

Biosynthesis and Biological Functions of Terpenoids in Plants

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Abstract Terpenoids (isoprenoids) represent the largest and most diverse class of chemicals among the myriad compounds produced by plants. Plants employ terpenoid metabolites for a variety of basic functions in growth and development but use the majority of terpenoids for more specialized chemical interactions and protection in the abiotic and biotic environment. Traditionally, plant-based terpenoids have been used by humans in the food, pharmaceutical, and chemical industries, and more recently have been exploited in the development of biofuel products. Genomic resources and emerging tools in synthetic biology facilitate the metabolic engineering of high-value terpenoid products in plants and microbes. Moreover, the ecological importance of terpenoids has gained increased attention to develop strategies for sustainable pest control and abiotic stress protection. Together, these efforts require a continuous growth in knowledge of the complex metabolic and molecular regulatory networks in terpenoid biosynthesis. This chapter gives an overview and highlights recent advances in our understanding of the organization, regulation, and diversification of core and specialized terpenoid metabolic pathways, and addresses the most important functions of volatile and nonvolatile terpenoid specialized metabolites in plants.

Keywords MVA pathway • MEP pathway • Prenyltransferase • Terpene synthase • Volatiles • Plant defense

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1 Introduction

Introductory chapters on terpenoid biosynthesis usually highlight the large number of terpenoid compounds found in nature. Indeed, the structural diversity associated with at least 40,000 compounds makes the class of terpenoids one of the most impressive examples in the divergent evolution of plant chemicals. The evolutionary success of this compound class is in part based on the simplicity of constructing different size molecules. According to the isoprene rule recognized by Wallach and Rutzicka in the late nineteenth and mid-twentieth centuries [1], all terpenoids are derived from the universal five-carbon building blocks, isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). The prenyl diphosphate intermediates built by condensation of these five-carbon units are used as precursors for the biosynthesis of terpenoids with fundamental functions in growth and development and for the formation of a large number of terpenoid compounds with more specialized roles in the interaction of plants with their environment. It is the latter group of terpenoids that is characterized by its tremendous structural diversity as a consequence of divergent biosynthetic gene evolution. Specialized terpenoids have a long history of being used as flavors, fragrances, pharmaceuticals, insecticides, and industrial compounds, several of which are addressed in this book. With the growing need for sustainable production platforms of plant-based drugs and the emerging use of terpenoids in the production of alternative fuels, substantial progress has been made in the engineering of terpenoid biosynthetic pathways in microbes and plants [2, 3]. Advanced functional genomics approaches provide unlimited access to the biosynthetic genes and molecular regulators of terpenoid-producing plants, and, at the same time, allow deeper insight to the complexity of plant terpenoid metabolism and regulation. In this chapter, I provide an overview of the organization of the early and core terpenoid metabolic pathways and give updates on the regulation and functional diversification of their genes and enzymes. Furthermore, I summarize the function

of terpene synthases and describe aspects of their coordinated and tissue-specific regulation in specialized metabolism prior to addressing the diverse roles of terpenoids in plant–environment interactions.

2 Core Terpenoid Biosynthetic Pathways and Their Regulation

Successful engineering of terpenoid products in plants critically depends on the flux of precursors delivered by the core isoprenoid biosynthetic pathways and, consequently, on the dynamic regulation of these biosynthetic routes. Plants use two independent pathways to produce IPP and DMAPP: the primarily cytosolic mevalonic acid (MVA) pathway and the plastidial methylerythritol phosphate (MEP) pathway (Fig. 1). The MVA pathway predominantly provides the precursors for the cytosolic biosynthesis of sesquiterpenoids, polyprenols, phytosterols, brassinosteroids, and triterpenoids, and for terpenoid biosynthesis in mitochondria (e.g., ubiquinones, polyprenols), and the five-carbon units derived from the MEP pathway are preferably used for the biosynthesis of hemiterpenoids (e.g., isoprene), monoterpenoids, diterpenoids, carotenoids and their breakdown products, cytokinins, gibberellins, chlorophyll, tocopherols, and plastoquinones (Fig. 2). It has become evident that both pathways are heavily regulated at multiple levels as was discussed in two recent reviews by Hemmerlin and coworkers [4, 5]. In addition to the transcriptional regulation of MVA and MEP pathway genes and their different paralogues, isoprenoid-pathway fluxes are controlled at posttranscriptional/translational levels and by feedback regulation. Recent studies have given a more global view of the dynamics and networks of the core isoprenoid pathways and the regulation of metabolic flux during plant development and in response to external stimuli (reviewed in [6, 7]). Therefore, this chapter primarily gives an overview of both pathways with some emphasis on those in *Arabidopsis* and provides updates on the different modes of regulation.

2.1 MVA and MEP Pathways—A Brief Summary of Their Biosynthetic Steps

The MVA pathway in plants (Fig. 1a) consists of six steps and starts with the Claisen-type condensation of two molecules acetyl-CoA to acetoacetyl-CoA (AcAc-CoA) catalyzed by acetoacetyl-CoA thiolase (AACT). In a subsequent aldol condensation reaction catalyzed by HMG-CoA synthase (HMGS), AcAc-CoA is combined with a third molecule of acetyl-CoA to form the C₆-compound S-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Response to different stresses, feedback regulation, and the role of HMGS in sterol metabolism (see below)

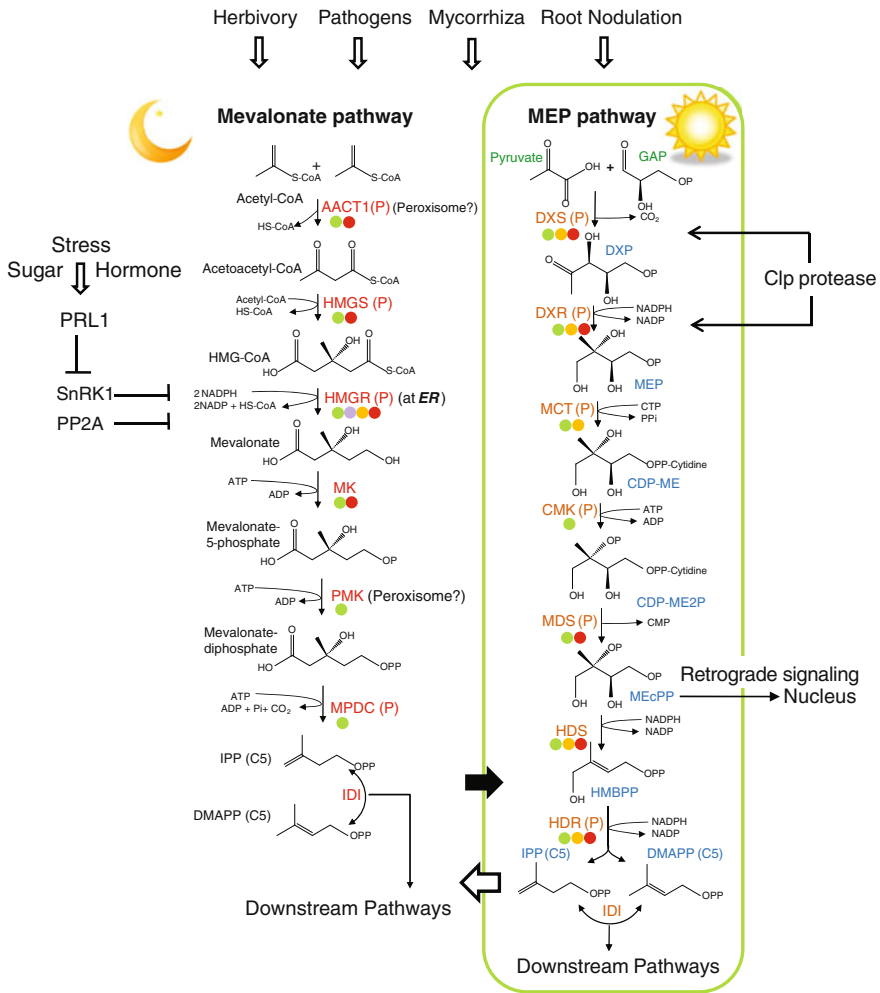


Fig. 1 Enzymatic steps of the MVA and MEP pathways and their regulation in isoprenoid precursor biosynthesis. Colored dots indicate the different levels of regulation for each enzyme according to the current status of knowledge [5]: green—transcriptional, purple—posttranscriptional, yellow—translational, red—posttranslational including feedback modulation. Other selected external and internal regulatory factors and posttranslational modifications of main regulatory enzymes are depicted as mentioned in the text. One or more gene paralogues as described from different plant species [4] are indicated by (P). Arrows indicate preferred trafficking of isoprenoid precursors between the cytosol and plastids in light (white) and dark (black) exposed tissues. Abbreviations for enzymes (red) and metabolites (black—MVA pathway; blue—MEP pathway) are as described in the text

support a key function of HMGS in the MVA pathway. In the following rate-limiting step, HMG-CoA reductase (HMGR) catalyzes the conversion of *S*-HMG-CoA to *R*-mevalonate in two NADPH-dependent reduction steps. All plant

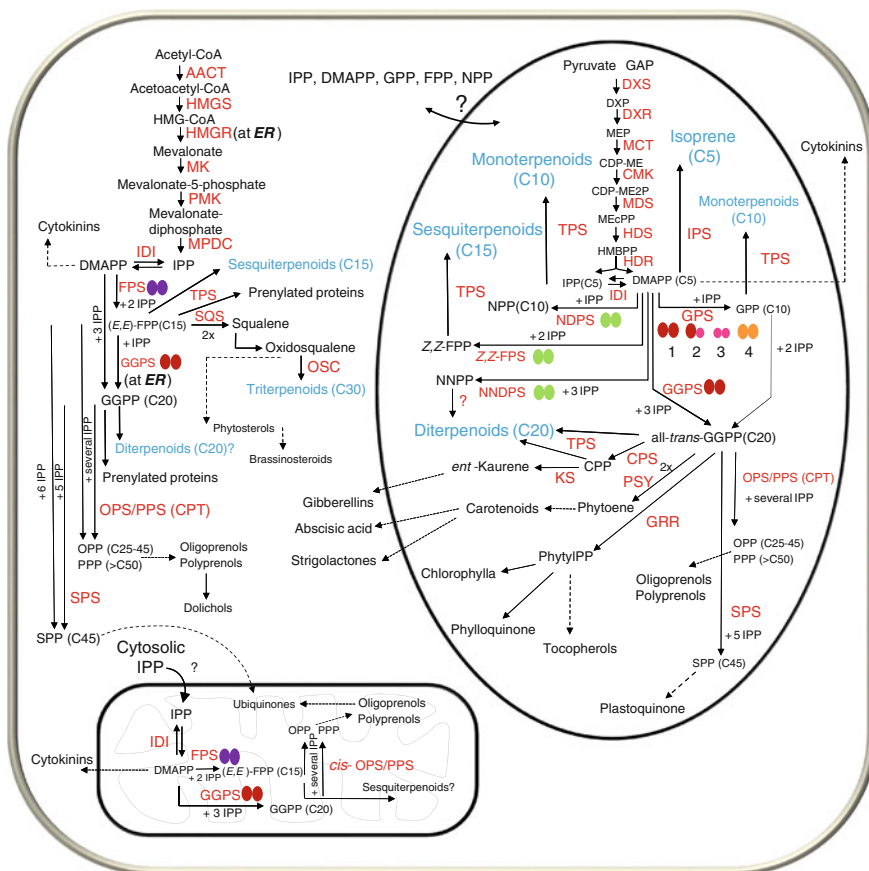


Fig. 2 Terpenoid biosynthetic pathways and their subcellular organization. Enzymes are marked in *red*; specialized terpenoids are marked in *blue*; all other intermediates and terpenoid end products are in *black*. *Solid and dashed arrows* indicate single and multiple enzymatic steps, respectively. *Colored ovals* indicate the homodimeric and heterodimeric composition of prenyltransferases involved in specialized terpenoid biosynthesis: GPS1—GGPS type, GPS2—heterodimer (heterotetramer) with large subunit (LSU) and small subunit (SSU I or SSU II), GPS3—homodimeric SSII type [136], GPS4—PPS type. Short-chain *cis*-prenyltransferases are marked in *green*. Abbreviations not mentioned in the text: *IPS* isoprene synthase; *GRR* geranylgeranyl reductase; *OPS* oligoprenyl diphosphate (*OPP*) synthase; *SPS* solanensyl diphosphate (*SPS*) synthase; *SQS* squalene synthase

HMGR proteins are membrane-bound with two membrane-spanning sequences and a highly conserved catalytic C-terminal domain. The presence of ER-specific retention motifs indicates a primary association of the membrane-spanning domain with the ER, whereas the N-terminal and C-terminal ends are positioned on the cytosolic side [8–13]. The association of HMGR to membranes seems to regulate its activity negatively, thereby limiting the accumulation of terpenoid end products such as sterols (e.g., [14, 15]). Many studies have reported on the critical regulatory

role of HMGR in the biosynthesis of phytosterols, triterpenoids, and sesquiterpenoid phytoalexins, although flux control often involves additional downstream enzymes such as sesquiterpene synthases (e.g., [16–21]). MVA produced by HMGR is finally converted into IPP via three enzymatic steps: two ATP-dependent phosphorylation steps, catalyzed by mevalonate kinase (MK) and phosphomevalonate kinase (PMK), and an ATP-driven decarboxylative elimination catalyzed by mevalonate diphosphate decarboxylase (MVD or MPDC).

The MEP pathway (Fig. 1b), which occurs in all photosynthetic eukaryotes and in cyanobacteria, apicomplexan protozoa, and most eubacteria [22–25] consists of seven enzymatic steps. In the first reaction, 1-deoxy-D-xylulose 5-phosphate (DXP) is formed by DXP synthase (DXS) from (hydroxyethyl) thiamine diphosphate, which is derived from pyruvate, and glyceraldehyde-3-phosphate (GAP) in a transketolase-like condensation. Plant DXS enzymes carry a highly conserved thiamine phosphate binding domain and are divided in the class-I type enzymes with primary expression in photosynthetic and floral tissues and the class-II type enzymes with more distinct roles in specialized metabolism (see below). Numerous studies have confirmed that DXS functions as an important regulatory and rate-limiting enzyme in the biosynthesis of plastidial terpenes [26–31]. Consequently, *DXS* mutants such as those of the single functional *Arabidopsis* class-I type *DXS* gene (*DXS1*) exhibit albino phenotypes [32–34].

The enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) catalyzes the second step of the MEP pathway, in which DXP is converted into 2-C-methyl-D-erythritol 4-phosphate (MEP) by an intramolecular rearrangement of DXP into 2-C-methyl-D-erythrose 4-phosphate, followed by an NADPH-dependent reduction [35, 36]. The reaction can be specifically inhibited by fosmidomycin, a structure analogue of the DXR substrate [37–39] thereby blocking the biosynthesis of downstream plastidial terpene biosynthesis [40–42]. The reaction catalyzed by DXR is in some cases considered a rate-limiting step depending on the species, tissue, and developmental stage. In *Arabidopsis*, *DXR1* is expressed in different plant organs [36] and *dxr* mutants show, similar to those of *DXS1*, an albino phenotype and deficiencies in gibberellin and abscisic acid (ABA) biosynthesis [43].

MEP is further converted in a CTP-dependent reaction to 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) by the enzyme 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (MCT or IpsD) [44, 45]. Phosphorylation of CDP-ME by the enzyme 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK, IpsE) then leads to the formation of 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate (CDP-ME2P) [46–48], which is subsequently cyclized by 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS, IpsF) into 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP) upon loss of CMP. In the last two steps of the MEP pathway, the enzyme 4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS, IpsG) first converts MEcPP in a two-electron reduction to 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP). In a final branching step, HMBPP is converted by 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR, IpsH) to a mixture of IPP and DMAPP with a ratio of 5 to 6:1 [49–51].

Mutants of *MCT*, *MDS*, and *CMK* exhibit similar albino phenotypes and downregulation of photosynthetic genes [52, 53]. Likewise, *hds* and *hdr-1* mutants have defects in chloroplast development [54, 55]. Interestingly, a partial loss-of-function mutant of *Arabidopsis* HDS, *hds-3 (csb3)*, was shown to be more resistant to biotrophic pathogens suggesting a link between the MEP pathway and plant defense responses [56].

2.2 Differential Expression of MVA and MEP Pathway Isozymes

Several enzymes of the MVA and MEP pathways, especially those with important regulatory roles, are encoded by small gene families, which allow for functional redundancy and divergence (summarized in [4]; Fig. 1). In the MVA pathway, paralogues have been identified for AACT, HMGS, HMGR, and MPDC, whereas the MEP pathway enzymes DXS, DXR, MCT, CMK, MDS, or HDR were found to be encoded by two or more isogenes [4]. The different roles of many of the MVA and MEP pathway isozymes depend on their expression in specific cellular tissues and are often divided into essential functions to provide terpenoid precursors in primary metabolism, growth, and development, and more specific functions in stress response and specialized metabolism. For example, in *Brassica juncea*, *HMGS* is represented by a four-member gene family. Two genes are highly expressed at early stages of floral development [57–59] and play a role in reproduction, as was also shown for the single *HMGS* gene in *Arabidopsis*, [60] whereas expression of the two other paralogues is restricted to leaves [58, 59].

Notably, paralogues of the *HMGR* family exhibit different developmental and tissue-specific expression patterns and can be distinguished by their response to endogenous molecules such as phytohormones and sterol metabolites as well as external stimuli that include light, wounding, elicitor treatment, and pest and pathogen attack (Fig. 1; reviewed in [4]). The differential expression of *HMGR* isozymes, as demonstrated by early studies of the *HMGR* gene families in Solanaceous plants (tomato, potato) [61, 62], is important for channeling and counterbalancing carbon flux to the different downstream pathways of stress response or development. This functional differentiation, however, does not seem to occur in all plants inasmuch as both *HMGR* genes in *Arabidopsis* do not respond to stress but are essential for the production of sterols for cell elongation, senescence, gametophyte development, and fertility [63, 64].

Functional divergence of MEP pathway genes has been primarily observed in the *DXS* gene family. Class II-type *DXS* genes respond to biotic interaction and are induced in the biosynthesis of apocarotenoids upon mycorrhizal colonization in legumes and other plant families [30, 65, 66]. Several studies also demonstrated that type II *DXS* genes are induced in response to pathogen and herbivore attack in association with the production of specialized metabolites (summarized in [4]).

2.3 Metabolic Regulation and Networks

There is clear evidence for the role of pathway intermediates and downstream metabolites in the regulation of the core terpenoid biosynthetic steps at transcriptional and posttranslational levels (Fig. 1). Feedback inhibition by free CoA has been demonstrated for AACT and HMGR and for the enzymatic products of HMGS [59, 67, 68]. Furthermore, plant MKs respond to feedback inhibition by the prenyl diphosphates, IPP, DMAPP, geranyl diphosphate (GPP), and farnesyl diphosphate (FPP) that modulate enzyme activity by acting as competitive inhibitors of ATP [69]. Similarly, in vitro feedback inhibition was found for a DXS protein from poplar by IPP and DMAPP and a structural analysis suggested possible binding of the prenyl diphosphates to the enzyme in competition with its thiamine pyrophosphate substrate [70]. This feedback inhibition has also been supported in vivo by recent metabolic flux studies in poplar [71].

The complexity of the regulatory network also becomes apparent when metabolic disturbances and changes in metabolic flux generated by overexpression or reduced expression of genes of the core isoprenoid pathways promote pathway feedback or feedforward signals that modify the expression of up- or downstream genes. For example, overexpression of *B. juncea* wild-type and mutated *HMGS1* in *Arabidopsis* caused an upregulation of *HMGR* and genes in sterol biosynthesis such as sterol methyltransferase 2, delta-24 sterol reductase, and C-22 sterol desaturase, which led to an elevated sterol content in leaves and seedlings and increased stress tolerance [57]. A similar response was observed for *HMGS* overexpression in tobacco resulting in improved sterol content, growth, pod size, and seed yield [72]. Conversely, knockdown of *AACT2* expression led to lower levels and altered profiles of sterols and caused reduced expression of downstream genes encoding FPP synthases and sterol methyltransferase [73]. *HMGR* activity also exhibits a positive feedback response to downstream metabolic changes such as reduced cycloartenol levels in transgenic tobacco expressing sterol methyltransferase type 1 (SMT1) and the depletion of endogenous sterols due to the inhibition of squalene synthase [74, 75].

The simultaneous response of several genes to pathway perturbations is further observed in mutants of the MEP pathway. For instance, silencing of *CMK* in *Arabidopsis* causes upregulation of *MCT*, *MDS*, and *HDS* expression [76]. Moreover, in rice, MEP pathway genes were found to be coexpressed with downstream genes in carotenoid and phytyl biosynthesis [77]. In line with these observations, detailed transcriptional coexpression network analyses in *Arabidopsis* demonstrated that gene modules in both MVA and MEP pathways are coregulated together with genes of downstream pathways and these findings have set the stage to identify regulatory elements of these gene modules [78–80]. Consequently, *cis* elements were mapped showing that the promoters of the *Arabidopsis* genes *DXS*, *DXR*, *CMK*, *HDR*, and phytoene synthase share a *cis*-regulatory element interacting with RAP2.2, a member of the ethylene response factor B-2 subfamily [6].

In conjunction with their regulation by light (see below), MEP and MVA pathways respond to regulators in sugar metabolism. *Arabidopsis* mutants of pleiotropic regulatory locus 1 (PRL1), a global regulator of sugar, stress, and hormone responses, accumulate MEP pathway-derived end products (Fig. 1a) [81]. The same mutants have reduced HMGR activity but no change in *HMGR* transcript or protein because of posttranslational modification. PRL1 inhibits the SNF1 (sucrose nonfermenting)-related protein kinase 1 (SnRK1), which negatively regulates HMGR1 by phosphorylation and inactivation of the catalytic domain (Fig. 1a) [82]. HMGR1 is also negatively regulated during normal development and in response to salt stress by protein phosphatase 2A (PP2A), which dephosphorylates the HMGR protein (most likely at a site different from the phosphorylation by SnRK1; Fig. 1a) [83]. Modulation of *HMGR* transcripts at the initiation of translation [84] and glycosylation of HMGR isoforms [11], respectively, have been discussed previously as other mechanisms of posttranscriptional or posttranslational regulation of stress-induced *HMGR* genes.

There are several possible connections of the isoprenoid pathway to other metabolic routes by delivery and competition for carbon precursors (e.g., amino acid degradation) [4], which will require further attention to gain a more comprehensive understanding of flux in terpenoid biosynthesis. A link of isoprenoid metabolism with lipid biosynthesis was described by Nieto et al. [85], who found that inhibition of sphingolipid biosynthesis in *Arabidopsis* caused posttranslational downregulation of HMGR activity decoupled from HMGR transcript and protein levels and a reduction in sterol content. Recently, an unexpected simultaneous downregulation of flavonoid and terpenoid metabolite levels was observed in trichomes of tomato mutants of the flavonoid biosynthetic enzyme chalcone isomerase (CHI) [86]. These results have led to several hypotheses about the regulatory connections between both pathways. It is possible that changes in the levels of flavonoids by accumulation (upstream of CHI) or depletion (downstream of CHI) modify terpenoid biosynthetic gene expression or directly inhibit biosynthetic and regulatory proteins [87, 88]. Based on previous findings, there is also the possibility that CHI itself might interact with proteins involved in terpenoid production or its regulation [89]. Furthermore, it will be important to examine regulatory factors that coordinate the metabolic flux through both pathways [90].

2.4 Regulation by Light and External Stimuli

Thanks to recent efforts to identify MVA and MEP pathway gene expression patterns by transcriptome and hierarchical cluster analyses it was shown that the genes of both pathways have opposite expression patterns during light or dark (Fig. 1) [6]. Whereas exposure to light leads to the downregulation of MVA pathway genes and reduced levels of sterols [79], it stimulates transcript accumulation of MEP pathway genes and genes in the carotenoid and chlorophyll biosynthetic pathways such as PSY (phytoene synthase) and HEMA1 (glutamyl-tRNA

reductase), which are essential for chloroplast differentiation [79, 80, 91–94]. Light also upregulates tocopherol, and plastoquinone biosynthetic genes such as VTE3 (vitamin E defective 3) [79]. The results are supported by studies that observed an increased carbon flux through the MEP pathway under enhanced light conditions by measuring the accumulation of MEcDP when 2-C-methyl-D-erythritol 2,4-cyclodiphosphate reductase activity was inhibited [95]. In contrast to the upregulation by light, expression of MEP pathway genes with the exception of HDR [55] is reduced during light–dark transition [6]. Dark exposure can induce HMGR activity as was shown in ginseng where HMGRs play a regulatory role in triterpene ginsenoside biosynthesis [96]. The light-dependent response of *Arabidopsis* MEP and MVA pathway genes is controlled by phytochrome B (PHYB) because *phyB* mutants have enhanced transcript levels and enzyme activity of HMGR but reduced levels of MEP pathway products [92]. Consequently, phytochrome interacting factors (PIFs) of the basic helix–loop–helix (bHLH) transcription factor family were identified as regulators that are involved in the light control of MEP and carotenoid biosynthetic pathway genes [93, 97]. Turnover of the MEP pathway enzymes DXS and DXR was also found to be correlated with the activity of Clp, a major plastid stromal protease (Fig. 1b) [98].

Downregulation of MEP pathway enzymes in the dark provides a dilemma for the biosynthesis of carotenoids and gibberellins required for the development of etiolated seedlings. Supported by observations from treatments with the MEP pathway inhibitor fosmidomycin, Rodriguez-Concepcion and coworkers suggested that during seedling germination in the dark, prenyl diphosphates derived from the MVA pathway are transported into etioplasts for gibberellin and carotenoid synthesis prior to the induction of MEP pathway enzymes upon illumination [92]. Given the responses of MEP and MVA pathway genes in light and dark, it is not surprising that the expression of several genes is under circadian control [7]. Coexpression analyses in *Arabidopsis* photosynthetic tissue connect several MEP pathway genes with core circadian oscillators (*LHY*, *CCA1*, *PRR9*) whereas only *AACT2* of the MVA pathway follows the expression of circadian regulators peaking in the dark [6]. However, in roots, expression of several MVA pathway genes such as *HMGR1* is correlated with that of circadian regulators (*TOC1*, *TIC*) showing clear differences in the circadian control of early pathway genes in above- and belowground tissues. Interestingly, in triple mutants of the *TOC1* related pseudo-response regulator (PRR) proteins *PRR9*, *PRR7*, and *PRR5*, genes and metabolites of carotenoid, chlorophyll, and tocopherol pathways are upregulated, which suggests a function of these proteins as negative regulators of the MEP pathway-dependent metabolic routes [99]. To what extent the oscillation of MVA and MEP pathway gene transcripts directly corresponds to changes in enzyme activity and downstream metabolites requires further attention. In snapdragon flowers, the rhythmic emission of volatile monoterpenes in plastids and sesquiterpenes in the cytosol depends on the MEP pathway that is controlled by the circadian clock [100].

In addition to their differential response to light, MVA and MEP pathways respond to multiple other external stimuli at gene transcript and posttranslational

levels (Fig. 1; summarized in [4]). To support the production of terpenoids for protection against temperature stress, carbon flux through the MEP pathway increases under elevated temperatures [95]. In the MVA pathway, not only HMGR but other enzymes such as AACT show induced responses under abiotic stress and appear to be involved in MVA pathway-mediated abiotic stress adaptation [68]. Changes in redox state also directly affect MVA and MEP pathway enzymes. Both HDS and HDR, which function as iron–sulfur reductases, have been identified as targets of the redox protein thioredoxin [101, 102] and thioredoxin-dependent regulation has also been suggested for DXR [102]. Moreover, it has been shown that HDS can receive electrons directly through the photosynthetic electron-transport chain via ferredoxin without any reducing cofactor, which is different from the flavodoxin/flavodoxin reductase and NADPH-dependent reducing system of HDS in bacteria [103].

Biotic stress such as pathogen attack often upregulates individual genes of *HMGR* families to direct flux toward the production of sesquiterpene phytoalexins under simultaneous downregulation of squalene synthase and sterol biosynthesis [62, 104]. Studies in tobacco showed that the regulation of pathogen-activated expression of HMGR involves the MEK2-SIPK/WIPK MAP kinase cascade [105, 106]. Another example highlights the importance of HMGR in root nodule development. The HMGR1 protein of *Medicago truncatula* directly interacts with NORK, which is a receptor-like kinase required for Nod factor signaling. Reduced expression of *HMGR1* in transgenic plants causes a severe decrease of root nodulation [107].

2.5 Regulation and Metabolite Exchange Across Subcellular Compartments

The compartmentalization of MEP and MVA pathways and associated downstream pathways allows for the subcellular regulation and coordination of photosynthesis-dependent and independent terpenoid biosynthetic routes. Despite the general notion that the MVA pathway enzymes are located in the cytosol or associated with the ER, peroxisomes have been discussed as localization sites for AACT (particularly AACT1 in *Arabidopsis*), PMK, and MVD based on the prediction of peroxisomal PTS targeting peptides and transient protein peroxisome import studies in *Catharanthus roseus* cells [108–110]. For MVD1 in *Arabidopsis*, however, mass spectrometry analysis suggests a cytosolic localization and MVD2 is predicted to reside in the cytosol [6]. In the absence of additional evidence for a partial localization of the MVA pathway in peroxisomes and possible transporters of isoprenoid precursors between the compartments, our current view on the subcellular organization of the MVA pathway remains incomplete.

The exchange of intermediates between the cytosol and plastids is usually not sufficient to rescue *Arabidopsis* mutants of biosynthetic enzymes in the MVA or

MEP pathways [31, 43, 64]. However, studies on *dxs2* mutants in tomato suggested that both pathways can, to some extent, compensate each other [66]. Moreover, in numerous cases, some degree of exchange of isoprenoid intermediates between plastids and the cytosol has been demonstrated based on the application of MEP and MVA pathway-specific inhibitors and the incorporation of stable-isotope precursors in primary and specialized terpenoid metabolites (e.g., [100, 111–117]). There is frequent evidence for trafficking of isoprenoid intermediates from the plastid to the cytosol in photosynthetic tissues (e.g., [113]). However, the contribution of the MVA pathway to the biosynthesis of plastidial isoprenoids can be substantial in the absence of light as was demonstrated by Opitz et al. [118] in roots of cotton seedlings or in dark-grown *Arabidopsis* seedlings [92].

To date, no specific transporters of isoprenoid precursors have been identified in the plastid membrane. The export of IPP from plastids to the cytosol was suggested to proceed by a plastidial proton symport system [119]. Studies by Flüge and Gao [120] indicated that IPP is not transported by plastidic phosphate translocators but depends on phosphorylated counter-substrates. In addition to the transport of IPP, there is evidence that longer prenyl diphosphates such as GPP and FPP are moved from plastids to the cytosol in tomato [121], the grape berry exocarp [122], and glandular trichomes of *Stevia rebaudiana* [116]. Genomic and proteomic analyses of single cells such as trichomes could be a promising approach to identify the isoprenoid transporter machinery between both compartments.

Despite some degree of exchange of isoprenoid intermediates between the plastid and the cytosol, the spatial separation of terpenoid biosynthetic pathways has been of benefit for the engineering of terpenoid end products. Expression and targeting of an FPP synthase and sesquiterpene synthase to plastids in tobacco did prevent carbon flux competition with sterol biosynthesis in the cytosol and promoted sesquiterpenoid yields by a thousandfold [123]. The same approach was successfully applied to produce high levels of the triterpene squalene in plastids and in tobacco trichomes although the latter case came at the cost of severely reduced growth [124]. Efforts have also been made to insert the entire MVA pathway in the tobacco chloroplast genome resulting in increased levels of mevalonate and carotenoids, but also squalene and sterols [125].

As mentioned above, expression of the MEP and MVA pathway genes is coordinately regulated by external stimuli. Other interdependent mechanisms of regulation between the pathways have been detected at posttranslational levels. Recent studies in tobacco demonstrated that blocking MEP pathway-dependent protein geranylgeranylation by treatment with the monoterpene *S*-carvone suppresses signaling to induce the MVA pathway-dependent formation of the sesquiterpene phytoalexin capsidiol [126]. Other possible roles of multicompartment networks in regulating the MVA pathway have been addressed by Verbitskiy et al. [127]. Work by these authors on proteins involved in RNA editing suggests that retrograde signaling between mitochondria and the cytosol might modify MVA pathway activity and, according to Tang et al. [128], this interaction seems to involve the mitochondrial respiratory pathway. Most notably, the MEP pathway intermediate, MEcPP, was found to function as a retrograde signaling molecule

between plastids and the nucleus. MEcPP elicits the expression of stress-responsive nuclear-encoded plastidial proteins which suggests that the MEP pathway functions in stress sensing and coordinating stress-induced nuclear genes [129].

3 Isomerization and Condensation of the C5 Building Blocks

The construction of terpenoids with more than five carbons requires a sufficient supply of IPP and its more reactive, electrophilic isomer DMAPP. Therefore, IPP derived from the MVA pathway needs to be converted to DMAPP by the activity of an IPP isomerase (IDI; Figs. 1 and 2). Type I IPP isomerase isoenzymes in plants have been localized to mitochondria and plastids and shorter isoforms have been predicted to remain in the cytosol [130]. In analogy to mammalian cells, an alternative localization of IPP isomerases in peroxisomes has been discussed [108] but additional evidence for the role of peroxisomes in plant isoprenoid metabolism is needed. Although the formation of DMAPP from IPP derived from the MVA pathway is essential for downstream reactions in the cytosol and mitochondria, IPP isomerization seems less important in plastids where both C5 building blocks are produced by the MEP pathway. However, plastidial IPP isomerase activity might be necessary to produce an optimal ratio of IPP and DMAPP for the downstream condensation reactions and to provide precursors for a possible transport to the cytosol.

In the second major stage of terpenoid biosynthesis, IPP and DMAPP units are fused by the catalytic activity of prenyltransferases (isoprenyl diphosphate synthases) to form prenyl diphosphates as the linear central precursors of all terpenoids (Fig. 2). The initial reaction catalyzed by a prenyltransferase is a head-to tail (1'–4) condensation of IPP with the allylic cosubstrate DMAPP based on an ionization–condensation–elimination mechanism to produce a C10-allylic diphosphate. Additional rounds of head-to-tail condensation of the allylic product with more IPP units lead to the formation of short-chain (C15–C25), medium-chain (C30–C35), and long-chain (C40–Cn) prenyl diphosphates. The *cis*- or *trans*-stereochemistry of the double bonds of the prenyl diphosphate product determines whether the enzyme operates as *cis*-prenyltransferase or *trans*-prenyltransferase, which belong to families of structurally unrelated enzymes [131]. Much knowledge has been gained on the biochemistry and evolution of short-chain *trans*-prenyltransferases, which synthesize C10-geranyl diphosphate (GPP), C15-*trans,trans*-farnesyl diphosphate ((*E,E*)-FPP), or C20-all-*trans*-geranylgeranyl diphosphate (all-*trans*-GGPP) as the main precursors in terpenoid metabolism, although more recent work has discovered similar roles of previously undetected short-chain *cis*-prenyltransferases (see below).

3.1 Geranyl Diphosphate Synthases

As a precursor in the biosynthesis of C10-monoterpenoids, GPP is synthesized from IPP and DMAPP by the activity of GPP synthase enzymes (GPSs), which are usually targeted to plastids (Fig. 2). Different classes of homodimeric and heterodimeric/tetrameric GPSs have been identified in plants [132–136] (Fig. 2). A heterotetrameric GPS from peppermint was the first GPS to be discovered in plants [137] and since then related heterodimeric proteins have been found in a variety of other species such as *Anthirrinum majus*, *Clarkia breweri*, and *Humulus lupulus* [137–139]. The enzymes consist of a large subunit (LSU), which has significant homology (~50 %) to GGPP synthases (GGPS, see below) and can exhibit GGPP synthase activity as a recombinant protein, and a small subunit (SSU I) that shares only ~20 % sequence similarity with homomeric prenyltransferases and is functionally inactive. It is generally thought that binding of SSU I modifies the activity of the LSU to produce GPP. The importance of the physical interaction of both subunits to make GPP has been confirmed by structural analysis of the heterotetrameric GPS from peppermint [134]. In *Arabidopsis*, Wang and Dixon [139] identified a separate lineage of SSU (SSU II) genes encoding GGPS-related proteins (GGR). *Arabidopsis* GGR modifies the in vitro activity of GGPS 11 to produce GPP and contains two conserved CxxxC motifs that are essential for the interaction of both subunits [139]. In contrast to the role of SSU I-containing GPSs in monoterpene formation in peppermint or hops, the function of heterodimeric GPSs carrying SSU II subunits is less clear because of the absence of a tight correlation between protein expression and the biosynthesis of monoterpenes in different tissues [139].

Engineering of GPS activity has been achieved by the expression of GPS.SSU I from snapdragon in tobacco and tomato fruits. The expressed subunit recruits plastidial GGPS proteins to form functionally active heterodimeric GPS proteins [121, 140]. The study on tomato also revealed that GPP produced in plastids is exported to the cytosol, where it can be used for monoterpene biosynthesis [121]. However, the exchange of GPP between both compartments might be limited in the absence of engineered GPP pools as was shown for a bifunctional *Arabidopsis* monoterpene/sesquiterpene synthase (TPS02), which is located in the cytosol and produces sesquiterpenes but no monoterpenes in planta [42].

Homodimeric GPS enzymes have been described from angiosperms and gymnosperms [135, 136, 141, 142]. These proteins belong to different lineages and are evolutionarily related to GGPSs (see below). The existence of a homodimeric GPS in *Arabidopsis* has been discussed controversially. A single *GPS1* gene was originally identified to encode a functionally active GPS enzyme [143]; however, more recently the GPS1 protein has been characterized as a multiproduct medium-/long-chain prenyl diphosphate synthase. The latter activity was observed when IPP was supplied in excess to the allylic substrates DMAPP, GPP, and FPP and was supported by the structural analysis of an active-site cavity with sufficient size to accommodate the medium-/long-chain products [144]. The GPS1 protein (renamed

by Hsieh et al. as polyprenyl di(pyro)phosphate synthase, PPS) is targeted to plastids [143] where IPP and DMAPP are produced at ratios of approximately 5:1 by the MEP pathway. Thus, it is possible that this enzyme exhibits a PPS activity in vivo.

3.2 Farnesyl Diphosphate Synthases

Trans-FPP synthases (FPSs) catalyze the formation of (*E,E*)-FPP as a central precursor in the biosynthesis of terpene primary metabolites (phytosterols, brassinosteroids, dolichols, ubiquinones), for protein prenylation, and in the production of specialized metabolites such as sesquiterpenoids and triterpenoids (Fig. 2). As type I (eukaryotic) FPSs, plant *trans*-FPSs build a superfamily of homodimeric enzymes that are often encoded by small species-specific gene families (e.g., [145–147]). FPS isozymes of different size that are produced as a result of differential gene transcription have been localized to the cytosol or the mitochondria where they produce FPP pools for the biosynthesis of cytosolic and mitochondrial downstream products [148] (Fig. 2). Targeting of FPSs to peroxisomes has been discussed based on YFP fusion experiments in *Catharantus roseus* cells [149]. However, no peroxisomal targeting has been demonstrated for fluorescent FPS fusion proteins in *Arabidopsis*, which is consistent with results from proteomic studies of the cytosol and purified peroxisomes [150, 151].

As with the isozymes of the MEP and MVA pathways, it has been a primary interest to elucidate the possible functional differences of prenyltransferase isoforms. In *Arabidopsis*, the two *FPS* paralogues, *FPS1* and *FPS2*, have overlapping expression patterns and can rescue each other's loss, whereas double mutants are impaired in male genetic transmission and arrested at early embryo development [152]. However, there is no complete functional redundancy between the two isozymes inasmuch as *FPS2* is the predominantly expressed isozyme in mature seeds and early seedling development, and *FPS1* appears to be only expressed in the maternal seed coat [153]. Consequently, seeds of *fps2* mutants have a reduced sterol content [152]. Keim et al. propose that the specific expression of *FPS2* in mature seeds is related to its higher enzymatic activity and thermal stability. The authors further speculate that during early development of the embryo (in the absence of *FPS2* expression), FPP might be imported from the seed tissue where *FPS1* is expressed [153].

3.3 Geranylgeranyl Diphosphate Synthases

Similar to (*E,E*)-FPP, all-*trans*-GGPP synthesized by all-*trans*-GGPSs is a major branching point for several downstream terpenoid pathways in primary and specialized metabolism. These include the biosynthesis of carotenoids and their

breakdown products (abscisic acid, strigolactones), chlorophylls, tocopherols, gibberellins, plastoquinones, and diterpenoids (all synthesized in plastids), geranylgeranylated proteins and poly-/oligoprenols (synthesized in the cytosol), and poly-/oligoprenols synthesized in the plastids and mitochondria (Fig. 2). Compared to FPSs, GGPS isozymes are represented by larger gene families. For example, the *Arabidopsis* genome contains 12 *GGPS* paralogues, of which 10 have been identified to encode functional GGPS proteins of most likely homodimeric architecture and with GGPP as the primary or sole product [154]. The different GGPS isozymes are located in the plastids, mitochondria, and the ER consistent with the subcellular compartmentalization of the diverse GGPP-dependent terpenoid pathways. With the exception of two of the *Arabidopsis* isozymes (GGPS1-mitochondrial, GGPS11-plastidial), which are expressed in the whole plant, the remaining family members exhibit distinct spatiotemporal expression patterns [154]. Seedling-lethal albino and embryo-lethal phenotypes are found in *gpps1* mutants, indicating that GGPS1 has essential functions in development and the chlorophyll biosynthetic pathway [155]. Although possible redundant or more specific functions of most of the GGPS isozymes are not well understood, it is apparent that the divergence in the *Arabidopsis* *GGPS* gene family is the result of functional specialization and fine-tuning of metabolic pathways in different cellular compartments and in tissues at different developmental stages or under different environmental conditions.

Both FPPS and GGPPS proteins have been expressed in modules with sesquiterpene synthases and diterpene synthases, respectively, to engineer the biosynthesis of sesquiterpenoids and diterpenoids in microbial systems and in planta [123, 156]. Specifically, the buildup of FPP pools in plastids improved the precursor supply and allowed for a substantial increase in yield of the desired sesquiterpene products [123]. Other strategies to improve pathway productivity include generating combinatorial mutations in prenyldiphosphate synthase and downstream terpene synthases. For example, prokaryotic expression of pathway variants of a GGPPS and a terpene synthase, which produces a levopimaradiene diterpene precursor in ginkgolide biosynthesis, led to a more than 2,000-fold increase in the levels of the levopimaradiene product thereby stressing the importance of protein engineering in these approaches [157].

3.4 Chain Length Regulation and Evolution of Prenyltransferases

Structural analysis combined with random or site-directed mutagenesis has provided substantial insight to the chain length regulation of short-chain prenyltransferase products [158]. Based on crystal structures of several homodimeric FPPs and GGPS from eukaryotes and prokaryotes [159–165], short-chain prenyltransferases share a common protein fold composed of 13 α -helices with 10 helices surrounding the active site cavity. IPP and the allylic substrate are bound by two highly

conserved aspartate-rich regions, a first DDx2-4D motif (FARM) and a second DDxxD motif (SARM), which are positioned on opposite walls of the cavity. Product chain length is in part regulated by amino acid residues upstream of the FARM motif (position -4, -5), which change the size of the hydrophobic substrate binding or elongation pocket of the polyisoprenoid chain [166, 167]. According to this mechanism, type I FPSs such as *Arabidopsis* FPS1 and FPS2 have a smaller binding pocket because of the presence of “bulkier” aromatic amino acid residues. In type II GGPSs, which comprise eubacterial and plant GGPSs, these aromatic amino acids are replaced by smaller residues such as alanine, serine, and methionine allowing the formation of a longer C20 chain. Studies of yeast GGPS indicated that chain termination at C20 depends on residues located deeper in the catalytic cavity [162]. Poulter and colleagues recently employed a large-scale bioinformatics approach combined with experimental enzyme characterization, protein crystallization, and computational modeling to predict the chain length specificity of a large number of putative polyprenyl transferases [168]. The approach, which resulted in a high rate of correctly predicted functions, largely supported the notion that steric hindrance in the elongation cavity is the main criterion determining chain length specificity. It is important to note that the study also suggested a chain-length-determining effect of “second shell” residues that are positioned in the vicinity of the residues lining the elongation pocket. Depending on their size, these neighboring residues may or may not provide flexibility for bulkier aromatic residues that protrude into the cavity to be moved or displaced by the growing polyprenyl chain [168].

Phylogenetic analyses of prokaryotic and eukaryotic prenyltransferases place plant FPSs in a clade with other eukaryotic FPSs that is distinct from a cluster containing plant GPS and GGPS proteins [158]. A comprehensive phylogenetic study of GGPS and GPS homologues of land plants and green algae demonstrated a lineage and species-specific expansion of GGPS families indicating gene duplication events and functional divergence [169]. The phylogeny shows several evolutionary transitions from proteins with GGPS to GPS activity. For example, gymnosperm homodimeric GGPSs, which form a distinct clade among plant GGPSs, can produce shorter prenyldiphosphates or synthesize exclusively GPP [133, 142]. In comparison to GGPSs from green algae and mosses that possess the FARM and SARM motifs and a conserved CxxxC motif, the gymnosperm GGPSs have acquired a second CxxxS (bifunctional GGPS) or CxxxC (GPS) motif. The two CxxxC motifs are characteristic of most proteins that are associated with GPS activity. Thus, they are present in the SSU I and SSU II subunits of heteromeric GPS proteins and critical in binding the LSU. The binding of both subunits limits access to the elongation cavity and terminates chain elongation at the formation of a C10-product [134]. SSU I and SSU II proteins have lost both aspartate-rich motifs or carry a mutated SARM, respectively, which is associated with the loss of prenyl diphosphate activity [135, 169]. Interestingly, an earlier study reported a flower-specific GPS from orchids with similarity to SSU II [136]. This protein lacks the SARM but maintains GPS activity as a homodimeric enzyme (Fig. 2).

Several proteins with homology to *Arabidopsis* PPS (former GPS1) have been reported from other plants and designated as homodimeric GPSs (Fig. 2). These proteins do not carry the CxxxC motifs and it remains to be determined whether they function as true GPSs in vivo or may exhibit medium-chain or long-chain polyprenyl diphosphate activity as was shown for the *Arabidopsis* enzyme. For instance, GPS activity was demonstrated for a protein in tomato, but assays were performed at a low IPP/DMAPP ratio [141]. Furthermore, silencing or mutation of this enzyme and of PPS in *Arabidopsis* resulted in dwarfed or embryo lethal phenotypes, which could be related to promiscuous GGPS activity to produce GGPP for gibberellin biosynthesis or the synthesis of longer precursors in plastoquinone biosynthesis. The formation of longer chain products by *Arabidopsis* PPS is also supported by the absence of aromatic amino acids near the FARM. Computational predictions such as those presented by Wallrapp et al. [168] should facilitate determining the chain length specificity of PPS homologues. In summary, GPS activity appears to be the result of promiscuity and neofunctionalization of GGPS (or PPS?) proteins in conjunction with the evolutionary adaptation of individual plant lineages to produce monoterpenes as constituents of floral scent or for chemical defense.

3.5 *Cis*-Isoprenyl Diphosphate Synthases

One of the surprising findings in the field of terpene biosynthesis in the past five years was the identification of short-chain *cis*-prenyltransferases (CPTs) and the conversion of their *cis*-prenyl diphosphate products to terpenoids by the activity of terpene synthases (see below). Prior to this discovery, it was generally believed that CPTs synthesize prenyl diphosphate products with a chain length of more than 50 carbons by using all-*trans* short-chain prenyl diphosphates as allylic primer substrates [170]. Such prenyltransferases in plants include enzymes that produce C70–C120 dehydrodolichol diphosphates or natural rubber (>C10,000) from (*E,E*)-FPP by head-to-tail condensations in a *cis* orientation [170–172]. Functional genomics studies of terpene biosynthetic genes in glandular trichomes of wild tomato then revealed the presence of a short-chain (*Z,Z*)-FPP synthase that produces (*Z,Z*)-FPP [173] (Figs. 2 and 3). Characterization of a nine-member CPT family in cultivated tomato gave additional evidence for short-chain enzyme activity by the identification of three genes encoding a neryl diphosphate (NPP) synthase (NDPS1 or SICPT1, expressed in trichomes), a (*Z,Z*)-FPP synthase (SICPT6, expressed in root and fruit), and a neryl neryl diphosphate (NNPP) synthase (NNDPS or SICPT2, expressed in the stem), respectively [174] (Figs. 2 and 3). All three proteins are targeted to plastids [174]. Notably, the *Z,Z*-FPP pool produced by (*Z,Z*)-FPP synthase in trichome-specific plastids in wild tomato is used by plastidic sesquiterpene terpene synthases (santalene/bergamotene sesquiterpene synthase [173] and 7-epizingiberene synthase [175, 176]), which are related to diterpene synthases. Engineering of (*Z,Z*)-FPP synthase and 7-epizingiberene synthase in trichomes of



levels in fruits because of feedback inhibition of GGPS by NPP. Based on these findings, it is plausible that NPP production is restricted primarily to trichomes to avoid inhibitory effects on carotenoid biosynthesis.

The association of the tomato *CPT* genes with terpenoid biosynthetic gene clusters [174] clearly indicates adaptive functional specialization in the tomato *CPT* gene family to provide short-chain prenyl diphosphates for different terpene biosynthetic pathways including trichome-specific terpene biosynthesis. In line with these findings, a *cis*-type prenyltransferase was identified in lavender that catalyzes the head-to-middle condensation of two DMAPP molecules to synthesize lavandulol diphosphate, the precursor of lavandulol [179]. Furthermore, in the nine-member *CPT* gene family of *Arabidopsis* a multiproduct prenyltransferase (AtCPT6) has been identified that makes polyisoprenoid diphosphates with six to eight isoprene units as precursors of polyisoprenoid alcohols in roots [180].

As with *trans*-prenyltransferases, efforts have been made to determine amino acid residues that control the chain length specificity of CPTs [170]. Sequences of CPTs share five conserved regions and employ residues for substrate binding and catalytic activity that are different from those of *trans*-prenyltransferases [170]. Kang et al. [181] exploited accession-specific sequence differences of NDPS and (*Z,Z*)-FPP synthase in tomato coupled with homology modeling and site-directed mutagenesis to identify four residues in region II that are important for product specificity. These residues are part of helix II, which, together with helix III, lines a hydrophobic cleft that influences product chain length [182, 183].

4 Conversion of Prenyl Diphosphates and Terpene Synthase Function and Regulation

Trans- and *cis*-prenyldiphosphates are the entry points to various downstream primary and specialized terpenoid biosynthetic routes in plastids, mitochondria, and the cytosol (summarized in Fig. 2). It is beyond the scope of this chapter to address all of these pathways and the reader is referred to other chapters in this series (e.g., carotenoid biosynthesis) or more specialized recent reviews in the field.

The tremendous diversity of terpenoids in specialized metabolism can to a large extent be attributed to the activity of terpene synthases (TPSs; Fig. 3a). TPS enzymes have, therefore, become a focus point of in planta and heterologous metabolic engineering of terpenoid end products with use as pharmaceuticals, flavors, biofuels, or plant chemical defenses [184] (see other chapters in this series). The TPS superfamily, which is divided into eight subfamilies (TPSa–h), comprises a large and still growing number of enzymes from almost all taxa in the plant kingdom [185]. TPSs convert acyclic C5 to C20 *cis*- or *trans*-prenyl diphosphate intermediates into C5-hemiterpenes such as isoprene, C10-monoterpenoids, C15-sesquiterpenoids, or C20-diterpenoids (Fig. 2). The primary enzymatic products are in most cases acyclic or cyclic hydrocarbons (Fig. 3a) that are frequently modified by secondary enzymatic

reactions such as hydroxylation, peroxidation, methylation, acylation, glycosylation, or cleavage to produce biologically active end products of even larger structural diversity [186]. TPS enzymes facilitate adaptations of terpene metabolism to the changing environment because their promiscuous activity often results in the production of more than a single compound (e.g., [187]) and TPS proteins easily acquire new catalytic properties by minor structural changes [187–192].

Mechanistically, TPS proteins are divided into class I and class II enzymes. The enzymatic reaction catalyzed by class I TPSs starts with the ionization of the prenyl diphosphate substrate by a divalent cation-dependent subtraction of the diphosphate moiety. The produced carbocation intermediate then enters different reactions that can include cyclizations, hydride shifts, and rearrangements prior to a termination of the reaction by proton loss or the addition of a nucleophile such as water [193] (Fig. 3). By contrast, class II TPSs, which include oxidosqualene cyclases (see below) and diterpene synthases, catalyze the ionization of their substrate by adding a proton to an epoxide ring or via protonation at the 14,15-double bond of GGPP, respectively. Class II diterpene synthases that fall into this category are *ent*-copalyl diphosphate (CPP) synthases (CPSs), which are involved in gibberellin and phytoalexin biosynthesis [194] (Fig. 2). In the gibberellin biosynthetic pathway, CPSs catalyze a protonation-induced bicyclization of the substrate GGPP to form *ent*-CPP, which is further ionized and converted to *ent*-kaure-16-ene by a class I *ent*-kaurene synthase (KS) activity. Detailed genomic studies of land plants revealed that the gibberellin biosynthetic pathway gave rise to the biosynthesis of an array of specialized labdane-related diterpenoids largely by gene duplication and divergence of CPS and KS homologues [194]. The ability to produce kaurene arose early in land plant evolution as can be assumed from the identification of a bifunctional class II/I CPS/KS in the moss *Physcomitrella patens*, which catalyzes the formation of *ent*-kaurene (and 16-hydroxykaurene) via a CPP intermediate in the biosynthesis of kaurenoic acid [195, 196]. Similar class II/I diterpene synthases such as abietadiene synthase occur in gymnosperms and can be considered early diterpene synthases. These enzymes produce (+)-CPP from GGPP prior to an ionization-initiated cyclization of (+)-CPP to the diterpene product [197]. An interesting new view on the evolution of plant TPS genes comes from a genomic study of a large TPS gene family in the fern *Selaginella moellendorffii* [198]. Two distinct types of TPS genes were identified: a group of diterpene synthases that represent a new plant TPS-h subfamily, and, surprisingly, a group of monoterpene synthases and sesquiterpene synthases that are more closely related to microbial TPSs and may be the first indication for a horizontal gene transfer of TPS genes [198].

It should be noted here that, recently, a new mechanism for the enzymatic formation of cyclic terpenes was discovered in the iridoid monoterpene biosynthetic pathway [199]. Iridoids have pharmaceutical and antibacterial activities and are also produced by aphids as pheromones [200, 201]. The iridoid synthase from *Catharantus roseus* is a short-chain reductase that most likely generates a C5-iridoid ring in the linear monoterpene 10-oxogeranial substrate by coupling a reduction step with a cyclization step via a Diels–Alder cycloaddition or a Michael addition [199].

This exciting finding may open the way for future discovery of similar reductase-type terpene cyclases in plants and other organisms.

More insight to the evolution of “regular” TPS enzymes has been gained from the analysis of an increasing number of crystal structures including those from an isoprene synthase [202], monoterpene synthases [203–206], sesquiterpene synthases [207, 208], a class I diterpene synthase (taxadiene synthase [209]), a class II CPP synthase [210, 211], and a class II/I diterpene synthase (abietadiene synthase [212]). Comparisons of the assembly of a class I type α -domain and class II type β and γ domains led to the prediction of an evolutionary scenario according to which an ancestral bifunctional classII/classI diterpene synthase (consisting of all three domains with a functional α - and β -domain) similar to the CPS/KS enzyme of *P. patens* gave rise to class II type diterpene synthases (consisting of all three domains with a functionally active β -domain and an inactive α -domain) and class I type TPSs (consisting of a nonfunctional β -domain and a functionally active α -domain) [213, 214]. A functionally active class I α -domain carries the highly conserved aspartate-rich motif, DDxxD, and a less conserved NSE/DTE motif, which are located on opposite sides of the entrance of the catalytic site and help position the diphosphate substrate by binding of a trinuclear magnesium cluster [215]. By contrast, functional class II β -domains carry a conserved DxDD motif, which is required for protonation-initiated carbocation formation [213].

Although TPS enzymes may convert more than one prenyl diphosphate substrate in vitro, their function in vivo is largely determined by the substrate pool that is available in the respective cellular compartment. In this regard, TPS enzymes localized in plastids generally produce monoterpenoids or diterpenoids from plastidial GPP and all-*trans*-GGPP, respectively, whereas TPSs in the cytosol primarily convert (*E,E*)-FPP to sesquiterpenes (or squalene in the biosynthesis of C30 terpenes). However, this general rule has recently been challenged by the discovery of plastidial (*Z,Z*)-FPSs and sesquiterpene synthases in tomato, the latter of which are more closely related to kaurene synthases in the TPS-e subfamily [177].

The existence of medium-size to large TPS families in *Arabidopsis* and many other plant species strongly supports the notion that TPS genes evolve by gene duplication and neofunctionalization [185, 216, 217]. Such duplication events combined with relocation in the genome can include other genes that encode modifying enzymes such as cytochrome P450s, and thus lead to the assembly of gene clusters. From the first discovery of a thalianol triterpene biosynthetic gene cluster in *Arabidopsis* [218], several such clusters have been found in the arabidiol, marneral, and avenacin triterpene biosynthetic pathways in *Arabidopsis* and oat, respectively [219, 220] (Sohrabi et al. in preparation), and for the biosynthesis of labdane-related diterpenoids in rice [221] or monoterpenoids and sesquiterpenoids in tomato [222]. The triterpene biosynthetic clusters carry genes for oxidosqualene cyclases (OSCs), which catalyze the cyclization of oxidosqualene to one or more cyclic triterpene alcohols via formation of a carbocationic intermediate [223, 224]. Coexpression with other cluster genes (e.g., P450s, desaturase, acyltransferase) in an operon-like manner then allows a consecutive derivatization of the triterpene precursor [218–220]. The evolutionary forces driving this coordinated gene cluster

assembly are believed to be twofold. Clustering of genes for pathway building facilitates the regulation of multiple genes at the level of chromatin and/or prevents the accumulation of possible cytotoxic products [219, 225, 226]. However, a strict coregulation of gene expression does not seem to be the case in all clusters as was shown for a diterpene biosynthetic cluster in rice containing P450s that are differentially regulated and function in two different pathways [194].

Clusters that exhibit a coordinated expression of their genes have allowed the identification of putative key regulators such as in the case of the basic leucine zipper transcription factor, OsTGAP1, which is involved in regulating a diterpenoid biosynthetic gene cluster in rice [227]. Another transcription factor that was identified previously to regulate terpene biosynthetic genes positively is a WRKY transcription factor in cotton, GaWRKY1, which regulates the transcription of a sesquiterpene synthase gene in the gossypol biosynthesis pathway [228]. More recent studies on *Artemisia annua* suggest that APETALA2/ethylene-response factors (AP2/ERF) are positive regulators of biosynthetic genes in the formation of the sesquiterpene artemisinin, an insect deterrent and antimalaria drug produced in leaf glandular trichomes [229]. However, these studies thus far do not place the identified transcription factors into regulatory networks related to development and cell specification.

A better understanding of the regulatory networks controlling terpene volatile formation has been gained in the process of flower maturation in *Arabidopsis*. Two R2R3 MYB transcription factors, MYB21 and MYB24, were identified that promote gynoecium growth and nectary development and positively affect expression of the major floral (*E*)- β -caryophyllene sesquiterpene synthase *TPS21* [230]. Both MYB TFs respond positively to jasmonic acid (JA), the levels of which are induced by the auxin response factor 6 (ARF6) and ARF8, both master regulators of flower maturation. *TPS21* and the second floral sesquiterpene synthase, *TPS11* [187], also respond more directly to JA by the direct binding of their promoters to the bHLH transcription factor MYC2 [231], which is a central regulator of the JA signaling pathway in developmental and stress responses [232, 233]. In addition, *TPS21* and *TPS11* gene expression is indirectly regulated by gibberellins through the binding of DELLA proteins (gibberellin signaling repressors) [231].

Similar to the tissue-specificity of terpene formation in flowers, terpene-specialized metabolism in roots appears to be a highly coordinated cell type-specific process. Genes of the thalianol and marnal triterpene biosynthetic gene clusters are coexpressed primarily in the root epidermis [218, 219]. Likewise, 14 genes of the *Arabidopsis* *TPS* family are expressed in different root tissues. For example, a recently identified rhizathalene diterpene synthase (*TPS08*; Fig. 4) was found to be primarily expressed in the root stele (see below) [234]. In addition, two 1,8-cineole monoterpene synthase genes are constitutively expressed in the stele of the root elongation zone and differentiation/maturation zone and in the epidermis and cortex of more mature roots; a similar expression pattern has been observed for two closely related (*Z*)- γ -bisabolene sesquiterpene synthases [188, 235]. However, no networks of temporal and spatial regulation have yet been defined for these root-specific genes.

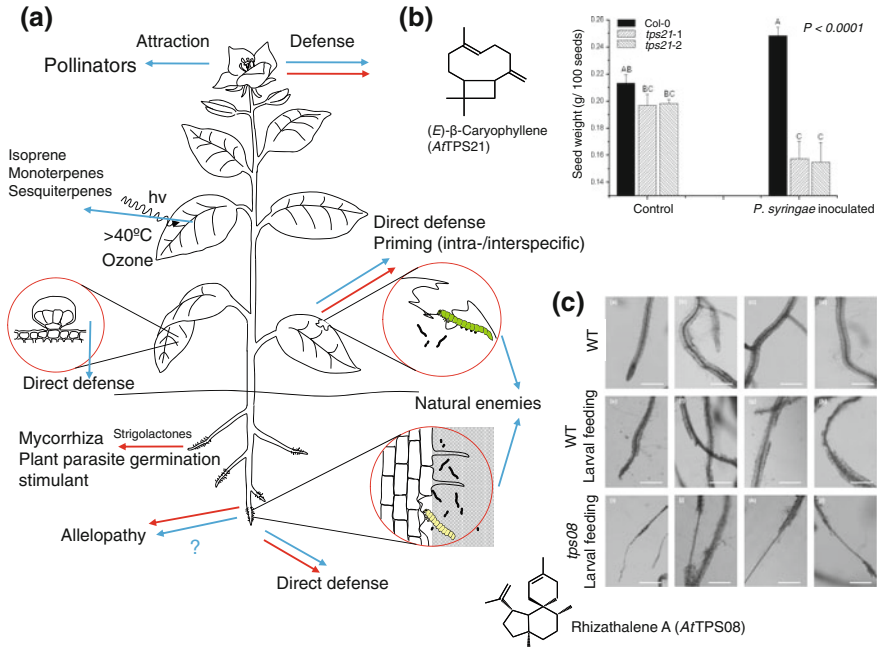


Fig. 4 Biological functions of plant terpenoids. **a** Functions of volatile terpenoids (blue arrows) and nonvolatile terpenoids (red arrows) in the interactions of plants with their environment. **b** Defensive activity of the volatile sesquiterpene, (*E*)- β -caryophyllene, against infection of *Arabidopsis* flowers by the microbial pathogen *Pseudomonas syringae*. Mutants deficient in (*E*)- β -caryophyllene biosynthesis in the floral stigmatic tissue (*tps21*) produce lighter seeds post inoculation of flowers with *P. syringae*. **c** Antifeedant activity of the semivolatile diterpenoid, rhizathalene, in *Arabidopsis* roots. Light microscopic pictures of roots of wild-type and rhizathalene biosynthetic mutants (*tps08*) with and without feeding by *Bradysia* (fungus gnat) larvae. Increased feeding damage is observed in the absence of the diterpenoid compound (**b, c** from Huang et al. 2012 [245] and Vaughan et al. 2013 [234], www.plantcell.org, Copyright American Society of Plant Biologists partially modified)

5 Multifunctionality of Plant Terpenoids

Although terpenoids serve important primary functions as photosynthetic pigments (carotenoids), electron carriers (side-chains of ubiquinone and plastoquinone), regulators of growth and development (gibberellins, abscisic acid, strigolactones, brassinosteroids, cytokinins), in protein glycosylation (dolichols), or as elements of membrane structure and function (phytosterols), specialized terpenoid metabolites (covered here), in particular, have been recognized for an array of biological roles. Volatile or semivolatile, low-molecular-weight terpenoids, which include isoprene, monoterpenoids, sesquiterpenoids, and diterpenoids, are implicated in the protection of plants against abiotic stress and in various biotic interactions above- and below-ground [236] (Fig. 4a). The substantial emissions of isoprene and monoterpenes from

various vascular and nonvascular plants have been associated with the protection against thermal stress. This process is presumably based on an intercalation of the volatile compounds with the photosynthetic membranes and thereby enhances membrane functionality [237–239]. Moreover, transgenic approaches in tobacco and poplar support a role of isoprene in oxidative stress protection [240–243] and are addressed in a separate chapter by Vickers et al.

Volatile terpenoids as constituents of floral scent are implicated in mutualistic interactions with plant pollinators. For instance, choice tests with bumblebees have indicated a role of monoterpenoids emitted by monkeyflowers in pollinator attraction [244]. Nevertheless, distinct evidence for a specific role of terpenoids in pollinator attraction by the use of biosynthetic mutants is still missing, but it can be assumed that attractive effects depend on mixtures of volatiles rather than individual compounds. The notion that floral volatile terpenoids serve multiple functions has been supported by their role in the defense of floral tissues against microbial pathogens. This interaction was demonstrated in flowers of *Arabidopsis* mutants, which lack the emission of (*E*)- β -caryophyllene from their stigmatic tissue. The mutant flowers were more susceptible to infection by *P. syringae*, which resulted in lighter and often misshaped seeds suggesting reduced plant fitness [245] (Fig. 4b). Similar findings were made by Junker et al. [246] demonstrating that floral volatiles play roles in the structuring of bacterial communities that colonize flower petals by providing compound-dependent niches.

Volatile terpenoids also serve important functions as constitutive or pathogen- and herbivore-induced compounds in the defense of photosynthetic tissues. For example, repellent activities have been reported for monoterpene volatiles that are emitted by leaves of *Chrysanthemum morifolium* and, notably, herbivore-deterrent effects have been observed for isoprene [247, 248]. Furthermore, volatile terpenoids that accumulate in glandular trichomes function as insect repellents as was, for example, found for the activity of sesquiterpenes in trichomes of wild tomato against white flies [249] (Fig. 4a). In conifers, the production of terpenoid oleoresin and terpenoid volatile emissions constitute an important chemical defense system [250]. In a search for resistance factors, the monoterpene (+)-3-carene was found to be associated with resistance of Sitka spruce (*Picea sitchensis*) to white pine weevil (*Pissodes strobi*) [251]. Variation of the (+)-3-carene production in resistant and susceptible trees was demonstrated to depend on the copy number of a (+)-3-carene *TPS* gene, differences in gene transcript and protein levels, and variation in catalytic efficiencies. Similarly, in *Arabidopsis*, ecotype-specific variation of the herbivore-induced volatiles, (*E*)-beta-ocimene and (*E,E*)-alpha-farnesene, is controlled by allelic variation and differences in subcellular targeting of the two terpene synthases, *TPS02* and *TPS03* [42].

The role of herbivore-induced volatile blends in the attraction of natural enemies of herbivores (Fig. 4a) and at higher trophic levels has been investigated in numerous studies (reviewed by [252, 253]). Work with transgenic *Arabidopsis* provided strong evidence for the role of volatile terpenes in these interactions [254–256]. However, as indicated for floral scent, the effect of these compounds has to be considered in the context of the entire herbivore-induced volatile blend, and actual fitness benefits to

the plant host under natural conditions are still debated [252, 257, 258]. Indirect defense responses mediated by volatile compounds also occur upon insect oviposition [259]. For example, egg deposition on the foliage of European field elm (*Ulmus minor*) by the elm leaf beetle (*Xanthogaleruca luteola*) leads to the emission of volatiles including the irregular homoterpene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), which play a role in the attraction of the specialist egg parasitoid, *Oomyzus gallerucae* [260].

In addition to their function in the interaction with herbivores and their enemies, constitutive and induced volatile mixtures (including volatile terpenes such as homoterpenes) can serve as interspecific, intraspecific, and intraplant “alarm” signals to prime or induce defense responses in neighboring plants or in unattacked tissues of the same plant [261–264]. In these interactions, volatiles may not necessarily need to enter the leaf tissue of the neighboring plant but remain on the leaf surface. This effect was observed for sesquiterpenoids that are emitted by rhododendron leaves and adsorbed on the leaves of birch trees, where they exhibit direct herbivore-repellent activities [265]. Moreover, terpenoids were suggested to be involved in parasitic plant interactions, specifically, in the attraction of the parasitic plant *Cuscuta pentagona* (dodder) to establish contact with tomato as its host [266]. The molecular mechanism of host plant detection in this response as in other volatile-mediated plant–plant interactions is still poorly understood.

The described functions of volatile terpenoids in aboveground plant defense are complemented by nonvolatile terpenoids. As an example, glycosides of geranylinalool serve as potent antifeedants in the wild tobacco, *Nicotiana obtusifolia* [267], and recently detected *ent*-kaurane-related diterpenoids in maize named kauralexins as well as acidic sesquiterpenes called zealexins function as pathogen-inducible phytoalexins [268, 269]. Similarly, metabolomics studies of *Barbarea vulgaris* revealed that triterpene saponins contribute to resistance against flea beetle attack [270].

An increased interest in the role of specialized metabolites belowground has shown that terpenoids serve functions similar to those aboveground. Recent studies in *Arabidopsis* roots discovered semivolatile diterpene hydrocarbons with an unusual tricyclic spiro-hydrindane structure called rhizathalenes [234] (Fig. 4c). These compounds are produced in the root stele, from where they diffuse through the surrounding cell layers to function as local antifeedants by reducing root herbivore damage on these cell layers [234] (Fig. 4c). The role of volatile terpenes in belowground indirect defense has been well established based on studies in maize showing that the sesquiterpene, (*E*)- β -caryophyllene, which is emitted from roots upon attack by the Western corn root worm *Diabrotica virgifera*, attracts entomopathogenic nematodes [271, 272]. These findings prompted attempts to engineer (*E*)- β -caryophyllene production in nonemitting American maize cultivars, which resulted in an increased attraction of nematodes and higher resistance to corn root worm attack [273]. However, constitutive emissions of (*E*)- β -caryophyllene were found to have additional costs inasmuch as they compromise seed germination, plant growth, and yield [274]. Therefore, more fine-tuned engineering

strategies considering herbivore-induced emissions may have to be developed to circumvent these cost effects.

Nonvolatile terpenoids can be exuded from roots into the rhizosphere and the surrounding soil environment where they are involved in different defense responses. Studies using rice mutants convincingly demonstrated that labdane-related diterpenoids named momilactones exhibit allelopathic effects on barnyard grass competitors [275]. Moreover, avenacins, which are triterpene saponins exuded by the roots of oat, are known for their potent activity as phytoalexins [276]. Excitingly, a recent study by Osbourn and colleagues revealed that common triterpene precursors have additional signaling functions in root development. Specifically, it was demonstrated that β -amyirin is involved with determining the patterns of epidermal root hair cells [277]. These findings indicate that the roles of specialized metabolites in biotic interactions and potential “primary” functions become increasingly blurred. Signaling functions have also been demonstrated for the abietane diterpenoid, dehydroabietinal, which is produced at picomolar concentrations in *Arabidopsis* leaf tissue and serves as a vascular signaling compound and potent activator of systemic acquired resistance [278]. This activity seems to depend on the association of dehydroabietinal with vascular sap proteins.

Finally, it should be noted that strigolactones have become an exciting model for the multifunctionality of small molecules. As carotenoid-derived compounds (reviewed by [279]), strigolactones have important roles as exogenous signals by recruiting arbuscular mycorrhizal fungi in the rhizosphere [280]. Parasitic plants such as *Striga lutea* (witchweed) eavesdrop on these compounds by using them as germination signals [281]. As internal signals, strigolactones function as growth and developmental hormones that suppress shoot branching [282, 283]. Other processes that involve strigolactone signaling functions include root growth and development, stem elongation, secondary growth, leaf expansion and senescence, and responses to drought and salinity [279, 284]. Rapid progress has been made in understanding the perception of strigolactones but many open questions remain about downstream targets and the role of strigolactone-related compounds [279].

6 Outlook

In the past years, research in terpenoid metabolism has received a boost from developments in synthetic biology to generate engineering platforms for the production of high-value terpenoid products. Production systems in microbes have been developed to result in substantial yields [285], however, engineering of terpenoids in plants still faces challenges because of the complexity of metabolic and regulatory networks. Nevertheless, strategies to avoid metabolic flux competition by targeting biosynthetic modules to different cellular compartments have proved to be promising. Likewise, establishing pathways in specialized cells such as trichomes helps avoid metabolic competition and phytotoxic effects that could negatively affect growth and yield. The discovery of trichome-specific CPTs and TPS

enzymes with substrate specificity for *cis*-prenyl diphosphates most likely will facilitate the engineering efforts in these tissues and provide new gene tools for building synthetic modules. Despite the successful use of distinct organelles such as plastids as “mini” subcellular factories, more efforts need to be made to understand the compartmentalization of the core terpenoid pathway. Especially, additional work should be performed to clarify the putative localization of the MVA pathway and prenyltransferase enzymes in peroxisomes, which would add yet another dimension to the compartmental complexity of terpenoid metabolism in plants. Genomics-based efforts to better understand the regulation of the early terpenoid pathways and terpenoid biosynthetic gene clusters are on their way and will be essential to gain a better understanding of the regulatory networks and epigenetic factors coordinating terpenoid metabolic routes in space and time. Finally, our knowledge of the biological roles of terpenoids is still far from complete. The recent findings of overlapping activities of terpenoids such as strigolactones or triterpenoids in biotic interactions and as internal signals indicate a need to use advanced mutant-based approaches for elucidating the multifunctionality of plant terpenoid compounds.

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