# Chapter 10

# The Role of the Methyl-Erythritol-Phosphate Pathway in Rhythmic Emission of Volatiles

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## Summary

Plants release an array of terpenoid compounds including hemiterpenes, mono- and sesquiterpenes, irregular terpenes and some diterpenes throughout their life cycles. These secondary metabolites play crucial roles in pollinator attraction, defense, communication and interaction with the surrounding environment. Release of these compounds from flowers and undamaged and herbivore attacked leaves follows a rhythmic profile, which is induced by illumination and often controlled by a circadian clock. In plants two distinct biochemical pathways localized in different cellular compartments, the cytosolic mevalonic acid (MVA) pathway and plastidial methyl-erythritol-phosphate (MEP) pathway, are responsible for the biosynthesis of basic carbon building blocks for terpenoid compounds. Mounting evidence suggests that the flux through the MEP pathway changes rhythmically over a daily light/dark cycle peaking during the day. In this chapter we discuss the contribution of the MEP pathway to the rhythmic emission of terpenoids released from different plant tissues and the regulatory steps controlling the flux through this pathway.

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## I Introduction

Volatile terpenoids represent more than 2% of the more than 20,000 different terpene molecules known to date (Knudsen and Gershenzon, 2006). Because of the physicochemical requirements for volatility, this group of natural products is restricted to low-molecular weight compounds (<300 Da) with low boiling points and high vapor pressure at ambient temperatures. All terpenoids are derived from the fusion of $C_5$ basic isoprene blocks and classified according to the number of $C_5$ units in their basic skeleton. The volatile fraction of terpenoids is represented by a simple five-carbon compound isoprene ($C_5$) as well as by monoterpenes ($C_{10}$), comprising the most abundant volatile constituents, followed by sesquiterpenes ($C_{15}$), irregular terpenes ($C_8$–$C_{18}$), and some diterpenes ($C_{20}$) (Knudsen and Gershenzon, 2006). These volatiles are produced in a wide range of plant species and released from flowers, fruits, and vegetative tissues into the atmosphere and from roots into the soil.

*Abbreviations:* aa – amino acid; ALA – alamethicin; CaMV – cauliflower mosaic virus; CCD – carotenoid cleavage dioxygenase; CDP – ME, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol; CDP-ME2P – 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate; CMK – 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase; CMS – 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthase; DMAPP – dimethylallyl diphosphate; DOX – 1-deoxy-D-xylulose; DXP – 1-deoxy-D-xylulose 5-phosphate; DXR – 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS – 1-deoxy-D-xylulose 5-phosphate synthase; FaNES – *Fragaria ananassa* nerolidol synthase; FPP – farnesyl diphosphate; FTC – Forest tent caterpillar; EST – expressed sequence tag; GA-3P – glyceraldehyde-3-phosphate; GGPP – geranyl geranyl diphosphate; GGPPS – geranyl geranyl diphosphate synthase; GPP – geranyl diphosphate; GPPS – geranyl diphosphate synthase; GPPS – SSU geranyl diphosphate synthase small subunit; HDR – 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase; HDS – 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase; HMBPP – 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate; HMG-CoA – 3-hydroxy-3-methylglutaryl-CoA; HMGR – 3-hydroxy-3-methylglutaryl-CoA reductase; IDI – isopentenyl diphosphate isomerase; IPP – isopentenyl diphosphate; ISPS – isoprene synthase; MCS – 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; ME-2,4cPP – 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate; MEP – 2-*C*-methyl-D-erythritol 4-phosphate; MeJA – methyl jasmonate; MVA – mevalonic acid; MVL – mevalolactone; RACE – rapid amplification of cDNA ends; RT-PCR – reverse transcriptase-polymerase chain reaction; TLC – thin layer chromatography; TPS – terpene synthase;

The primary functions of volatile terpenoids are to defend plants against herbivores and pathogens or to provide a reproductive advantage by attracting pollinators and seed dispersers (Gershenzon and Dudareva, 2007; Dudareva et al., 2006). Within a species the blend of emitted terpenoids differ quantitatively and qualitatively with some compounds in common (reviewed in Dudareva et al., 2006). Moreover, in some species the release of volatile terpenoids from flowers and undamaged and herbivore-attacked leaves displays a rhythmic pattern throughout the photoperiod (Dudareva et al., 2006).

In plants two distinct biochemical pathways localized in different subcellular compartments are responsible for the biosynthesis of the universal five carbon building blocks, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Chappell, 1995; Lichtenthaler et al., 1997b; Lange et al., 2000) (Fig. 1). In the cytosol, IPP is synthesized from three molecules of acetyl-CoA by the classical mevalonic acid (MVA) pathway (Qureshi and Porter, 1981; Newman and Chappell, 1999), while in plastids, it is derived from pyruvate and glyceraldehyde-3-phosphate via the methyl-erythritol-phosphate (MEP) pathway (Lichtenthaler et al., 1997a, b; Eisenreich et al., 1998; Lichtenthaler, 1999; Rohmer, 1999). The cytosolic MVA pathway and the plastidic MEP pathway are reviewed in Chapter 10 of this book (Lichtenthaler, 2008). [AU1]

DMAPP generated from the MEP pathway in plastids is used for isoprene formation (Schwender et al., 1997; Zeidler et al., 1997) via isoprene synthases (Silver and Fall, 1995; Schnitzler et al., 1996; Miller et al., 2001). It is also used by methylbutenol synthase to produce the hemiterpene methylbutenol emitted by ponderosa pines (Zeidler and Lichtenthaler, 2001). In both compartments, IPP and DMAPP undergo condensation catalyzed by short-chain prenyltransferases to form precursors for monoterpene and diterpene biosynthesis in plastids and sesquiterpenes in the cytosol (Dudareva et al., 2004). Although subcellular compartmentation of the MVA and MEP pathways in plants allows both pathways to operate independently and contribute to sesquiterpene, and to monoterpene and diterpene formation, respectively, the biosynthesis of certain monoterpenes and sesquiterpenes in some plant species occurs via the cooperation of both

[AU2]

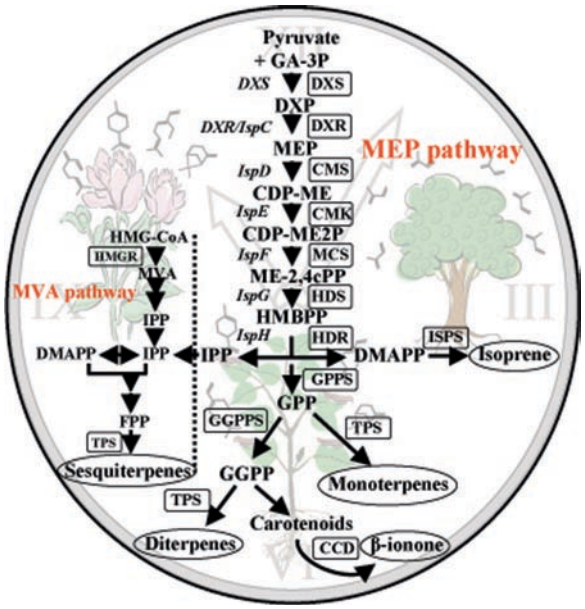


*Fig. 1.* MEP pathway and rhythmic emission of terpenoids in plants. CCD, carotenoid cleavage dioxygenase; CMK/IspE, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; CMS/IspD, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthase; CDP-ME, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol; CDP-ME2P, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, DXP reductoisomerase; DXS, DXP synthase; FPP, farnesyl diphosphate; GA-3P, glyceraldehyde-3-phosphate; GGPP, geranyl geranyl diphosphate; GGPPS, GGPP synthase; GPP, geranyl diphosphate; GPPS, GPP synthase; HDS/IspG, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate synthase; HDR/IspH, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase; HMBPP, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; IPP, isopentenyl diphosphate; ISPS, isoprene synthase; MCS/IspF, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; ME-2,4cPP, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; TPS, terpene synthase. Names of the enzymes are *boxed* and the corresponding genes in MEP pathway are *italicized* on the *left*. Volatile compounds are indicated within the *ovals*.

pathways (Adam et al., 1999; Piel et al., 1998; Dudareva et al., 2005). Although all the genes and enzymes responsible for each step in these pathways have been isolated and functionally characterized, to date very little is known about the complex regulatory mechanisms controlling the flux through these pathways and their cooperation in the biosynthesis of volatile terpenoids and their emission. In this chapter we discuss the contribution of the MEP pathway to the biosynthesis of terpene volatiles and the regulation involved in their rhythmic emission.

## II The MEP Pathway and Rhythmic Emission of Floral Volatiles

Terpenoids represent the largest class of floral volatiles and include such well known and widely distributed constituents of floral scents as the monoterpenes, linalool, limonene, myrcene, ocimene, geraniol, and the sesquiterpenes, farnesene, nerolidol, caryophyllene, and germacrene. Emission of these compounds from flowers of many plant species occurs at similar levels over a daily light/dark cycle (e.g. *Clarkia breweri* (Pichersky et al., 1994)), while in some flowers their emission exhibits distinct diurnal or nocturnal patterns. Nocturnal monoterpene emission was observed in five *Nicotiana* species, *N. rustica*, *N. alata*, *N. forgetiana*, *N. bonariensis*, and *N. langsdorffii* (Raguso et al., 2003) in contrast to diurnal oscillations in emission of terpenoids from *Rosa hybrida* L. cv Honesty (Helsper et al., 1998), *Antirrhinum majus* (Dudareva et al., 2003), *Petunia hybrida* (Simkin et al., 2004), and *Arabidopsis thaliana* (Aharoni et al., 2003) (Table 1).

Moreover, within a single scent bouquet emission of various monoterpene compounds can follow different profiles. In *Hesperis matronalis* flowers, for example, 1,8-cineole is released primarily during the day, *(E)*-β-ocimene mainly near dusk whereas the levels of linalool emission remain constant over a daily light/dark cycle (Nielsen et al., 1995).

The rhythmic release of floral volatiles can be induced by illumination or darkness, or can be controlled by a circadian clock. Nocturnal oscillations in emission of volatiles were reported to be controlled by a circadian clock in contrast to diurnal rhythmicity in volatile emission which is controlled by irradiation levels. However, a circadian nature of diurnal rhythmicity in emission of terpenoids was recently shown in rose and snapdragon flowers (Helsper et al., 1998; Dudareva et al., 2003). Moreover, snapdragon flowers which emit three monoterpenes, myrcene, *(E)*-β-ocimene, and linalool and a sesquiterpene, nerolidol, were used as a model system to investigate the contribution of both IPP biosynthetic pathways to the regulation of

*Table 1.* Terpenoid compounds showing rhythmic emission in plants.

| Major terpenoid compound(s) | Plant species | Source | Reference |
|---|---|---|---|
| *Constitutively emitted terpenoids* | | | |
| Myrcene and (*E*)-β-ocimene | *Antirrhinum majus* | Flower | Dudareva et al., 2003 |
| Linalool and nerolidol | *Antirrhinum majus* | Flower | Dudareva et al., 2003 |
| Myrcene, limonene, (*E*)-β-ocimene, β-caryophyllene, thujopsene and α-humulene | *Arabidopsis thaliana* | Flower | Aharoni et al., 2003 |
| β-Pinene | *Artemisia annua* | Leaf | Lu et al., 2002 |
| 1,8 Cineole and (*E*)-β-ocimene | *Hesperis matronalis* | Flower | Nielsen et al., 1995 |
| 1,8-Cineole, limonene, myrcene, sabinene | *Nicotiana* spp.[a] | Flower | Raguso et al., 2003 |
| β-Pinene | *Petunia hybrida* | Leaf and flower | Simkin et al., 2004 |
| *trans*-β-ocimene, linalool and 1,8-cineole | *Pinus pinea* | Leaf | Staudt et al., 1997 |
| α-Pinene, linalool and β-pinene + sabinene | *Pistacia lentiscus* | Leaf | Hansen et al., 1997 |
| Geraniol, citronellol, nerol, *E*-citral, *Z*-citral, methylgeranylate | *Rosa hybrida* | Flower | Helsper et al., 1998 |
| 1,8-Cineol, α-pinene and β-pinene | *Rosmarinus oficinalis* | Leaf | Hansen et al., 1997 |
| *Induced terpenoids* | | | |
| 4,8,12-Trimethyltrideca-1,3,7,11-tetraene (TMTT) | *Capsicum annuum* | Leaf | Kunert et al., 2002 |
| (*E*)-β-ocimene, (*E*)-4,8- dimethyl 1,3,7-nonatriene (DMNT), (*E*)-β-farnesene and (*E,E*)-α-farnesene | *Gossypium hirsutum* | Leaf | Loughrin et al., 1994 |
| (*E*)-β-ocimene and DMNT | *Gossypium hirsutum* | Leaf | Loughrin et al., 1997 |
| DMNT and TMTT | *Phaseolus lunatus* | Leaf | Kunert et al., 2002 |
| Linalool, 1,8-cineole, and (*E*)-β-farnesene, terpinen-4-ol | *Picea abies* | Leaf | Martin et al., 2003 |
| (-)-Linalool | *Picea sitchensis* | Leaf | Miller et al., 2005 |
| (−)-Germacrene D | *Populus trichocarpa* | Leaf | Arimura et al., 2004a |
| *Isoprene* | | | |
| Isoprene | *Elaeis guineensis* | Leaf | Wilkinson et al., 2006 |
| | *Eucalyptus* spp | Leaf | |
| | *Ficus bengalensis* | Leaf | |
| | *Ficus religiosa* | Leaf | Padhy and Varshney, 2005 |
| | *Mangifera indica* | Leaf | |
| | *Melia azedarach* | Leaf | |
| | *Populus* × *canescens* | Leaf | Loivamäki et al., 2007 |
| | *Populus deltoides* | Leaf | Funk et al., 2003 |
| | *Quercus alba* | Leaf | Geron et al., 2000 |
| | *Quercus rubra* | Leaf | Funk et al., 2003 |
| | *Syzygium jambolanum* | Leaf | Padhy and Varshney, 2005 |
| | *Ulex europaeus* | Leaf | Cao et al., 1997 |

[a]Nocturnal emission



rhythmic emission of terpenoids (Dudareva et al., 2005). Treatment of cut snapdragon flowers with pathway-specific inhibitors (fosmidomycin for the MEP pathway and mevinolin for the MVA pathway) revealed that fosmidomycin inhibits emission of both monoterpenes and the sesquiterpene nerolidol, while mevinolin has virtually no effect on the amount of emitted compounds. These results suggested that the MVA pathway does not contribute to nerolidol formation and that both monoterpene and sesquiterpene biosynthesis in snapdragon flowers relies on the plastidial supply of IPP precursors via the MEP pathway. Consistent with these results, exogenously supplied stable isotope-labeled 1-deoxy-[5,5-$^2H_2$]-D-xylulose ([$^2H_2$]-DOX), a specific precursor of the MEP pathway, was incorporated into both monoterpenes and nerolidol but the level of labeling of these compounds was greater at night than during the day. The oscillations in the level of labeling of these compounds could be the result of the rhythmic operation of the endogenous MEP pathway with greater flux during the light period thus reducing the incorporation of exogenous substrate.

The biosynthesis of plastidial IPP precursors is directly linked to photosynthesis. The two immediate precursors of the MEP pathway, pyruvate and glyceraldehyde 3-phosphate, are derived from the

Calvin cycle, suggesting that the rhythmic changes in the flux through this pathway could be induced either by light or controlled by an endogenous clock. The exposure of snapdragon flowers to continuous darkness for 3 days revealed a persistence in the oscillations in emission and in the levels of labeling of monoterpenes and nerolidol, indicating that the flux through the MEP pathway follows a diurnal rhythm which is controlled by a circadian clock (Dudareva et al., 2005). Moreover, the elimination of the contribution of the MEP pathway to nerolidol biosynthesis by the inhibitor fosmidomycin in the presence of exogenous deuterium labeled [2,2-$^2H_2$]-mevalolactone ([$^2H_2$]-MVL) resulted in the elimination of rhythmicity in nerolidol emission and the level of its labeling. Thus, the diurnal fluctuations in terpenoid emission in snapdragon flowers are likely the result of rhythmicity in the flux through the MEP pathway.

Headspace analysis of terpenoids released from Arabidopsis flowers revealed that nearly all monoterpenes (e.g., myrcene, limonene, and *(E)*-β-ocimene) and sesquiterpenes (β-caryophyllene, thujopsene, and α-humulene) also exhibit a clear diurnal pattern in their emission (Aharoni et al., 2003) (Table 1). Moreover, the overexpression in Arabidopsis of a strawberry linalool/nerolidol synthase (*FaNES1*) targeted to plastids under the control of a constitutive 35S CaMV promoter resulted in production of linalool, whose emission followed diurnal oscillations as well. These results suggest that the diurnal emission of compounds synthesized by endogenous and introduced terpene synthases may be determined by the availability of precursors, the biosynthesis of which could occur rhythmically within the biosynthetic pathways. Other factors like glycosidase or glycosyltransferase activities as well as the light activation of the 35S CaMV promoter (Schnurr and Guerra, 2000) in the case of the introduced linalool, can not be excluded (Aharoni et al., 2003). Although the contribution of plastidial MEP and cytosolic MVA pathways to the biosynthesis of terpenoid precursors was not analyzed in Arabidopsis, the existence of crosstalk between these two pathways and the trafficking of isoprenoid intermediates from the plastid to the cytosol has been demonstrated in Arabiodopsis seedlings (Laule et al., 2003).

The initial step of the MEP pathway includes the condensation of pyruvate and glyceraldehyde 3-phosphate with the formation of 1-deoxy-D-xylulose-5-phosphate (DXP) in a reaction catalyzed by the transketolase DXP synthase (DXS) (Fig. 1). The produced DXP is then used in plants as a precursor for IPP biosynthesis as well as for the biosynthesis of the cofactors, thiamin pyrophosphate and pyridoxal phosphate (Julliard and Douce, 1991; Himmeldirk et al., 1996). The first step specific for IPP production is catalyzed by DXP reductoisomerase (DXR) and involves the conversion of DXP to methylerythritol phosphate (Fig. 1). While DXS is thought to be an important rate-controlling step of the MEP pathway (Lois et al., 2000; Estévez et al., 2001), DXR may also serve as a significant control point of the metabolic flux through the pathway since it catalyzes the first committed step of the MEP pathway towards terpenoid biosynthesis (Takahashi et al., 1998; Mahmoud and Croteau, 2001; Carretero-Paulet et al., 2006). The post-transcriptional regulation of the MEP pathway is described in chapter 11 of this book (Boronat, 2008). Analysis of DXS and DXR expression in snapdragon flowers over a daily light/dark cycle revealed that only DXS transcripts show a rhythmic pattern which peaks during the light period and strongly correlates with the pattern of diurnal monoterpene and nerolidol emissions (Dudareva et al., 2005) (Table 2). These results suggest that transcriptional regulation of DXS expression determines the rhythmic profile of the flux through the MEP pathway in snapdragon flowers. The lack of diurnal oscillations in DXR transcript levels could be due to a minor role of DXR in the regulation of the MEP pathway in snapdragon flowers or post-transcriptional regulation of DXR activity. Additionally, the generic DXR probe used in these experiments could have recognized more than one possible DXR isoform, and so masked the correlation of expression of a specific DXR isogene with monoterpene and sesquiterpene emission (Dudareva et al., 2005).

The fact that exogenously supplied [$^2H_2$]-DOX did not affect the total amount of emitted terpenoids and did not eliminate rhythmicity in their emission provides evidence that some additional regulatory mechanisms also take place downstream of DXS. While the expression of other genes in the MEP pathway downstream of DXR was not analyzed in snapdragon flowers, expression analysis of geranyl diphosphate synthase (GPPS), which catalyzes

*Table 2.* MEP pathway and downstream genes showing rhythmic expression.

| Gene | Plant species | Reference |
|---|---|---|
| 1-Deoxy-D-xylulose 5-phosphate synthase (*DXS*) | *Antirrhinum majus* | Dudareva et al., 2005 |
| 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR*) | *Populus trichocarpa* | Arimura et al., 2004a |
| 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR*) | *Artemisia annua* | Lu et al., 2002 |
| 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR/IspC*) | | |
| 4-Diphosphocytidyl-2-*C*-methyl-D-erythritol synthase (*CMS or IspD*) | | |
| 4-Diphosphocytidyl-2-*C*-methyl-D-erythritol kinase (*CMK/IspE*) | | |
| 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase (*MCS/IspF*) | *Arabidopsis thaliana* | Hsieh and Goodman, 2005 |
| 1-Hydroxy-2-methyl-2-(*E*)-butenyl | | |
| 4-Diphosphate synthase (*HDS/IspG*) | | |
| 1-Hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase (*HDR/IspH*) | | |
| Isoprene synthase (*ISPS*) | *Populus* × *canescens* | Loivamäki et al., 2007 |
| Pinene synthase (*QH6*) | *Artemisia annua* | Lu et al., 2002 |
| Myrcene and (*E*)-β-ocimene synthases | *Antirrhinum majus* | Dudareva et al., 2003 |
| (−)-Germacrene D synthase | *Populus trichocarpa* | Arimura et al., 2004a |
| Carotenoid cleavage dioxygenase (*PhCCD1*) | *Petunia hybrida* | Simkin et al., 2004 |

a single condensation of IPP and DMAPP to form GPP (Fig. 1), the substrate for monoterpene biosynthesis, and the monoterpene synthases responsible for myrcene and *(E)*-β-ocimene formation was performed in petal tissue during a daily light/dark cycle. In snapdragon, only a small subunit (GPPS.SSU) of a heterodimeric GPP synthase controls the rate of GPP production (Tholl et al., 2004). Levels of transcripts for GPPS.SSU and monoterpene synthases exhibited similar weak diurnal oscillations which are retained under continuous dark, suggesting that their cyclic expression is under circadian control (Dudareva et al., 2003; Tholl et al., 2004). Although the expression of DXS, GPPS.SSU and monoterpene synthases all positively correlated with monoterpene emission, the molecular mechanisms involved in the regulation of the flux towards terpenoids still remain unknown.

In Arabidopsis, the high levels of DXS and DXR gene expression were also found in the inflorescences, which was consistent with the high emission of terpenoids from this part of the plant (Carretero-Paulet et al., 2002). The expression of the DXR gene closely paralleled that of DXS, but exhibited a slightly more restricted pattern. Its expression begins later than DXS in emerging inflorescences, suggesting that in contrast to snapdragon, DXR instead of DXS might be limiting for the onset of isoprenoid biosynthesis in Arabidopsis flowers (Carretero-Paulet et al., 2002) (Table 2). Consistent with diurnal emission of terpenoids, the expression of both genes as well as the rest of the MEP pathway genes was significantly induced by light (Carretero-Paulet et al., 2002; Hsieh and Goodman, 2005). The only exception is the 1-hydroxy-2-methyl-2-*(E)*-butenyl 4-diphosphate reductase (HDR) which catalyzes the last step of the MEP pathway and is expressed constitutively regardless of light/dark conditions (Hsieh and Goodman, 2005). Although the expression of the MEP pathway genes was not analyzed in the inflorescences over a daily light/dark cycle, their expression in 2-week-old Arabidopsis seedlings exhibited diurnal oscillations and was significantly suppressed during the transition from light to dark. Moreover, DXS and DXR expression followed two different diurnal profiles with DXS peaking earlier in the light cycle (Hsieh and Goodman, 2005). Whether a circadian clock is involved in the regulation of expression of the MEP pathway genes as well as the flux through the MEP pathway in Arabidopsis still remains to be determined.

Experiments with pathway-specific precursors and inhibitors in snapdragon flowers revealed that the endogenous MVA pathway does not contribute to nerolidol formation and is blocked before the formation of mevalonic acid. The fact that [$^2H_2$]-MVL supplied to snapdragon flowers was efficiently incorporated into nerolidol and led

to an increase of its emission during both day and night indicated that the later enzymes of the MVA pathway were active. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR, EC 1.1.1.34), which catalyzes the NADPH-dependent reduction of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonic acid, is the key regulatory enzyme of the MVA pathway and has been extensively studied in some plant species. *Arabidopsis thaliana*, for example, contains two differentially expressed HMGR genes which encode three isoforms (Lumbreras et al., 1995). In contrast to Arabidopsis, three HMGR genes have been identified in snapdragon. To isolate these genes we used two different approaches, RT-PCR in combination with 5′ and 3′ rapid amplification of cDNA ends (RACE) and cDNA library screening. Since a search of 11,600 nonreduntant expressed sequence tags (ESTs) from a normalized snapdragon cDNA library constructed from mRNAs isolated from different vegetative and floral organs revealed no clones with homology to the HMGRs, degenerate primers were designed in the HMGR conserved domain and used in RT-PCR experiments with total RNA isolated from upper and lower lobes of snapdragon flowers. RT-PCR resulted in a fragment of ~450 nucleotides which showed homology to known HMGRs. 5′ and 3′ RACE was used to recover the corresponding full-length clone. The obtained full-length cDNA clone (designated as AmHMGR1) is 2,195 nucleotides in size and encodes an open reading frame of 1,758 nucleotides, corresponding to a protein of 586 aa (Fig. 2). It has 174 and 263 nucleotides in its 5′ and 3′ untranslated regions, respectively.

For functional characterization of the protein encoded by AmHMGR1 cDNA, the coding region was subcloned into the pET-28a expression vector and expressed in *Escherichia coli* Rosetta cells. The HMGR activity of the isopropylthio-β-galactoside-induced bacterial crude extracts and recombinant His-tag purified protein was determined by measuring the $^{14}$C-mevalonate formation from the $^{14}$C labeled substrate 3-hydroxy-3-methylglutaryl-CoA. Thin layer chromatography (TLC) separation of the reaction products showed a single band with an $R_f$ value corresponding to that of mevalonic acid, thus confirming the HMGR activity of the enzyme (Fig. 3).

Since HMGRs are known to belong to a multigene family (Bach et al., 1999), the AmHMGR1 cDNA was used as a probe to screen a snapdragon petal specific cDNA library to identify other HMGR genes, if any. This screening resulted in two truncated clones with sequence homology to AmHMGR1. Full length cDNAs for each clone were obtained by RACE amplification and designated as AmHMGR2 and AmHMGR3. AmHMGR2 and AmHMGR3 cDNAs were 2,080 and 2,071 nucleotide in size and encoded proteins of 548 aa and 555 aa, respectively. Sequence alignment and phylogenetic analysis of the AmHMGR deduced amino acid sequences revealed that AmHMGR2 and AmHMGR3 are closely related with 85% aa identity and both share about 70–72% identity with HMGR1 (Figs. 2 and 4). All three AmHMGRs exhibited high aa sequence identity to HMGRs from *Andrographis paniculata* (73–81%), *A. thaliana* (72–75%) and *Hevea brasiliensis* (76–77%) (Figs. 2 and 4).

To determine the contribution of these genes to nerolidol biosynthesis and emission, their expression was analyzed by semi-quantitative RT-PCR in different floral tissues of 5 day-old flowers, a developmental stage with high levels of terpenoid emission (Dudareva et al., 2003). The highest level of expression of all three genes was found in stamens (Fig. 5). AmHMGR1 and AmHMGR2 were also highly expressed in tubes and pistils of snapdragon flowers, while low levels of expression of all three genes were observed in ovaries and upper and lower petal lobes, the tissues primarily involved in the formation and emission of terpenoid volatiles (Dudareva et al., 2003). In green tissues, AmHMGR2 was expressed at a low level in leaves, while low levels of both AmHMGR1 and AmHMGR3 expression were found in sepals (Fig. 5). Low levels of expression of all three AmHMGRs in scent producing parts of the flower (upper and lower petal lobes) suggest that the MVA pathway contributes little if any to nerolidol biosynthesis in snapdragon flowers. This statement is also supported by our earlier feeding experiments with [$^2H_2$]-MVL in the presence of fosmidomycin (Dudareva et al., 2005) where in the absence of the MEP pathway [$^2H_2$]-MVL feeding led to almost complete deuterium labeling of nerolidol.

[AU3]

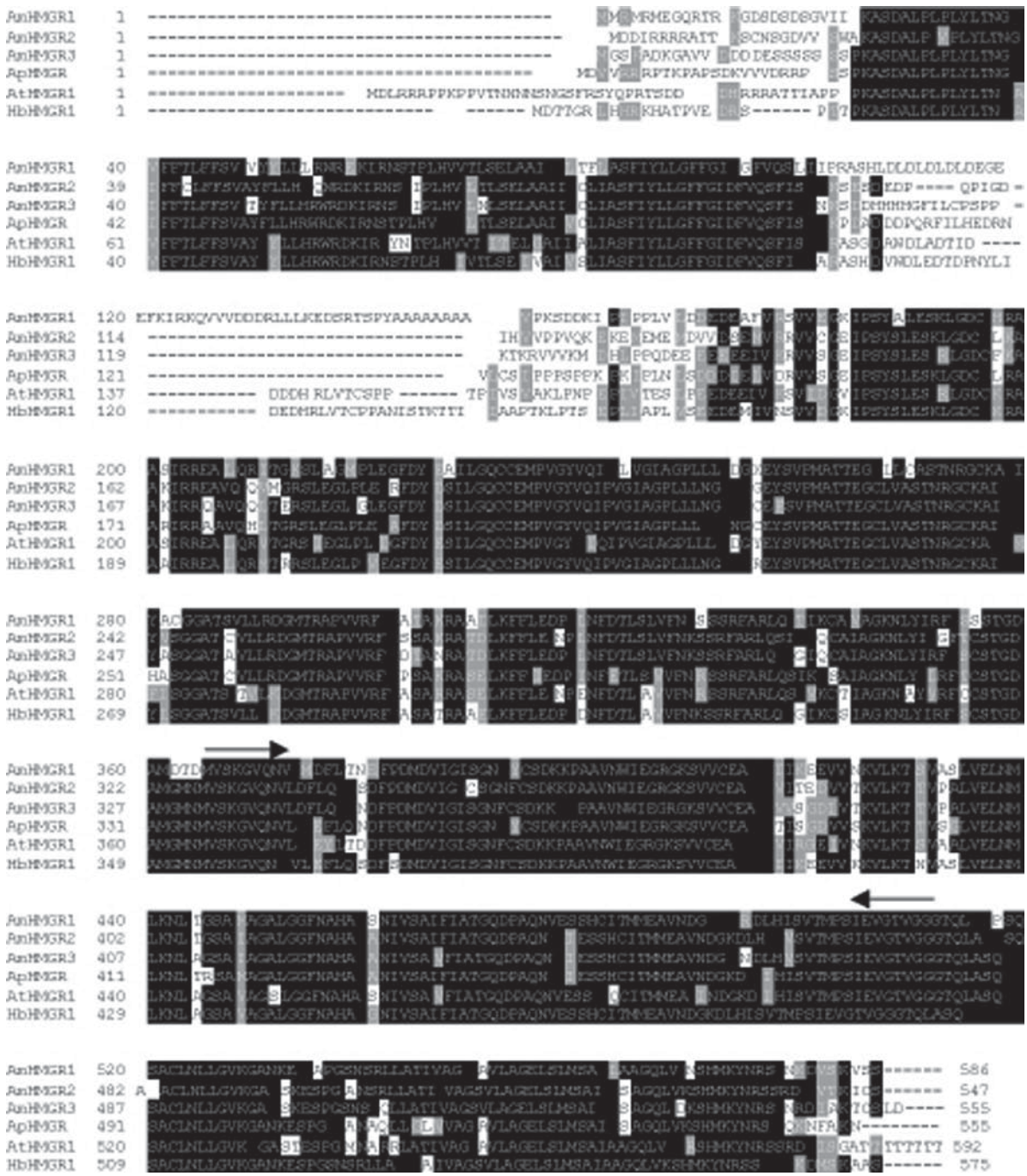


*Fig. 2.* Alignment of deduced AmHMGR amino acid sequences to other plant HMGRs. Alignment of AmHMGR predicted amino acid sequences with HMGRs from *Andrographis paniculata* (AAP14352), *Arabidopsis thaliana* HMGR1 (P14891) and *Hevea brasiliensis* (AAQ63055). Alignment was performed using ClustalW and *shaded* using the BoxShade Version 3.21 software program (Human Genome Sequencing Center, Houston, TX). Residues *shaded in black* indicate conserved residues (identical in at least four out of six sequences shown), and residues *shaded in gray* are similar in at least two of six sequences shown. *Dashes* indicate gaps that have been inserted for optimal alignment. The positions of degenerate forward and reverse primers used for obtaining ~450 bp of *AmHMGR1* are indicated by *arrows.*

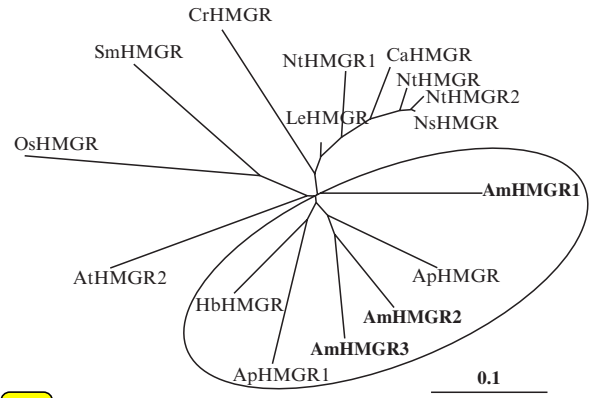


*Fig. 3.* Analysis of HMGR activity of a recombinant *E. coli* expressed AmHMGR1. Reactions were carried out using crude cell extracts from pET28-Rosetta (control, lane 1), pET28-HMGR1-Rosetta (lane 2) and Ni-NTA purified fraction from pET28-HMGR1-Rosetta cells (lane 3) in the presence of $^{14}$C-3-hydroxy-3-methylglutaryl-CoA. Product identification was performed by thin layer chromatography. The $R_f$ value of the product formed by AmHMGR1 corresponds to that of mevalonate.

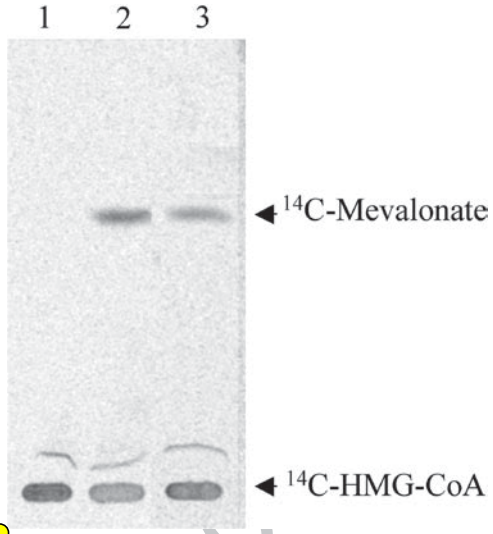


*Fig. 4.* Phylogenetic tree illustrating the relatedness of AmHMGR proteins to other plant HMGRs. The unrooted neighbor joining tree was created using ClustalX and TreeView for visualization. AmHMGR1, AmHMGR2 and AmHMGR3, *Antirrhinum majus* HMGR1 (EF666139), HMRG2 (EF666140) and HMGR3 (EF666141); ApHMGR, *Andrographis paniculata* HMGR (AAP14352); AtHMGR1 and AtHMGR2, *Arabidopsis thaliana* HMGR1 (P14891) and HMGR2 (P43256); CaHMGR, *Capsicum annuum* HMGR (Q9XEL8); CrHMGR, *Catharanthus roseus* HMGR (AAT52222); HbHMGR, *Hevea brasiliensis* HMGR (AAQ63055); LeHMGR, *Lycopersicon esculentum* HMGR (AAL16927); NtHMGR, NtHMGR1 and NtHMGR2, *Nicotiana tabacum* HMGR (AAL54879), HMGR1 (AAB87727) and HMGR2 (AAL54878); NsHMGR, *Nicotiana sylvestris* HMGR (Q01559); OsHMGR, *Oryza sativa* HMGR (AAA21720); SmHMGR, *Solanum melongena* HMGR (AAQ12265).

## III The MEP Pathway and Rhythmic Emission of Leaf Volatiles

Rhythmic release of terpenoids is not only limited to flowers, but is also found in vegetative tissue. An evergreen holm oak *Quercus ilex* widespread in the Mediterranean forests emits 14 terpenes, out of which α-pinene is the most abundant (Loreto et al., 1996; Staudt and Bertin, 1998). Emission of these compounds is light-dependent and three distinct classes were identified based on their responses to light induction: a rapidly induced class including α-pinene, a more slowly induced class including *cis*-β-ocimene, and the most slowly induced class with 3-methyl-3-buten-1-ol as a representative (Loreto et al., 1996). Diurnal oscillations in the emission of terpenoids with peak emission during the day were also reported in Norway spruce (*Picea abies* L.) (Martin et al., 2003), Stone pine (*Pinus pinea* L.) (Staudt et al., 1997), rosemary (*Rosmarinus officinalis* L.), pistachio (*Pistacia lentiscus* L.) (Hansen et al., 1997), and Chinese wormwood (*Artemisia annua*) (Lu et al., 2002) (Table 1). Although the involvement of both the MVA and MEP pathways in the regulation of diurnal terpenoid emission in these species was not investigated, the expression of DXR and β-pinene synthase was analyzed

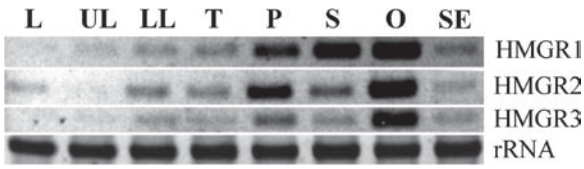


*Fig. 5.* Tissue specific *AmHMGR* mRNA expression in snapdragon flowers. Semiquantitative quantitative RT-PCR analysis of *AmHMGR* in young leaves (L), upper (UL) and lower (LL) petal lobes, tubes (T), pistils (P), stamens (S), ovaries (O), and sepals (SE) of 5-day-old snapdragon flowers is shown. One μg of total RNA from each tissue was used for cDNA preparation and RT-PCR was performed for 25 cycles.

in *A. annua* during a daily light/dark cycle (Lu et al., 2002). The levels of mRNA transcripts for both genes peaked shortly after noon showing a diurnal rhythm in their expression. Moreover, diurnal oscillations in the expression of β-pinene synthase

were retained in plants exposed to constant light or constant dark, suggesting the involvement of a circadian clock in the regulation of its expression (Lu et al., 2002) (Table 2).

## IV The MEP Pathway and Rhythmic Emission of Herbivore-Induced Plant Volatiles

Terpenoids, monoterpenes, sesquiterpenes and homoterpenes, are often released from plant vegetative tissues in response to damage and herbivore attack. These emitted volatiles can directly repel (De Moraes et al., 2001; Kessler and Baldwin, 2001) or intoxicate (Vancanneyt et al., 2001) microbe and animals, or attract natural predators of attacking herbivores, indirectly protecting the signaling plant from further damage via tritrophic interactions (Mercke et al., 2004; Arimura et al., 2004b; Degen et al., 2004). The monoterpenes linalool, *(E)*-β-ocimene and α-pinene, as well as the sesquiterpenes germacrene D, caryophyllene and α- and β-farnesene are the compounds most often released from herbivore-damaged tissues. In most cases, the release of herbivore-induced terpenoids follows diurnal cycles with a peak of emission during the day (Table 1). Examples include emission from grape leaves (*Vitis labrúsca* L.) infested by Japanese beetles (*Popillia japonica*) (Loughrin et al., 1997), cotton plants (*Gossypium hirsutum*) injured by beet armyworms (*Spodoptera exigua* Hübner) (Loughrin et al., 1994), poplar trees (*Populus trichocarpa* × *deltoides*) infested with forest tent caterpillars (FTC) (*Malacosoma disstria*) (Arimura et al., 2004a), Sitka spruce (*Picea sitchensis*) attacked by white pine weevils (*Pissodes strobi*) (Miller et al., 2005), and lima beans (*Phaseolus lunatus*) infested with the Egyptian cotton leafworms (*Spodoptera littoralis*) (Arimura et al., 2005) (Table 1). Moreover, induced diurnal emission of mono-and sesquiterpenoids was found in Sitka spruce and Norway spruce after exposure of intact plants to methyl jasmonate (MeJA), an elicitor simulating insect or pathogen attack, and in lima bean treated with alamethicin (ALA), an ion channel-forming fungal elicitor (Miller et al., 2005; Martin et al., 2003; Kunert et al., 2002).

Similar to the situation with the constitutive emission of volatile terpenoids from vegetative tissues, the contribution of the MVA and MEP pathways to the regulation of rhythmic emission of herbivore-induced volatiles was not investigated. The only example includes hybrid poplar where the expression of (−)-germacrene D synthase and one DXR gene (PtdDXR1) was analyzed in local FTC-infested and systemic leaves (Arimura et al., 2004a). While the FTC-induced expression of (−)-germacrene D synthase exhibited an obvious diurnal profile that peaked during the light period and closely matched the actual pattern of FTC-induced volatile release, FTC did not affect the abundance of PtdDXR1 transcripts which displayed only slight diurnal fluctuations (Arimura et al., 2004a) (Table 2).

## V The MEP Pathway and Rhythmic Emission of Isoprene

Vegetative tissues of many plant species including mosses, ferns, gymnosperms and angiosperms release a highly volatile five-carbon terpene isoprene into the atmosphere (Kesselmeier and Staudt, 1999; Sharkey and Yeh, 2001). Although the biological function of isoprene is still unclear, this hemiterpene may act to increase the tolerance of photosynthesis to high temperatures by stabilizing the thylakoid membranes (Sharkey et al., 2001; Peñuelas et al., 2005; Velikova and Loreto, 2005), protect plants against extensive light (Peñuelas and Munne-Bosch, 2005), serve as an antioxidant by quenching reactive oxygen species (Loreto and Velikova, 2001; Affek and Yakir, 2002) or as an overflow valve for carbon and energy excess (Rosenstiel et al., 2002; Magel et al., 2006). Isoprene emission displays a clear diurnal pattern as was found in many trees including gorse *Ulex europaeus* (Cao et al., 1997), some oak species *Quercus alba*, *Q, rubra*, *Q. robur* (Geron et al., 2000; Funk et al., 2003; Brüggemann and Schnitzler, 2002a), eastern cottonwood *Populus deltoides* (Funk et al., 2003), eucalyptus *Eucalyptus* sp., banyan *Ficus bengalenis*, peepul *Ficus religiosa*, mango *Mangifera indica*, chinaberry *Melia azedarach*, jambolan *Syzygium jambolanum* (Padhy and Varshney, 2005), poplar *Populus* spp. (Mayrhofer et al., 2005), and oil palm *Elaeis guineensis* (Wilkinson et al., 2006) (Table 1). These diurnal oscillations in isoprene emission can be induced by light or controlled by

a circadian clock. Circadian control of isoprene biosynthesis was shown in oil palm and grey poplar (Wilkinson et al., 2006; Loivamäki et al., 2007) raising the issue of its widespread reach in the plant kingdom.

Isoprene is synthesized from DMAPP in chloroplasts in a reaction catalyzed by isoprene synthase (ISPS) (Fig. 1) (Silver and Fall, 1995; Schnitzler et al., 1996; Sharkey et al., 2005; Sasaki et al., 2005) and shares the MEP pathway with monoterpenes, diterpenes and carotenoids as tetraterpenes. Within the plastid, isoprene synthase competes with GPP synthase for DMAPP utilization and has much higher $K_m$ values for DMAPP (in the millimolar range) (Wildermuth and Fall, 1998) when compared with that of GPP synthases (in the micromolar range) (Tholl et al., 2001; 2004; Burke and Croteau, 2002). Rhythmic emission of isoprene can be regulated by the availability of DMAPP substrate and/or by the activity of isoprene synthase. Although the regulatory mechanisms controlling rhythmic isoprene emission are not completely understood, analysis of *PcISPS* and *PcDXR* gene expression in shoot cultures of grey poplar over a daily light/dark cycle revealed diurnal oscillations which were retained in continuous light only for *PcISPS* indicating that its expression is controlled by a circadian clock while the *PcDXR* expression is light-dependent (Loivamäki et al., 2007) (Table 2). However, light was found to be a trigger of *PcISPS* gene expression as well, since a twofold increase in the *PcISPS* transcript levels was observed under constant light conditions. Consistent with the observed results circadian regulatory elements and putative light elements were found in the promoter region of PcISPS gene (Wilkinson et al., 2006; Loivamäki et al., 2007). Despite the circadian rhythm in *PcISPS* expression, the levels of PcISPS protein and its activity did not display diurnal fluctuations suggesting that the availability of DMAPP might be an important factor controlling circadian changes in isoprene emission (Loivamäki et al., 2007). Indeed, leaf DMAPP levels and isoprene emission were closely coordinated and showed similar diurnal variations (Mayrhofer et al., 2005). Light-dependent DMAPP production with highest levels from predawn to midday was also found in all isoprene and methylbutenol (a $C_5$ terpenoid similar to isoprene) emitting and nonemitting species (Brüggemann and Schnitzler, 2002a; Rosenstiel et al., 2002; Magel et al., 2006). Although the capacity to emit isoprene was clearly associated with elevated DMAPP levels (Rosenstiel et al., 2002), diurnal variations in cellular DMAPP levels may be a general characteristic of plant metabolism.

The last step in the MEP pathway is catalyzed by the IspH protein which converts 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphospate into IPP and DMAPP (Fig. 1) at a 5:1 ratio (Adam et al., 2002). This IPP:DMAPP ratio within the cell is adjusted by isopentenyl diphosphate isomerase (IDI) which catalyzes the isomerization of IPP to DMAPP and might play a regulatory role in determining DMAPP levels. Analysis of IDI activity in oak leaves revealed that it is always higher than ISPS activity and that both activities can fully account for the observed isoprene emission. However, like ISPS, IDI activity does not display diurnal oscillations and cannot be responsible for diurnal variations in DMAPP levels and isoprene emission (Brüggemann and Schnitzler, 2002b), which can thus be attributed to circadian diurnal changes in the flux through the MEP pathway.

Feeding experiments with dideuterated deoxyxylulose (DOX-$d_2$) were performed to understand the regulatory mechanisms controlling the flux through the MEP pathway in *Eucalyptus globulus* (Wolfertz et al., 2004). The exogenous DOX-$d_2$ displaced the endogenous sources of carbon for isoprene biosynthesis but did not lead to an increase in isoprene emission suggesting that the DXS activity is negatively feedback modulated by the intermediates of the MEP pathway downstream from deoxyxylulose 5-phosphate (Wolfertz et al., 2004).

DMAPP is the last precursor of isoprene and can be formed not only from the plastidial MEP pathway but also from extrachloroplastic courses of carbon via the cytosolic MVA pathway. The contribution of different sources of DMAPP to isoprene emission was analyzed by comparing the labeling patterns of DMAPP and emitted isoprene in mature leaves of *Populus nigra* and *Phragmites australis* exposed to $^{13}CO_2$ (Loreto et al., 2004). A rapid, high level of $^{13}C$ labeling of emitted isoprene (90% in 15 min) along with a partial DMAPP labeling (28–36%) indicates that the labeled DMAPP represents a chloroplastic DMAPP which contributes to isoprene emission. Pretreatment of

leaves with fosmidomycin resulted in residual isoprene emission and a very low $^{13}C$ labeling of the DMAPP pool suggesting that the residual isoprene is formed from the extra-chloroplastic sources and that at the very least, the MEP and MVA pathways are not cross-linked following inhibition of the plastidial pathway (Loreto et al., 2004; Nogués et al., 2006).

## VI Conclusions

The crucial role of volatile terpenoids in the plant life cycle highlights the importance of understanding their biosynthesis and regulation of their formation and emission. Terpenoid emission from flowers and undamaged and herbivore attacked leaves often exhibit rhythmic patterns. However, reports concerning rhythmicity in terpenoids concentrate mainly on in planta chemical composition and emission profiles leaving the regulatory mechanisms of rhythmic emission still unknown. The recent discovery of the MEP pathway revealed that IPP and DMAPP could be synthesized not only in the cytosol but also in plastids and the exchange of intermediates between subcellular compartments could exist, thus adding an additional level of complexity to the investigation of the regulation of the flux towards volatile terpenoids. Despite the discovery of all the genes of the MEP pathway (see Fig. 1) the contribution of these genes to the regulation of the rhythmic emission of terpenoids in plants still remains to be determined. Further investigations of the MEP pathway enzymes and direct measurements of their levels and activities will provide new insights into the complex regulatory network of isoprenoid biosynthesis in plants. The determination of IPP and DMAPP pools in different cellular compartments in combination with feeding experiments with pathway specific precursors and inhibitors will uncover the contribution of each of the IPP biosynthetic pathways to the rhythmic emission of terpenoids. The integration of metabolic profiling with transcriptomic and proteomic datasets will help to elucidate the regulatory aspects of the isoprenoid network in plants. At present it is not known at what level the circadian clock controls terpenoid emission. Thus, the discovery of principles underlying circadian clocks and potential connections between circadian oscillations in gene expression and oscillations in metabolic activity are expected to yield important new insights into the role of the endogenous biological clock in the regulation of rhythmic emission of terpenoids.

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# Author Queries

Chapter No.: 10

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