factors.

Although biosynthetic gene clusters are not known to include MVA or MEP pathway genes, the clustering of genes encoding downstream steps of the terpenoid biosynthetic network such as prenyltransferases, TPSs and modifying enzymes likely provides two major benefits to plants. First, the close genetic linkage of clustered genes favors inheritance of the essential steps of a biosynthetic pathway, while minimizing the risk that genes are separated by crossover which could lead to the release of potentially deleterious intermediates due to an incomplete biosynthetic pathway [286]. Second, such gene clusters allow potential co-regulation through epigenetic mechanisms in response to long-term or inherited stress responses enabling efficient adaptation of a plant to the surrounding environment. Remarkably, enrichment of the histone variant H2A.Z mediated by ARP6, a part of the chromatin remodeling complex, was demonstrated to be positively correlated with the expression of both the thalianol and marneral triterpenoid biosynthetic gene clusters in Arabidopsis [297]. Both of these gene clusters contain oxidosqualene cyclase genes and two non-homologous P450 genes, as well as an additional acyltransferase gene in the case of the thalianol cluster. Chromatin modifications limited to the gene clusters enabled the coordinated expression of these biosynthetic genes. Further analysis of these biosynthetic gene clusters in Arabidopsis revealed that they are embedded in three-dimensional domains and their conformation differs between transcriptionally active and silent clusters with the latter being associated with heterochromatic chromosomal regions at the nucleolar periphery [284].

6.3. Metabolic reactivation of IP and DMAP by isopentenyl phosphate kinase

Recently, it was discovered that besides the two classical MVA and MEP pathway enzymes, MDD and HDR, respectively, plant genomes encode an additional IPP-generating enzyme, IPK, originally identified in archaebacteria (Fig. 1). While MDD and HDR are respectively located in the peroxisomes and plastids, IPK resides in the cytoplasm and is involved in a metabolite reactivation process via ATP-dependent phosphorylation of isopentenyl phosphate (IP) and dimethylallyl phosphate (DMAP) to their corresponding diphosphates IPP and DMAPP [228]. In contrast to plants, archaea rely exclusively on the MVA pathway in which IPK catalyzes its final step resulting in IPP formation. This alternative archaebacterial MVA pathway bifurcates from the canonical MVA pathway after the intermediate MVAP. In the canonical MVA pathway, MVAP undergoes phosphorylation catalyzed by PMK to produce MVAPP, which is subsequently decarboxylated by MDD (Fig. 1). In the archaebacterial MVA pathway, the order of reactions is reversed wherein MVAP undergoes initial decarboxylation to IP catalyzed by a phosphomevalonate decarboxylase (MPD) followed by IP phosphorylation catalyzed by IPK [298]. While in archaea IPK is a part of the alternative MVA pathway, in plants it acts synergistically with the MVA pathway and plays an important role in regulating the formation of both MVA and MEP pathway-derived terpenoid compounds by controlling the ratio of IP/DMAP to IPP/DMAPP [228]. Unlike in archaea, IP and DMAP in plants originate from the active dephosphorylation of IPP (DMAPP) by dedicated Nudix hydrolases that, together with IPK, regulate the concentration of IPP destined for higher-order terpenoid biosynthesis [42]. To date it remains an open question of how prenyl diphosphates are protected from endogenous phosphatase activity and are instead specifically utilized by Nudix hydrolases.

6.4. The role of protein complexes and metabolic channeling in the regulation of terpenoid biosynthesis

Within the terpenoid metabolic network the simultaneous production, within a single cell or cellular compartment, of an array of terpenoids, all derived from a small number of prenyl diphosphate precursors, (Fig. 1) raises the question of how cells specifically produce the needed spectrum and amounts of compounds in an ever-changing metabolic context. One way is to form supramolecular protein complexes that contain sequential metabolic enzymes, often linked to structural cellular elements like membranes [299]. When the formation of such complexes results in metabolic channeling they are considered as metabolons. This structural arrangement of enzymes can increase the local concentration of substrates thereby accelerating reaction rates, protect metabolites from competing enzymes or degradation, and sequester pathway intermediates thus preventing the release of possibly toxic intermediates into the surroundings [300,301]. Additionally, it prevents the exposure of incorporated enzymes to compounds throughout the cell that may act as enzyme inhibitors. The transient and dynamic nature of the protein-protein interactions allows such complexes to efficiently respond to changing environmental conditions and channel carbon to accommodate cellular requirements. To date, the presence of many complexes of metabolic enzymes has been proposed in plants based on observed protein-protein interactions, however metabolite channeling has only been proven in a limited number of cases (reviewed in [301]). Several multiprotein complexes have been already identified in the terpenoid network, however, despite some indirect genetic evidence additional detailed analyses are still required to further verify the role of such complexes for substrate channeling in terpenoid biosynthetic pathways.

GGPP biosynthesis represents an essential branch point in plant metabolism as this precursor is required for the formation of tocopherol, plastoquinone, phylloquinone, chlorophyll, carotenoids, gibberellins and diterpenes (Fig. 1). In Arabidopsis it was found that the “hub GGPPS”, AtGGPPS11, can interact with distinct GGPP modifying enzymes to direct flux towards different end products. AtGGPPS11 interaction with solanesyl diphosphate synthase 2 (SPPS2) favors plastoquinone biosynthesis [271] (Fig. 5A). Alternatively, to direct flux towards chlorophyll formation, AtGGPPS11 can directly interact with geranylgeranyl reductase (GGR) [271] that either reduces GGPP to form phytyl diphosphate for chlorophyll and tocopherol formation or yields chlorophyll directly from geranylgeranyl-chlorophyll [302]. In rice, out of 12 short-chain prenyltransferase candidates, only one, OsGGPPS1, is a functional GGPPS in the plastid. It forms a complex with GGPPS recruiting protein (GRP), which belongs to the GGPPS SSU-II family [159], and recruits OsGGPPS1 to the thylakoid membrane while also enhancing its activity [179]. The GGPPS-GRP heteromer coexists in a multiprotein complex consisting of GGR, light harvesting-like protein 3 (LIL3), protochlorophyllide oxidoreductase (PORB) and chlorophyll synthase (CHLG) [179] (Fig. 5B). These results suggest that interaction of GRP with OsGGPPS1 provides a mechanism for recruiting the GGPPS to a potential metabolon for chlorophyll biosynthesis localized to the thylakoid membrane, while reducing flux towards competing terpenoid pathways.

Apart from its role in chlorophyll biosynthesis, GGPPS can also interact with phytoene synthase (PSY) to direct carbon flux towards carotenoid formation (Fig. 5C). The interaction of GGPPS with PSY has been reported in Arabidopsis, as well as in red pepper (*Capsicum annuum*) and tobacco (*Nicotiana tabaccum*) where SSU-II is additionally involved in protein complex formation, suggesting this molecular mechanism is likely conserved across plant species [180,271,303,304].

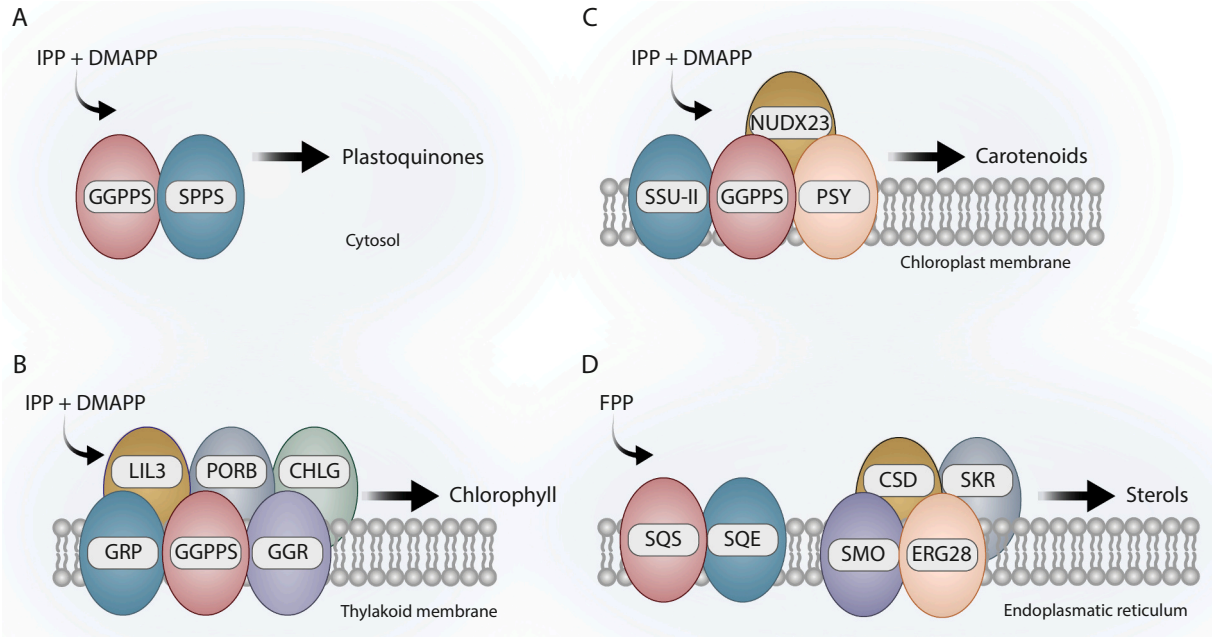

Fig. 5. Protein complexes identified in plant terpenoid metabolism.
Protein complexes identified that may represent potential metabolons involved in metabolic channeling in the formation of (**A**) plastoquinones, (**B**) chlorophyll, (**C**) carotenoids and (**D**) sterols. Abbreviations: dimethylallyl diphosphate (DMAPP); isopentenyl diphosphate (IPP); solanesyl diphosphate synthase (SPPS); geranylgeranyl diphosphate synthases (GGPPS); geranylgeranyl reductase (GGR); GGPPS recruiting protein (GRP); light harvesting-like protein 3 (LIL3), protochlorophyllide oxidoreductase (PORB) and chlorophyll synthase (CHLG); phytoene synthase (PSY); small subunit II (SSU-II); Nudix hydrolase 23 (NUDX23); farnesyl diphosphate (FPP); squalene epoxidase (SQE); squalene synthase (SQS); sterol 4α-methyl oxidase (SMO); 4α-carboxysterol-C3-dehydrogenase/C-4 decarboxylase (CSD); sterone ketoreductase (SKR); ergosterol biosynthetic protein 28 (ERG28).

Moreover, recent studies in Arabidopsis showed that GGPPS and PSY physically interact with Nudix hydrolase 23 (NUDX23), which is required for assembly of PSY and GGPPS in a large enzyme complex and enhances their stability (Fig. 5C) [305]. While demonstrating complex formation, all the above studies lack direct biochemical evidence for substrate channeling. However, since PSY on its own cannot effectively access freely diffusible GGPP, this suggests the necessity for channeling of substrate via a protein complex. A chimeric mini-metabolon was generated by translationally fusing AtGGPPS11 and PSY, analysis of which, in vitro and *in planta*, revealed that indeed, complex formation and channeling is required for efficient carotenoid production [306]. In cauliflower, tomato, carrot and Arabidopsis, another enzyme of carotenoid biosynthesis, phytoene desaturase (PDS), which acts downstream of PSY, was found to be a part of multiprotein complexes located in both plastidial membranes as well as the stroma, although the other members were not identified [307].

In the cytosol, similar to the GGPP situation in plastids, FPP is a required substrate for multiple competing pathways responsible for the formation of sesquiterpenes, sterols, triterpenes and brassinosteroids (Fig. 1). To date there is no evidence for FPPS containing multiprotein complexes in plants, however, early data in yeast suggested the existence of protein complexes downstream of FPPS. Indeed, feeding assays of yeast microsomal fractions with squalene showed only limited conversion to 2,3-oxidosqualene by squalene epoxidase (SQE) [308], whereas when FPP was supplied instead of squalene, efficient biosynthesis of 2,3-oxidosqualene occurred. This suggests that squalene synthase (SQS), catalyzing the first committed step in sterol biosynthesis, may channel its product to the immediate downstream enzyme SQE and form a complex (Fig. 5D).

Protein-protein interactions of enzymes downstream of SQE further support the involvement of a metabolon in sterol biosynthesis in both plants and yeast. It has been shown that formation of C-4 sterol intermediates is catalyzed by the sterol C4 demethylase (SC4DM) multienzyme complex which consists of three catalytic enzymes including sterol 4α-methyl oxidase (SMO), 4α-carboxysterol-C3-dehydrogenase/C-4 decarboxylase (CSD) and sterone ketoreductase (SKR), and is tethered to the ER by the non-catalytic ergosterol biosynthetic protein28 (ERG28) (Fig. 5D) [309–311]. In addition, yeast contains another ergosterol biosynthetic enzyme ERG11, lanosterol C-14 demethylase, which yields 4-dimethylcholesta-8,14,24-trienol, within the SC4DM complex [312]. Knock-down or knock-out of Arabidopsis ERG28 promoted the accumulation of 4-carboxy-4-methyl-24-methylenecycloartanol (CMMC) which inhibits polar transport of auxin resulting in aberrant growth phenotypes [310]. In contrast to plants, down-regulation of ERG28 in yeast interrupts sterol C4-demethylation and leads to accumulation of the 3-keto and carboxylic acid sterol intermediates [309]. Taken together, these results suggest ERG28 restricts release of pathway intermediates and plays a scaffolding role that facilitates the sequential transfer of these intermediates within the SC4DM complex.

6.5. *Flux regulation at the enzyme level*

In addition to transcriptional regulation, plants modulate flux through the MEP and MVA pathways by controlling the stability and activity of their enzymes. Post-translational modifications and feedback mechanisms allow the plant to modulate enzymatic activity in response to changes in precursor supply, product demand, physiological conditions and environmental stimuli. Moreover, allosteric feedback regulation by up- or downstream metabolites can prevent the accumulation of toxic products or pathway intermediates. Significant progress has been made in the last decade, uncovering several mechanisms controlling the stability and activity of MVA and MEP pathway enzymes in plants.

6.5.1. *Feedback mechanisms in the MEP pathway*

Within the MEP pathway, DXS is subject to multiple layers of post-translational and allosteric regulation (Fig. 6). Studies in various plant species showed that *DXS* transcript abundance, its protein amount and enzymatic activity do not always correlate. For example, when the flux

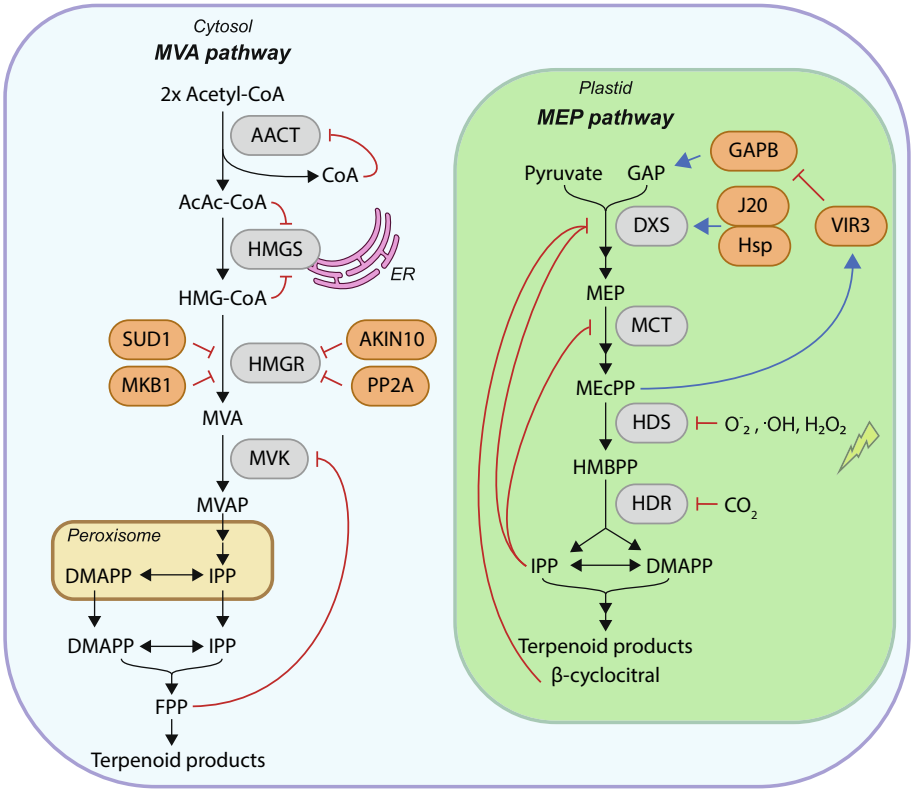

Fig. 6. Post-translational regulation of flux through the MVA and MEP pathways. Highlighted in gray are biosynthetic enzymes targets for activation or inhibition by pathway intermediates or end products. Regulatory proteins that post-translational control the activities of these biosynthetic enzymes are depicted with an orange background. Positive regulatory interactions are indicated by blue arrows, whereas inhibitory effects are indicated by red blunt arrows. Multiple enzymatic steps are indicated by the presence of more than one arrowhead. Post-translational modifications involve phosphorylation (AKIN10), dephosphorylation (PP2 A), proteasomal degradation (MKB1, SUD1), as well as protein quality control mechanisms such as refolding/degradation of damaged proteins (J20/Hsp). Metabolite intermediates can allosterically interfere with the enzyme's active site, proper folding or dimerization while molecules formed in response to stresses including ROS and atmospheric CO_2 can modulate flux through the pathway by interference with proteins. Metabolite abbreviations: acetyl Coenzyme A (Acetyl-CoA); acetoacetyl CoA (AcAc-CoA); dimethylallyl diphosphate (DMAPP); farnesyl diphosphate (FPP); glyceraldehyde 3- phosphate (GAP); (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP); 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA); isopentenyl diphosphate (IPP); mevalonic acid (MVA); mevalonate 5-diphosphate (MVAP); 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP); 2-*C*-methyl-D-erythritol 4-phosphate (MEP). Protein abbreviations: AcAc-CoA thiolase (AACT); 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS); 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR); mevalonate kinase (MVK); Suppressor of Dry2 Defects 1 (SUD1); Makibishi 1 (MKB1); Arabidopsis SNF1-related protein kinase 1 (AKIN10); protein phosphatase 2 A (PP2A); 1-deoxy-D-xylulose 5-phosphate synthase (DXS); 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase (MCT); (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS); (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; (HDR); DnaJ-like protein (J20); heat shock protein (Hsp); Virescent 3 (VIR3); GAP dehydrogenase B (GAPB). ER depicts the endoplasmic reticulum. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

through the MEP pathway is inhibited, like in the Arabidopsis *clb6* mutant that contains a mutation in *HDR*, DXS protein levels were higher compared to wild-type plants while mRNA levels were lower or showed only minor changes [60,313–315]). Similarly, no changes in *DXS* expression but higher corresponding protein levels were observed in Arabidopsis plants after blocking the pathway with the DXR inhibitor fosmidomycin [313], suggesting that a MEP-pathway intermediate(s) downstream of DXP negatively regulates the stability of DXS. Moreover, analysis of the catalytic properties of DXS revealed that its activity is inhibited by IPP and DMAPP [316,317] and this inhibition could be reduced by thiamine diphosphate (ThPP), a cofactor of DXS [317]. Recent cryo-EM, modeling, and isothermal titration calorimetry assays showed that DXS functions as a homodimer [318], and IPP/DMAPP likely binds to an allosteric site, which is distinct from the predicted ThPP binding within the enzyme active site [319]. IPP/DMAPP appear to interfere with dimerization of the DXS monomers, thus preventing formation of the catalytically active homodimers, and serving as a rapid feedback mechanism to excess IPP/DMAPP [319]. In addition to inhibition by IPP and DMAPP, it has been shown that β-cyclocitral (βCC), the product of β-carotene oxidation in response to leaf mechanical wounding or herbivory, simultaneously inhibits DXS activity by blocking the pyruvate binding site, and reduces *DXS* transcription, thus suppressing flux through the MEP pathway [320]. Recently, it has been shown that molecular chaperones stabilize catalytically active DXS homodimers [321,318]. Damaged and misfolded DXS proteins are tagged by Arabidopsis DnaJ-like protein J20 for recognition by heat shock proteins Hsp70 and Hsp100 [322,323]. This complex formation contributes to maintaining an active pool of DXS in the plastid through either reactivation by refolding, or degradation of DXS by plastidial Clp proteases [323].

Similar to DXS, the third enzyme of the MEP pathway (Figs. 1 and 6), MCT, is feedback inhibited by IPP/DMAPP [324]. Unfortunately, the mechanism of this inhibition is currently unknown since IPP/DMAPP neither compete with MCT's substrate, MEP, for the active site, nor bind a previously identified allosteric binding-site that is targeted by the synthetic inhibitor triazolopyrimidine [324,325]. The last two enzymatic steps of the MEP pathway catalyzed by HDS and HDR (Fig. 1) both contain $[4Fe–4S]^{2+}$ clusters, making these enzymes hypersensitive to inactivation by molecular oxygen and reactive oxygen species (ROS) [326–330]. HDR activity is also inhibited by elevated atmospheric CO_2 levels, resulting in an accumulation of its substrate HMBPP and decreased terpene emission in poplar [331].

Over the past decade, increasing evidence shows that the MEP pathway ultimately regulates supply of its own substrate through the

retrograde signaling intermediate MEcPP [332] which accumulates in response to a variety of stress conditions including wounding, high temperature, heavy metals, and different light intensities [333,334]. MEcPP has widespread effects on plant physiology with proposed roles in general stress response, endoplasmic reticulum homeostasis, calcium signaling, stromule development, light response and growth [332,333,335–338]. Recently, MEcPP was predicted to negatively regulate flux through the MEP pathway by controlling GAP supply via a feedback loop involving Virescent3 (VIR3), a putative metalloprotease localized in the stroma lamella, and degradation of GAP dehydrogenase B (GAPB) [332] (Fig. 6). Additionally, MEcPP positively correlates with DXS enzyme abundance suggesting further involvement of MEcPP in the fine-tuning of MEP pathway flux via feedback regulation to DXS through a yet unknown mechanism [339].

6.5.2. Feedback mechanisms in the MVA pathway

The first enzyme of the MVA pathway, AACT (Fig. 1), releases free CoA upon condensation of two molecules of acetyl-CoA. Analysis of alfalfa (*Medicago sativa*) AACT activity in vitro showed that it is inhibited by free CoA (Fig. 6) [340]. Overexpression of *MsAACT1* in alfalfa roots did not increase sterol formation under optimal growth conditions, however under saline conditions these plants exhibited enhanced production of squalene, without altering HMGR activity, and increased stress tolerance compared to wild-type plants [340]. Taken together, these data may suggest a role of AACT and its CoA dependent post-translational regulation in controlling flux through the MVA pathway under abiotic stress conditions [340]. Kinetic analysis of HMGS, which catalyzes the second step of the MVA pathway, revealed that high concentrations of acetoacetyl-CoA, one of its substrates, inhibits enzyme activity in vitro by competing with acetyl-CoA for the active site (Fig. 6) [341]. Further site-directed mutagenesis of *Brassica juncea* BjHMGS revealed that a single amino acid, H188, is responsible for substrate inhibition, and H188N substitution relieves the substrate inhibition while decreasing V_{max} [341]. Follow up overexpression of the gene encoding this mutated enzyme *in planta* did not increase production of sterols, likely due to non-inhibitory levels of endogenous acetoacetyl-CoA and/or low catalytic activity of the overexpressed protein [342].

Product feedback inhibition was also found for HMGR in Arabidopsis seedlings grown on media supplied with mevalonate which reduced HMGR activity without affecting *AtHMGR1* and *AtHMGR2* mRNA levels [343]. Moreover, it has been shown that the activity of HMGR can be regulated by post-translational modifications. Indeed, the catalytically active C-terminus of HMGR, which resides in the cytosol, can be phosphorylated at the S577 position by a SNF1-related protein kinase 1 (AKIN10) leading to inactivation of the enzyme (Fig. 6) [344,345]. In addition, the N-terminus of HMGR1, but not HMGR2, can be dephosphorylated by Protein Phosphatase 2 A (PP2 A) at a position other than S577 resulting in a protein with reduced enzymatic activity (Fig. 6) [346]. This indicates that coordinated (de)phosphorylation modulates HMGR activity. Furthermore, downstream terpenoid products can indirectly feedback regulate HMGR activity and stability through activation of the endoplasmic reticulum associated degradation (ERAD) quality control system. It has been shown that in alfalfa, HMGR stability is controlled by an endoplasmic reticulum localized E3 ubiquitin ligase (MKB1) which recruits the ERAD system [347,348]. Indeed, roots of *MKB1* knockdown lines have higher HMGR protein levels, in particular in response to jasmonic acid, and over-accumulate bioactive triterpenoid saponin monoglucosides causing aberrant root growth [347]. However, the mechanistic details of plant-specific ERAD recruitment by terpenoid products or intermediates, and regulation of proteasome dependent degradation of HMGR remain to be discovered. In contrast to MKB1, SUPPRESSOR OF DRY2 DEFECTS 1 (SUD1), a (putative) E3 ubiquitin ligase, was found to positively regulate HMGR activity (Fig. 6) [349]. Protein extracts of Arabidopsis *sud1* mutants showed decreased HMGR catalytic activity without changes in HMGR protein abundance, indicating that SUD1 likely degrades a negative regulator of HMGR [349]. As a final example of feedback regulation within the MVA pathway, MVK activity is regulated by the downstream prenyl diphosphates. Indeed, increasing in vitro concentrations of FPP, GPP, and GGPP were all able to inhibit activity of MVK from several plant species including Madagascar periwinkle, melon, common bean, and rubber plant (Fig. 6) [350,351].

7. Connection with other pathways of specialized metabolism

Many plants produce non-terpenoid specialized metabolites, sometimes in the same specific tissue or cell type as terpenoids, however, little is known about the connectivity between and the regulation of flux allocation towards the biosynthesis of terpenoids and other specialized metabolic pathways. The phenylalanine-derived phenylpropanoids are a large class of non-terpenoid specialized metabolites holding a diverse array of physiological functions. In plants, phenylpropanoid biosynthesis relies on the plastidial shikimate pathway which starts with the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) derived from glycolysis and the pentose phosphate pathway, respectively [352]. Therefore, the shikimate as well as the MEP and MVA pathways ultimately compete for carbon supply (Fig. 2), reducing power and ATP. Indeed, a negative correlation between production of terpenoids and phenylpropanoids was found in glandular trichomes of basil varieties (*Ocimum* sp.) that produce blends of these classes of compounds at different ratios [353]. Moreover, total terpenoid content was correlated positively with terpene synthase activities, and negatively with phenylalanine ammonia-lyase (PAL), a key enzyme in the core phenylpropanoid pathway. This allows maximized flux towards terpenoids by fine-tuning the activity of terpenoid biosynthetic enzymes in conjunction with the restriction of the phenylpropanoid pathway [353]. In addition, the ratio of phenylpropanoid and terpenoid products is controlled by expression of genes at major metabolic branch points including *3-deoxy-D-arabino-heptulosonate-7-phosphate synthase* (*DAHPS*), *PAL*, and *DXS/DXR*, for the shikimate, phenylpropanoid, and terpenoid pathways, respectively [354]. Differential post-translational modification of specific proteins involved in metabolism of both terpenoids and phenylpropanoids, detected through proteomic analysis, was suggested to further contribute to determining the ratio between these products in basil glandular trichomes [354].

The increasing availability of transcriptomic and metabolomic data from several different plant species has revealed numerous TFs, a small number of which have already been tested for their ability to coordinate the expression of terpenoid and phenylpropanoid biosynthetic genes. When *Mentha spicata* TFs, YABBY *MsYABBY5* or R2R3-MYB *MsMYB,* both of which repress terpenoid biosynthesis in spearmint, were ectopically expressed in sweet basil (*O. basilicum*), slightly different effects on terpenoid and phenylpropanoid production were observed. In the case of *MsYABBY5*, the levels of both mono- and sesquiterpenes as well as the phenylpropanoid eugenol, showed a marked decline [355], while *MsMYB* led to a reduction in terpenoid formation without affecting the eugenol levels [356]. In *Ophiorrhiza pumila*, OpMYB1 suppressed expression of genes for both the phenylpropanoid and terpenoid pathways [357] in contrast to expression of *Arabidopsis* R2R3-MYB TF, *PRODUCTION OF ANTHOCYANIN PIGMENT1* (*PAP1*), which in rose flowers resulted in increased emission of both mono−/ sesquiterpenes and eugenol [358]. Additionally, MYB and WRKY TFs have been shown to inversely affect the phenylpropanoid and terpenoid pathways albeit with opposite outcomes. Overexpression of *MYB5B* from grapevine in tomato led to an increase in carotenoid levels with a simultaneous decrease in flavonoids [359], whereas OsWRKY13 in rice has been shown to upregulate expression of phenylpropanoid biosynthetic genes while instead suppressing transcription of those involved in terpenoid production [360]. Together, these examples demonstrate the ability of a single TF to modulate both pathways, but each may be just one of the components in a more complex regulatory network of which the details and molecular mechanism remain unknown.

The ratio between terpenoids and phenylpropanoids could also be affected by feedback regulation of the phenylpropanoid pathway intermediates or end products. The application of *trans*-cinnamic acid, a central intermediate in the phenylpropanoid biosynthesis, to leaves or flowers of yarrow (*Achillea millefolium*) downregulated *PAL* and *chalcone synthase* (*CHS*), while increasing the expression of terpenoid biosynthetic genes *DXR*, *GPPS* and *linalool synthase* (*LIS*) [361]. In *Artemisia annua*, when *trans*-cinnamic acid levels were increased either by exogenous application or downregulation of *cinnamate-4-hydroxylase* (*C4H*), the former led to increased expression of *CYP71AV1* [362] encoding a key P450 enzyme for the synthesis of the sesquiterpene lactone artemisinin [363], and *C4H* downregulation increased artemisinin levels [364], possibly via elevated levels of salicylic acid [365]. This antagonistic relationship between terpenoid and phenylpropanoid biosynthesis is not consistent however, and tomato trichomes harboring the *anthocyanin free* (*af*) mutation are defective in both flavonoid and terpenoid biosynthesis [366]. The *af* mutant carries a deficient *chalcone isomerase* (*CHI*) gene making it unable to produce naringenin from naringenin chalcone, thereby halting the flux through the flavonoid biosynthetic pathway, but the mechanism behind the reduction in terpenoid levels remains unclear. The coupled regulation of both pathways in this context was confirmed by the reduced expression of MEP and MVA pathway, prenyltransferase and terpene synthase genes in trichomes of the *af* mutant and by the recovery of both flavonoid formation and terpene biosynthesis upon complementation with wild-type *CHI* [366,367]. The suppression of terpenoid biosynthesis in the *af* mutant could be the result of direct feedback regulation by naringenin chalcone or upstream intermediates, or indirect stress-response due to excessive ROS buildup in the absence of flavonoids [367]. Similarly, isoprene, a common hemiterpene frequently emitted by conifers, can affect transcription of phenylpropanoid pathway genes including *PAL* [368,369].

8. Open unsolved questions and conclusions

The utility of terpenoids to humans as flavorings, fragrances, preservatives, pesticides, biofuels, and medicines, along with their ecological and physiological significance in plants makes a complete understanding of their biosynthesis of great value. Recent research has supported the presence of a complex terpenoid biosynthetic network with numerous branches and points of regulation instead of a simple linear pathway [370]. Although substantial progress has been made towards elucidating the individual biosynthetic steps of multiple pathways, we still have limited knowledge of the interplay between terpenoid precursor pathways and the multiple regulatory mechanisms that control carbon flux towards distinct end products under different conditions.

Terpenoid precursor pathways rely on general primary carbon metabolism for substrate supply which is dependent on the metabolic context within a cell or compartment that is driven by plant developmental stage, tissue, organ, and cell type. To date, little is known about the relative contributions of different primary metabolic pathways to terpenoid biosynthesis and in particular of the regulatory crosstalk between them. Therefore, further systematic profiling of primary and secondary metabolites in different tissues, developmental stages, and even single cells, combined with stable isotope labeling and flux analysis, along with genetic validation, will unveil the regulatory network that allocates substrates to the MVA and MEP pathways. As terpenoid pathways produce both essential primary and important secondary metabolites, another open question is how plants regulate the allocation of carbon to distinct groups of compounds. Within the MEP and MVA pathways several steps are catalyzed by multiple orthologs, however we still lack a comprehensive understanding of their differential functions and roles in regulating flux towards terpenoid end products. Further systematic analysis of *cis*-acting elements, as well as other regulatory features including epigenetic modifications and transcription factors, along with their integration in the overall terpenoid biosynthetic network will reveal the biological significance and metabolic roles of these different pathway genes and their orthologs.

Moreover, recent results show that, as opposed to being strictly compartmentally separated, different chain length prenyl diphosphates are colocalized in multiple cellular compartments raising the question of if and how they are prevented from interfering with prenyltransferases, and are efficiently utilized by specific downstream terpene synthases, often with substrate promiscuity, to produce the required compounds for a given metabolic context. The control of local concentrations and shielding of toxic or unstable intermediates could be the missing links in our understanding of the terpenoid network. Until now, direct proof for metabolite channeling in terpenoid biosynthesis is still lacking. In the future, more sophisticated analysis such as isotopic labeling studies, competition assays, as well as metabolite-protein and protein-protein interaction studies should illuminate the role of supraprotein complexes and metabolite channeling in plant terpenoid metabolism. Considering the terpenoid metabolic network is distributed between multiple compartments, transport of MEP and MVA pathway intermediates, IPP, DMAPP, and possibly other prenyl diphosphates across membranes of organelles, including plastids, mitochondria and peroxisomes, requires the presence of transporters, for which the nature and mechanism(s) of action remain unknown.

CRediT authorship contribution statement

Matthew E. Bergman: Writing – original draft. **Ruy W.J. Kortbeek:** Writing – original draft. **Michael Gutensohn:** Writing – original draft. **Natalia Dudareva:** Writing – original draft.

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