11 Involvement of Compartmentalization in Monoterpene and Sesquiterpene Biosynthesis in Plants

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Abstract

Terpenoids play numerous vital roles in basic plant processes with volatile monoterpenes and sesquiterpenes contributing to plant defense and reproduction. The biosynthesis of terpenoids in plants occurs in different subcellular compartments, which until recently were believed to include the cytosol, plastids, and mitochondria. The plastidic MEP pathway and the cytosolic MVA pathway give rise to IPP and DMAPP, which are subsequently utilized by prenyltransferases to produce prenyl diphosphates. It has been accepted that GPP and monoterpenes are synthesized in plastids, whereas FPP and sesquiterpenes are produced in the cytosol. Here we discuss how compartmentalization contributes to the formation of terpenoid diversity in plants in light of recent reports on new subcellular localizations for some enzymatic steps as well as on bifunctional terpene synthases capable of producing both mono- and sesquiterpenes.

Keywords

Monoterpenes • Sesquiterpenes • Mevalonic acid pathway • Methylerythritol-phosphate pathway • Prenyltransferases • Terpene synthases • Subcellular compartmentalization

11.1 Introduction

Terpenoids represent the largest and most diverse class among plant secondary metabolites. They are involved in various basic plant processes, such as photosynthesis, respiration, growth, development, and adaptation to environmental conditions (Gershenzon and Kreis 1999; Rodríguez-Concepción and Boronat 2002). Volatile terpenoids, monoterpenes (C10), sesquiterpenes (C15), and some diterpenes (C20) play

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important roles in direct and indirect plant defense against herbivores and pathogens, as well as in reproduction by attracting pollinators and seed disseminators (Dudareva et al. 2006). Biosynthesis of terpenoids in plants occurs in different subcellular compartments including the cytosol, plastids, and mitochondria, which is consistent with their various functions.

All terpenoids originate from the universal five-carbon precursors, isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP), which are derived from two alternative biosynthetic pathways localized in different subcellular compartments. The classical mevalonic acid (MVA) pathway, which until recently has been believed to operate in the cytosol, gives rise to IPP from three molecules of acetyl-CoA (McCaskill and Croteau 1995; Newman and Chappell 1999). In contrast, the methylerythritol-phosphate (MEP) pathway takes place in plastids and produces IPP from pyruvate and glyceraldehyde 3-phosphate (cf. Eisenreich et al. 1998; Lichtenthaler 1999; Rohmer 1999; and elsewhere in this volume). Although the subcellular compartmentalization allows the MVA and MEP pathways to operate independently, metabolic "crosstalk" between them has been reported (Schuhr et al. 2003), particularly in the direction from plastids to the cytosol (Hemmerlin et al. 2003a; Laule et al. 2003; Dudareva et al. 2005).

In both subcellular compartments, IPP and DMAPP are subsequently utilized by prenyltransferases to produce prenyl diphosphates. In the cytosol, farnesyl diphosphate synthase (FPPS) catalyzes the condensation of one DMAPP molecule and two IPP molecules to produce FPP (C15), the precursor of sesquiterpenes (McGarvey and Croteau 1995). In plastids, a head-to-tail condensation of one IPP and one DMAPP molecule catalyzed by geranyl diphosphate synthase (GPPS) forms GPP (C10), the universal precursor of monoterpenes (cf. Ogura and Koyama 1998; Poulter and Rilling 1981), whereas condensation of one DMAPP molecule with three IPP molecules by the action of geranylgeranyl diphosphate synthase (GGPPS) yields GGPP (C20), the precursor of diterpenes (Koyama and Ogura 1999). Upon the formation of the prenyl diphosphate precursors GPP, FPP, and GGPP, a wide range of structurally diverse cyclic and acyclic monoterpenes, sesquiterpenes, and diterpenes are generated through the action of a large family of terpene synthases/cyclases (TPSs) (Cane 1999; Wise and Croteau 1999; and literature cited therein).

It has generally been accepted that GPP and monoterpenes are synthesized in plastids, whereas FPP and sesquiterpenes are produced in the cytosol. Here we will discuss how the subcellular compartmentalization contributes to the formation of terpenoid diversity in plants in light of recent reports on bifunctional terpene synthases capable of producing both mono- and sesquiterpenes as well as on metabolic engineering of the terpenoid profile by switching the subcellular localization of terpene synthases.

11.2 IPP and DMAPP Are Formed in Various Subcellular Compartments

11.2.1 The Mevalonic Acid Pathway

The MVA pathway consists of six enzymatic steps, which lead to the formation of IPP (Fig. 11.1) and provide the precursors for sesquiterpenes, sterols, and ubiquinone in plants (Newman and Chappell 1999; Disch et al. 1998). Generally, the MVA pathway in plants (in contrast to mammals, see Kovacs et al. 2002, 2007) is considered to operate in the cytosol; however, only fragmented experimental data existed until recently regarding the subcellular compartmentalization of the enzymes involved. The initial step of the pathway, the condensation of two molecules of acetyl-CoA, is catalyzed by acetoacetyl-CoA thiolase (AACT). The first plant AACT was cloned from radish, by functional complementation of a yeast mutation (Vollack and Bach 1996). Biochemical characterization of two *Arabidopsis* homologs, AACT1 and AACT2, and the analysis of T-DNA insertion mutants for both genes revealed that only AACT2 is involved in the MVA pathway, while the metabolic role of AACT1 still remains to be determined (Jin and Nikolau 2007; Ahumada et al. 2008). Transient

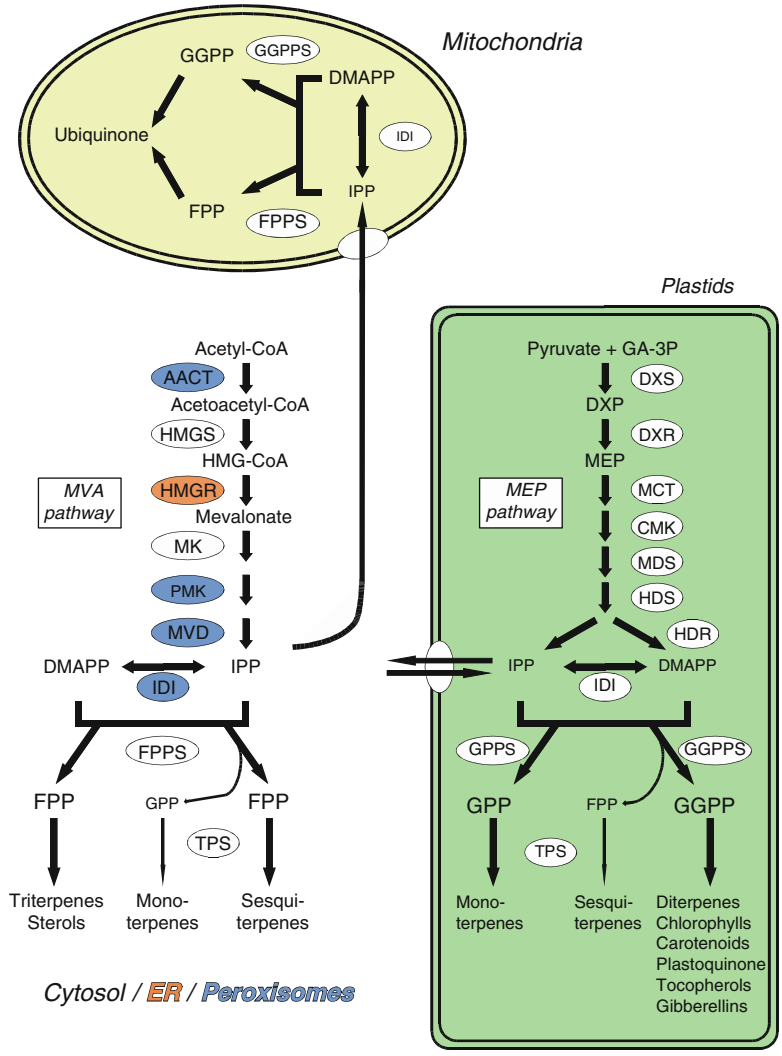

Fig. 11.1 Compartmentalization of metabolic pathways involved in the terpenoid biosynthesis in plants. Plastids and mitochondria are highlighted in green and yellow, respectively. Enzymes of the MVA pathway localized in peroxisomes and at ER/ER-derived membranes are labeled in blue and orange, respectively. The enzymatic steps are indicated by *arrows* and the enzymes involved are depicted as *circles* with the abbreviation of their names. Abbreviations: *AACT* acetoacetyl-CoA thiolase, *CMK* 4-(Cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase, *DMAPP* dimethylallyl diphosphate, *DXP* 1-deoxy-D-xylulose 5-phosphate, *DXR* 1-deoxy-D-xylulose 5-phosphate reductoisomerase, *DXS* 1-deoxy-D-xylulose 5-phosphate synthase, *FPP* farnesyl diphosphate, *FPPS* farnesyl diphosphate synthase, *GA-3P* D-glyceraldehyde 3-phosphate, *GGPP* geranylgeranyl diphosphate, *GGPPS* geranylgeranyl diphosphate synthase, *GPP* geranyl diphosphate, *GPPS* geranyl diphosphate synthase, *HDR* (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase, *HDS* (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase, *HMG-CoA* 3-hydroxy-3-methylglutaryl-CoA, *HMGR* 3-hydroxy-3-methylglutaryl-CoA reductase, *HMGS* 3-hydroxy-3-methylglutaryl-CoA synthase, *IDI* isopentenyl diphosphate isomerase, *IPP* isopentenyl diphosphate, *MCT* 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase, *MDS* 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase, *MEP* 2-*C*-methyl-D-erythritol 4-phosphate, *MK* mevalonate kinase, *MVA* mevalonate, *MVD* mevalonate diphosphate decarboxylase, *PMK* phosphomevalonate kinase, *TPS* terpene synthases (including monoterpene synthases, sesquiterpene synthases, and bifunctional terpene synthases)

expression of AACT1 and AACT2 fused in frame to the C-terminus of a green fluorescent protein (GFP) showed that *Arabidopsis* AACT2 is localized in the cytosol, whereas AACT1 is located in the peroxisomes (Carrie et al. 2007; Ahumada et al. 2008). A peroxisomal/glyoxysomal localization suggests the potential involvement of AACT1 in fatty acid degradation (Hartmann et al., this volume). However, recent proteomic analyses identified AACT2 in *Arabidopsis* leaf peroxisomes (Reumann et al. 2007, 2009), which could be due to the existence of a yet unidentified splicing variant of AACT2 being targeted to this organelle. The second step of the MVA pathway is catalyzed by HMG-CoA synthase (HMGS) and includes the condensation of one molecule of acetyl-CoA with acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Subcellular localization of HMGS has been studied only in *Brassica juncea*, which contains four HMGS isoforms with 97% amino acid identity (Nagegowda et al. 2005). Despite the presence of putative peroxisome targeting PTS2-like signals in all *Brassica juncea* HMGS isoforms, GFP localization studies showed that at least BjHMGS1 is a cytosolic enzyme (Nagegowda et al. 2005).

In the next step, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes the formation of mevalonic acid from 3-hydroxy-3-methylglutaryl-CoA, which is considered to represent the rate-limiting step in the MVA pathway leading to phytosterols (cf. Bach 1986; Chappell et al. 1995). Plant HMGRs have two hydrophobic transmembrane regions at their N-terminus, while the highly conserved catalytic domain is exposed to the cytosol (Campos and Boronat 1995). Earlier *in vitro* studies using isolated microsomal membranes suggested that *Arabidopsis* and tomato HMGRs are capable of integrating into the endoplasmic reticulum (ER) membrane (Campos and Boronat 1995; Denbow et al. 1996). Heterologous expression of two isoforms of radish HMGR in a yeast *hmgr*$^-$ mutant led to microsome-bound enzyme (Vollack et al. 1994). However, more recent extensive investigation of subcellular localization of HMGR in *Arabidopsis* revealed its dual localization: in the ER where it is synthesized and inserted into the membrane as well as within spherical vesicular structures derived from subdomains of the ER and located in the cytosol and the central vacuole (Leivar et al. 2005). Membrane domains of two tobacco HMGR isozymes fused to GFP were also targeted differentially: The domain belonging to one HMGR isoform was targeted to ER, while that of the second isoform was found in globular structures and seemed to be directed by the actin skeleton (Merret et al. 2007).

The downstream steps from mevalonate to IPP involve two phosphorylation reactions and a single decarboxylation reaction that are catalyzed by mevalonate kinase (MK), phosphomevalonate kinase (PMK), and mevalonate diphosphate decarboxylase (MVD), respectively. While these enzymes are well studied in other eukaryotic systems (Kovacs et al. 2007), very little is known about their subcellular localization in plants. Recently, cDNAs encoding these three MVA pathway enzymes were cloned from *Catharanthus roseus*, and their activities were confirmed by functional complementation of yeast *erg12*, *erg8*, and *mvd1* mutants defective in MK, PMK, and MVD, respectively (Simkin et al. 2011). Since these *C. roseus* enzymes and their respective *Arabidopsis* homologs contain N-terminal PTS2 consensus or PTS2-related sequences, their subcellular localization was analyzed by transient expression of fusion constructs with yellow fluorescent protein (YFP). While the MK-YFP was exclusively localized in the cytosol, the PMK-YFP and MVD-YFP were co-localized to a large extent with a peroxisomal marker and only a small portion remained in the cytosol (Simkin et al. 2011). These results suggest that in plants PMK and MVD are potentially peroxisomal enzymes; however, additional analyses using different techniques will be necessary to further confirm this subcellular localization.

11.2.2 The MEP Pathway

The mevalonate-independent pathway known as the MEP pathway involves seven enzymes to form IPP and DMAPP from pyruvate and

D-glyceraldehyde 3-phosphate and provides the precursors for monoterpenes, diterpenes, carotenoids, tocopherols, and the prenyl moiety of chlorophyll (Fig. 11.1). In plants, the MEP pathway has been fully elucidated using a combination of biochemical and genomic approaches (see Rodríguez-Concepción and Boronat 2002; Rodríguez-Concepción et al., this volume). The following unified nomenclature has been proposed recently for the MEP pathway enzymes (Phillips et al. 2008b): 1-deoxy-D-xylulose 5-phosphate synthase (DXS), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase (MCT), 4-(Cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase (CMK), 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS), (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS), and (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR). In contrast to questions concerning subcellular localization of enzymes involved in the MVA pathway, all enzymes of the plant MEP pathway are known to possess transit peptides for plastid targeting and their plastidic localization has been demonstrated experimentally in numerous reports (Bouvier et al. 2000; Carretero-Paulet et al. 2002; Querol et al. 2002; Hsieh and Goodman 2005; Hsieh et al. 2008).

11.2.3 Isopentenyl Diphosphate Isomerases

While the cytosolic MVA pathway produces IPP and requires its subsequent isomerization to DMAPP, the plastidic MEP pathway results in the synthesis of both IPP and DMAPP. However, HDR, catalyzing the last step in the MEP pathway and responsible for the so-called pathway "branching" (Hoeffler et al. 2002; Tritsch et al. 2010), produces IPP and DMAPP in a ratio of approximately 6:1 (Rohdich et al. 2003; Eisenreich et al. 2004), suggesting that isomerization of IPP is also required in plastids to optimize the substrate formation for the subsequent steps. Thus, both cytosolic and plastidic formation of terpenoid compounds relies on isopentenyl diphosphate isomerase (IDI) activity (Fig. 11.1). IDI is a divalent metal ion-requiring enzyme interconverting IPP and DMAPP found in all living organisms (cf. Gershenzon and Kreis 1999). The *Arabidopsis* genome contains two genes, *IDI1* and *IDI2* (Campbell et al. 1997), both encoding proteins with N-terminal sequences that were shown to target a fused GFP to the plastids in the case of IDI1 and to the mitochondria in the case of IDI2 (Okada et al. 2008; Phillips et al. 2008a; Sapir-Mir et al. 2008). While these IDIs provide DMAPP for plastid- and mitochondria-derived isoprenoids (Fig. 11.1), it has remained unclear until recently how the MVA-derived cytosolic IPP undergoes isomerization to DMAPP. Remarkably, shorter transcripts for both *Arabidopsis IDI* genes have been identified that code for IPP isomerase proteins lacking the N-terminal extensions (Okada et al. 2008; Phillips et al. 2008a). Both short versions of IDIs were shown to be localized to peroxisomes, suggesting that this particular step downstream of the MVA pathway takes place in this organelle (Sapir-Mir et al. 2008). However, more studies in different plant systems are needed to confirm this.

11.3 Prenyl Diphosphate Synthases/Prenyltransferases Function in Different Subcellular Compartments

The steps following the synthesis of the basic isoprene units IPP and DMAPP involve head-to-tail condensation of DMAPP with one or more IPP residues, catalyzed by short-chain prenyl diphosphate synthases/prenyltransferases, leading to the formation of the prenyl diphosphate precursors GPP, FPP, and GGPP (Fig. 11.1) for the various terpenoid families (Koyama and Ogura 1999; Liang et al. 2002).

11.3.1 Geranyl Diphosphate Synthase

A head-to-tail condensation of one molecule of IPP and DMAPP in a reaction catalyzed by gera-

nyl diphosphate synthase (GPPS) leads to the formation of GPP, the precursor of monoterpenes (Poulter and Rilling 1981; Ogura and Koyama 1998). GPPSs were isolated from a diverse range of plant species and found to exist in two fundamentally different dimeric structures. Heterodimeric GPPSs were found in peppermint (*Mentha piperita*), snapdragon (*Antirrhinum majus*), Clarkia (*Clarkia breweri*), and hop (*Humulus lupulus*) (Burke et al. 1999; Tholl et al. 2004; Wang and Dixon 2009), while in grand fir (*Abies grandis*), Norway spruce (*Picea abies*), and the orchid *Phalaenopsis bellina* (Burke and Croteau 2002a; Schmidt and Gershenzon 2008; Schmidt et al. 2010; Hsiao et al. 2008) GPPS is a homodimeric enzyme.

In the heterodimeric GPPS, a small subunit alone is catalytically inactive, while the large subunit alone could be inactive as well, as was found in peppermint (Burke et al. 1999), or represent a functional geranylgeranyl diphosphate synthase (GGPPS) on its own, as was shown in snapdragon and hop (Tholl et al. 2004; Wang and Dixon 2009). Only the formation of a heterodimer between these two subunits leads to an active enzyme producing GPP. A prenyltransferase from *Arabidopsis thaliana* as well as its tomato homolog were originally proposed to represent homodimeric GPPSs (Bouvier et al. 2000; van Schie et al. 2007a). However, further detailed characterization of this *Arabidopsis* enzyme revealed that it rather is a polyprenyl pyrophosphate synthase (AtPPPS) catalyzing the formation of C_{25} to C_{45} medium-/long-chain products (Hsieh et al. 2011). Likewise, two AtPPPS homologs from *P. abies* and *Quercus robur* were shown to catalyze the synthesis of larger prenyl diphosphate products (Schmidt and Gershenzon 2008). Remarkably, a new subtype of GPPS small subunit (SSU-II) was identified recently in *Arabidopsis,* which is capable of interacting with endogenous GGPPS, and the resulting heterodimer catalyzes the synthesis of GPP (Wang and Dixon 2009). Biochemical studies also showed that GPPS small subunits can interact with GGPPS from phylogenetically distant plant species, thus changing their GGPPS activity to efficient GPP production *in vitro* (Burke and Croteau 2002b; Tholl et al. 2004; Wang and Dixon 2009). The formation of such chimeric enzyme *in planta* was recently demonstrated by overexpression of snapdragon small subunit of GPPS in tobacco plants (*Nicotiana tabacum*) (Orlova et al. 2009). The total GPPS activity and monoterpene emission from leaves and flowers was increased in these transgenic plants, indicating the formation of functional heterodimers with the endogenous large subunit partners. The formation of chimeric GPPS in transgenic plants led to leaf chlorosis, increased light sensitivity, and dwarfism, most likely due to a diminished synthesis of geranylgeranyl diphosphate (GGPP) needed for formation of photosynthetic pigments and gibberellic acid (Orlova et al. 2009). The observed decrease in sesquiterpene emission suggested that an increase in flux toward GPP formation in plastids reduced the IPP pool and its transport to the cytosol (Orlova et al. 2009).

The GPPS subunits are known to contain transit peptides required for their plastid targeting, and numerous reports support their plastidic localization (Fig. 11.1). The first evidence came from biochemical studies in *Vitis vinifera* which demonstrated the presence of GPPS activity in isolated intact plastids after tryptic digestion (Soler et al. 1992). Thereafter, an *in situ* localization study using antibodies generated against GPPS confirmed plastidic localization of GPPS in *Marchantia polymorpha* (Suire et al. 2000). Moreover, immunogold localization studies using antibodies generated against the small subunit of GPPS showed that it is localized exclusively within the leucoplasts of epidermal cells of snapdragon petals (Tholl et al. 2004). Transient expression of the large (LSU) and small subunits (SSU) of hop GPPS fused in frame to the N-terminus of GFP demonstrated that LSU is plastid localized, whereas the SSU-GFP fusion protein aggregated around the plastids (Wang and Dixon 2009). In contrast to all other characterized plant GPPS, a cytosolic localization was demonstrated for GPPS in *Lithospermum erythrorhizon* cell cultures, using cell fractionation, marker enzyme assays, and immunoblotting with antibodies against GPPS (Sommer et al. 1995). These results were in agreement with *in vivo*

feeding experiments with ^{13}C-labeled glucose and the MVA pathway inhibitor mevinolin showing that the GPP-derived hemiterpenoid shikonin is formed via the cytosolic MVA pathway in *L. erythrorhizon* cells (Li et al. 1998).

11.3.2 Farnesyl Diphosphate Synthase

The sequential condensation of two molecules of IPP with one DMAPP molecule in a reaction catalyzed by farnesyl diphosphate synthase (FPPS) leads to the formation of FPP, the precursor of sesquiterpenes (cf. McGarvey and Croteau 1995). Genes encoding FPPS, a homodimeric enzyme, have been isolated and characterized from various plant species, and it has been shown that some plants contain at least two genes encoding different FPPS isoforms (Delourme et al. 1994; Attucci et al. 1995; Adiwilaga and Kush 1996; Cunillera et al. 1996; Li and Larkins 1996; Matsushita et al. 1996; Pan et al. 1996; Hemmerlin et al. 2003b). Two FPPS genes, *FPS1* and *FPS2,* were identified in *Arabidopsis* (Cunillera et al. 1996; Cunillera et al. 1997). *FPS1* alone produces two different isoforms of the enzyme, FPPS1S and FPPS1L, derived from two *FPS1* transcripts with alternative transcription start sites. The corresponding FPPS1L protein contains an additional 41 aa at its N-terminus, missing in a shorter FPPS1S version, which target this isoform into the mitochondria (Fig. 11.1), where it provides FPP for the mitochondrial isoprenoid compounds such as ubiquinone (Campbell et al. 1997). In contrast, FPPS1S and FPPS2 are localized in the cytosol (Fig. 11.1) with FPPS1S providing FPP for general plant cell functions and FPPS2 being involved in the isoprenoid synthesis for more specialized functions (Cunillera et al. 2000). In addition to the above-mentioned subcellular localizations of FPPSs, immunocytochemical studies suggested that FPPS is localized in the chloroplasts of rice mesophyll cells (Sanmiya et al. 1999). Immunoblot analysis of subcellular fractions as well as trypsin-treated chloroplasts also detected FPPS in chloroplasts of tobacco and wheat leaves (Sanmiya et al. 1999). However, the reaction of those antibodies exclusively with FPPS, and not with other prenyltransferases, needs to be confirmed.

All plant FPPSs characterized to date catalyze the head-to-tail condensation of one DMAPP molecule and two IPP molecules in the trans (*E*) configuration; however, an FPP synthase (zFPPS) recently identified from the wild tomato *Solanum habrochaites* was shown to catalyze the condensation of IPP and DMAPP in the *cis* (*Z*) configuration resulting in *Z,Z*-FPP (Sallaud et al. 2009; Tissier et al. 2013, this volume). This enzyme carries a 45-aa N-terminal transit peptide which mediates the transport of a fused GFP into chloroplasts, suggesting that zFPPS is localized in plastids and uses IPP and DMAPP provided by the plastidic MEP pathway.

11.4 Compartmentalization of Mono- and Sesquiterpene Biosynthesis

Following the formation of prenyl diphosphate precursors, low-molecular-weight terpene metabolites are formed by the action of a large family of enzymes known as terpene synthases (TPS) (cf. Cane 1999; Wise and Croteau 1999; Nagegowda 2010; Chen et al. 2011). The large diversity of TPSs seems to have originated from repeated duplications and subsequent divergence of an ancestral TPS involved in primary metabolism (Bohlmann et al. 1998b; Trapp and Croteau 2001). One of the most outstanding properties of TPSs is their proclivity for making multiple products from a single prenyl diphosphate substrate (Tholl 2006). These enzymes can be classified into three functional classes, monoterpene synthases, sesquiterpene synthases, and diterpene synthases, the first two being the subject of our discussion.

11.4.1 Monoterpene Synthases

The biosynthesis of monoterpenes, C10 compounds, is catalyzed by specialized monoterpene synthases, which utilize GPP as a substrate. In the last decade, a number of monoterpene

synthases have been isolated and characterized from various plant species (see for references: Nagegowda and Dudareva 2007; Dudareva and Pichersky 2008; Nagegowda 2010). Many of the monoterpene synthases catalyze the formation of a single product; however, several multiproduct monoterpene synthases have also been identified. For example, *Arabidopsis* myrcene/ocimene synthase converts GPP into myrcene, *(E)*-β-ocimene and small amounts of cyclic monoterpenes (Bohlmann et al. 2000), while LtMTS2, a monoterpene synthase from tomato (*Solanum lycopersicum*), produces β-phellandrene, β-myrcene, and sabinene from GPP (van Schie et al. 2007b). Another tomato monoterpene synthase, phellandrene synthase (PHS1), is unique in its use of neryl diphosphate (NPP) as preferred substrate to form primarily β-phellandrene as well as other monoterpenes, δ-2-carene, α-phellandrene, and limonene. It can also use GPP to form myrcene, ocimene, and linalool (Schilmiller et al. 2009). To date, it is believed that monoterpene biosynthesis takes place in plastids. Indeed, all isolated monoterpene synthases have a typical transit peptide at their N-terminus responsible for chloroplast targeting and therefore are 50–70 aa longer than sesquiterpene synthases (Bohlmann et al. 1998b; Williams et al. 1998). Despite the large number of isolated monoterpene synthases, their subcellular localization was investigated for only a few enzymes, among which (4*S*)-limonene synthase (LS) represents the most widely studied. Immunogold labeling studies using antibodies generated against LS combined with *in vitro* protein import experiments with isolated pea chloroplasts provided direct evidence that LS is localized to the leucoplasts of the secretory cells of peppermint (*Mentha* x *piperita*) oil glands (Turner et al. 1999). LS was also found in plastids of *Arabidopsis*, tobacco, and *Citrofortunella mitis* by *in situ* localization studies with antibodies generated against LS (Bouvier et al. 2000; Ohara et al. 2003). Moreover, GFP localization experiments showed that the N-terminal part of a lemon LS synthase directs the GFP protein to tobacco plastids (Aharoni et al. 2004). In contrast to the general agreement that all monoterpenes are synthesized in plastids, recent reports indicate that monoterpene biosynthesis might occur in the cytosol (Aharoni et al. 2004) or can have a dual, plastidic and mitochondrial, localization (Aharoni et al. 2004; Lee and Chappell 2008).

11.4.2 Sesquiterpene Synthases

Sesquiterpene synthases are responsible for the biosynthesis of C15 sesquiterpenoid compounds from FPP. Several sesquiterpene synthases have been cloned and biochemically characterized from various plant species and are believed to be located in the cytosol, consistent with the sesquiterpene biosynthesis in this cellular compartment (Chappell 1995; Bohlmann et al. 1998a). As in the case of monoterpene synthases, only a few studies were devoted to the subcellular localization of sesquiterpene synthases. *In vitro* protein import experiments with two putative *Medicago truncatula* sesquiterpene synthases, MtTps1 and MtTps2, demonstrated that these two proteins were not imported into the isolated chloroplasts, suggesting their cytosolic localization (Gomez et al. 2005). Recently, it was also shown in GFP fusion experiments that two sesquiterpene synthases, (+)-germacrene D synthase and (*E,E*)-α-farnesene synthase, responsible for the volatile profile of kiwifruit (*Actinidia deliciosa*) flowers are localized in the cytoplasm (Nieuwenhuizen et al. 2009).

Although sesquiterpene synthases are expected to be cytosolically localized, the presence of a putative plastid targeting sequence at the N-terminus, similar to monoterpene synthases, was recently reported for some sesquiterpene synthases. *Pinus sylvestris* PsTPS2, responsible for the formation of 1(10),5-germacradiene-4-ol and other products with a germacrene skeleton, contains a putative N-terminal transit peptide of 37 aa in size (Köpke et al. 2008). However, to date, there is no experimental evidence for its plastidic localization. Also, an atypical terpene synthase, santalene and bergamotene synthase (SBS), was isolated from the wild tomato *Solanum habrochaites* that is responsible for the synthesis of type II sesquiterpenes from *Z,Z*-FPP (Sallaud et al. 2009). Like the zFPPS described earlier, the SBS contains an N-terminal transit

peptide that mediates transport of a fused GFP into the plastids.

11.5 Bifunctional Terpene Synthases Involved in Mono- and Sesquiterpene Synthesis

It is generally accepted that GPP and FPP, the precursors for monoterpenes and sesquiterpenes, respectively, are compartmentally separated and that monoterpene biosynthesis takes place in plastids, where GPP is synthesized, whereas sesquiterpene formation occurs in the cytosol, where FPP is formed (Aharoni et al. 2005). However, it has been well documented that sesquiterpene synthases from various plant species are able to accept both GPP and FPP (Pechous and Whitaker 2004; Tholl et al. 2005; Green et al. 2007), with sesquiterpene synthase activities significantly exceeding their monoterpene synthase activities. Recently, bifunctional enzymes capable of efficient formation of both monoterpenes and sesquiterpenes depending on substrate availability were discovered. It has been shown that such bifunctional enzymes could be directed to different subcellular compartments, thus extending the range of available substrates for enzyme utilization and increasing the diversity of the metabolites produced. We have recently isolated two nerolidol/linalool synthases (AmNES/LIS-1/-2) from snapdragon (*A. majus*) (Nagegowda et al. 2008). Further examples include two nerolidol synthase genes, *FaNES1* and *FaNES2,* from strawberry (*Fragaria ananassa*) (Aharoni et al. 2004) and *terpene synthase 1* from maize (*Zea mays*) (Schnee et al. 2002).

In snapdragon, AmNES/LIS-1 and AmNES/LIS-2 enzymes share 95% identity and are both capable of producing linalool and nerolidol from GPP and FPP, respectively, with very similar catalytic efficiencies (Nagegowda et al. 2008). However, AmNES/LIS-1 is localized in the cytosol and is responsible for nerolidol biosynthesis, whereas AmNES/LIS-2 has a 30 aa transit peptide in its N-terminus and was shown to be located in plastids and accounts for linalool formation. The presence of both monoterpene/sesquiterpene synthase activities in plastids was further confirmed using purified leucoplasts, which produced nerolidol from FPP and linalool from GPP (Nagegowda et al. 2008). The coexistence of AmNES/LIS-1 and AmNES/LIS-2 enzymes with dual monoterpene/sesquiterpene activities in the cytoplasm and leucoplasts does not rule out that minute quantities of linalool and nerolidol can be made in the cytosol and plastids, respectively, as a result of the possible presence of GPP and FPP in trace amounts in the corresponding cellular compartments. However, feeding of cut snapdragon flowers with exogenously supplied [$^2H_2$] mevalolactone efficiently labeled nerolidol and showed no detectable incorporation into linalool (Dudareva et al. 2005), suggesting that if a GPP pool exists in the cytosol, it is small and does not contribute significantly to linalool formation.

Similar to snapdragon, the cultivated strawberry *Fragaria ananassa* contains two nerolidol synthases, FaNES1 and FaNES2, which efficiently convert GPP and FPP into the monoterpene and sesquiterpene alcohols linalool and nerolidol, respectively (Aharoni et al. 2004). As in snapdragon, one of the two enzymes, FaNES1, is localized in the cytosol and the other, FaNES2, has an N-terminal extension, which can target GFP to mitochondria and plastids upon transient expression in tobacco protoplasts. In contrast to snapdragon flowers, only one of these two genes, *FaNES1* encoding the cytosolic enzyme, is highly expressed in ripe strawberry, while *FaNES2* expression was barely detectable. This observation implies that both linalool and nerolidol in strawberry fruits are exclusively formed by FaNES1 in the cytosol and that sufficient levels of both substrates exist in the cytoplasm to support the biosynthesis of roughly similar quantities of linalool and nerolidol produced by fruits (Aharoni et al. 2004). Although FaNES1 might be involved in the synthesis of linalool in the cytosol due to its dual enzymatic activity, these data do not exclude the simultaneous presence of a yet unknown linalool synthase localized in plastids and being predominantly responsible for linalool synthesis in strawberry fruits.

Bifunctional terpene synthases like those found in the dicotyledons snapdragon (Nagegowda et al. 2008) and strawberry (Aharoni et al. 2004) have also been identified in monocotyledons.

The TPS1 enzyme, encoded by the maize *terpene synthase 1* gene, is capable of producing (*E*)-nerolidol, (*E*)-β-farnesene, and (*E*,*E*)-farnesol from FPP, as well as linalool from GPP *in vitro* (Schnee et al. 2002). After herbivore damage of maize plants, *tps1* expression was increased by almost eightfold leading to the emission of a volatile blend with (*E*)-β-farnesene, linalool, and the (*E*)-nerolidol metabolite (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) as the prominent compounds. Initially, no transit peptide was detected in the TPS1 protein, suggesting its cytosolic localization and involvement in sesquiterpene formation (Schnee et al. 2002). However, more recent bioinformatic analysis of TPS1 using various algorithms predicted its plastidic targeting (Nagegowda et al. 2008). Thus, compartmentalization of TPS1 remains to be determined experimentally, which should show the sites of linalool, (*E*)-β-farnesene, and DMNT biosynthesis within the cells, as well as the contribution of TPS1 to their formation.

11.6 Metabolic Engineering of Mono- and Sesquiterpene Synthesis Reveals Small GPP and FPP Pools in Cytosol and Plastids, Respectively

In the case of bifunctional enzymes localized in one subcellular compartment, the level of available substrates will play a crucial role in determining the type and relative amounts (monoterpenes versus sesquiterpenes) of products formed. To date, little is known about the endogenous pools of GPP and FPP in different subcellular compartments including the cytosol and plastids. However, recent metabolic engineering of the terpenoid spectrum (cf. Dudareva and Pichersky 2008) has greatly contributed to the evaluation of precursor pool sizes in different compartments of plant cells. When N-terminally truncated monoterpene synthases, limonene synthases from *Perilla frutescens* and *Citrus limon*, were ectopically expressed in tobacco, transgenic plants produced low but measurable levels of limonene, indicating the presence of a small GPP pool in the cytosol (Ohara et al. 2003; Wu et al. 2006). Similarly, the overexpression of basil sesquiterpene synthase, α-zingiberene synthase (ZIS) (which can also utilize GPP and produce a number of monoterpenes *in vitro*), in tomato fruits under the control of a fruit ripening-specific promoter led to the accumulation of a number of monoterpenes including α-thujene, α-pinene, β-phellandrene, and γ-terpinene in addition to expected high levels of α-zingiberene and several other sesquiterpenes (Davidovich-Rikanati et al. 2008), indicating that a small pool of GPP is available in the cytosol of tomato fruits as well. On the other hand, the direction of patchoulol synthase (PTS), a sesquiterpene synthase from *Pogostemon cablin*, to the plastids of transgenic tobacco using an N-terminal transit peptide of the RubisCO small subunit yielded plants that accumulated low levels of patchoulol and several other sesquiterpenes, showing the presence of a small FPP pool in tobacco plastids (Wu et al. 2006). These results were consistent with the previously reported detection of FPPS in tobacco chloroplasts (Sanmiya et al. 1999)

In many cases, FPP, which is expected to be produced in relatively large amounts in the cytosol, needed for sterol biosynthesis, is not readily available for catalysis by introduced sesquiterpene synthases (Aharoni et al. 2005). Only very low levels of the respective sesquiterpenes were obtained in *Arabidopsis* plants overexpressing a chicory germacrene A synthase (Aharoni et al. 2003) and in tobacco plants expressing the amorpha-4,11-diene synthase from *Artemisia annua* or a fungal trichodiene synthase (Hohn and Ohlrogge 1991; Wallaart et al. 2001). To date, the constitutive overexpression of the cytosolically localized maize TPS10 and PTS in *Arabidopsis* and tobacco, respectively, represent the two most successful attempts at producing high levels of volatile sesquiterpenes by enzymes targeted to the cytosol (Schnee et al. 2006; Wu et al. 2006), suggesting a sufficient supply of FPP for cytosolic sesquiterpene synthesis in these cases.

The ectopic expression of bifunctional terpene synthases in transgenic plants is of particular interest since these enzymes can efficiently use both GPP and FPP substrates and may allow for

the estimation of the relative ratio of these two substrate pools in a distinct subcellular compartment. Recently, cytosolically localized bifunctional linalool/nerolidol synthase FaNES1 from strawberry was targeted to *Arabidopsis* and potato plastids (Aharoni et al. 2003; Aharoni et al. 2006) as well as to *Arabidopsis* mitochondria (Kappers et al. 2005) by the addition of the respective targeting signals. Both transgenic *Arabidopsis* and potato plants expressing the plastid-targeted version of FaNES1 produced high levels of linalool and its glycosylated and hydroxylated derivatives (Aharoni et al. 2003; Aharoni et al. 2006). Surprisingly, these *Arabidopsis* plants also produced some nerolidol, although at levels 100- to 300-fold lower than those of linalool, thus once again suggesting that a small pool of FPP is present in plastids (Aharoni et al. 2003). Targeting of FaNES1 to mitochondria resulted in transgenic *Arabidopsis* plants emitting nerolidol at levels that were 20- to 30-fold higher than those from transgenic plants with the plastid-targeted FaNES1 in addition to the nerolidol derivative (*E*)-DMNT (Kappers et al. 2005). Thus, these results show that plant mitochondria indeed have a readily available FPP pool, which is generated by the mitochondria-localized FPPS isoform (Cunillera et al. 1997) or alternatively imported from the cytosol (Hartmann and Bach 2001) and is normally used for ubiquinone biosynthesis. Overall, the above-described metabolic engineering studies suggest the presence of substrates for mono- and sesquiterpene biosynthesis in both plastids and cytosol, although at different levels: high GPP/trace FPP in plastids versus high FPP/trace GPP in cytosol. These substrate pools allow endogenous (bifunctional) enzymes to produce their respective products in both subcellular compartments, however, at a level representative of that of the precursors.

11.7 Summary and Future Perspectives

The past decade has witnessed significant progress in the identification of genes and enzymes involved in terpenoid biosynthesis in plants. However, to date, only limited knowledge exists about the subcellular localization of enzymes involved in the terpenoid network and the contribution of different cellular compartments to terpenoid formation. While only plastids are believed to be involved in IPP and DMAPP biosynthesis via the MEP pathway, at least four different compartments, cytosol, peroxisomes, ER, and spherical vesicular structures, contribute to the formation of IPP and DAMPP via the MVA pathway (Fig. 11.1). Further analysis of subcellular localization of the enzymes involved in the MVA pathway and in reactions downstream of IPP will provide new insights on the role of compartmentalization in the regulation of the flux toward terpenoid precursors and allow us to understand the FPP allocation for sterol and sesquiterpene biosynthesis. Given the involvement of various subcellular compartments in the biosynthesis of terpenoid precursors, it seems obvious that numerous transport processes across the organellar membranes are required (Fig. 11.1). This might include the transport of intermediates of the MVA pathway between the cytosol and peroxisomes, IPP as well as other up- and downstream metabolites between plastids and cytosol, and IPP from the cytosol to mitochondria. However, to date, very little is known about these transport processes, the transporters involved, and their substrate specificities.

The existence of small pools of GPP and FPP in the cytosol and plastids, respectively, also raises the question about their origin. They might originate from incomplete reactions catalyzed by the cytosolic FPPS and the plastidic GGPPS, releasing small quantities of the reaction intermediates GPP and FPP, respectively (Fig. 11.1). Alternatively, they can be the products of cytosolic GPPS and plastidic FPPS, which have been identified in limited plant species. Future functional genomic, transcriptomic, and proteomic analysis will show whether one of these two scenarios is prevalent in the plant kingdom. Moreover, such analysis will also lead to the identification of putative transporters involved in the transport of terpenoid precursors across the organellar membranes described above. Knowledge about the endogenous pools of GPP

and FPP in different subcellular compartments will be even more crucial for rational metabolic engineering in the light of the ability of many sesquiterpene synthases as well as bifunctional terpene synthases to produce both mono- and sesquiterpene compounds. This knowledge will provide a foundation for future successful metabolic engineering of plant terpenoid profiles to boost plant defense, increase pollinator attraction, and heighten the production of biologically valuable compounds (Aharoni et al. 2005, 2006; Dudareva and Pichersky 2008).

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