



Review

Isoprenoid generating systems in plants – A handy toolbox how to assess contribution of the mevalonate and methylerythritol phosphate pathways to the biosynthetic process



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ABSTRACT

Isoprenoids comprise an astonishingly diverse group of metabolites with numerous potential and actual applications in medicine, agriculture and the chemical industry. Generation of efficient platforms producing isoprenoids is a target of numerous laboratories. Such efforts are generally enhanced if the native biosynthetic routes can be identified, and if the regulatory mechanisms responsible for the biosynthesis of the compound(s) of interest can be determined.

In this review a critical summary of the techniques applied to establish the contribution of the two alternative routes of isoprenoid production operating in plant cells, the mevalonate and methylerythritol pathways, with a focus on their co-operation (cross-talk) is presented. Special attention has been paid to methodological aspects of the referred studies, in order to give the reader a deeper understanding for the nuances of these powerful techniques. This review has been designed as an organized toolbox, which might offer the researchers comments useful both for project design and for interpretation of results obtained.

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Abbreviations: Ac-CoA, acetyl CoA; AACT, acAc-CoA thiolase; CLM, clomazone (2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone); DMAPP, dimethylallyl diphosphate; DMNT, (3E)-4,8-dimethyl-1,3,7-nonatriene; DOXP, deoxyxylulose phosphate; DX, 1-deoxy-D-xylulose; DXK, D-xylulose kinase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; ER, endoplasmic reticulum; FOH, farnesol; FPP, farnesyl diphosphate; FSM, fosmidomycin; GAP, D-glyceraldehyde 3-phosphate; GOH, geraniol; GGOH, geranylgeraniol; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; HMGR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; INST-MFA, isotopically non-stationary state metabolic flux analysis; IPP, isopentenyl diphosphate; IRMS, Isotope Rate Mass Spectrometry; ME, 2C-methyl-D-erythritol; MEcPP, 2C-methyl-D-erythritol 2,4-cyclodiphosphate; ME-glc, glucosylated 2C-methyl-D-erythritol; MVA, mevalonic acid/mevalonate; MVK, mevalonate kinase; MVL, mevalonolactone; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Pyr, pyruvate; SnRK-1, sucrose non-fermenting-1-related protein type kinases.

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1. Introduction

Plants exhibit a remarkably high level of biochemical complexity and flexibility which, despite their sessile lifestyle, allows them to survive in different environments and quickly adapt to various fluctuating conditions. This metabolic plasticity is to great extent achieved due to the isoprenoids, the most structurally and functionally diverse class of plant natural products which includes essential primary metabolites (e.g., phytosterols, chlorophylls, ubiquinone) as well as a broad range of functional secondary metabolites. These specialized natural products play a vital role in plant adaptive responses to biotic and abiotic stress, attract pollinators, repel predators, modulate allelopathic interactions (e.g. isoprene, monoterpenes, diterpenes). Isoprenoids exert either synergistic (photosynthetic pigments) or antagonistic (phytohormones: gibberellins and cytokinins) effects in cellular processes. Some isoprenoids are produced constitutively (e.g. phytosterols) while synthesis of others is induced upon particular cues such as wounding, attack of predator, elicitation or upon elevated concentration of methyl jasmonate (e.g., sesquiterpenoids, triterpenoids). All these features add up to the exquisite modulatory potential of the so-called plant terpenome [1]. Evidently, to fully exploit this prospective, the plant cell requires precise and strictly regulated metabolic network.

The use of plant isoprenoids as pharmaceuticals, fragrances, flavours, colorants, and dietary supplements (Fig. 1) makes them the most commercially exploited group of plant-derived natural products with the market worth millions of dollars. Those economically priceless isoprenoids are present in plant tissues in rather small amounts and their acquisition from natural resources is in most cases very expensive, insufficient and may pose a threat for biodiversity (e.g. isolation of paclitaxel from Pacific yew). Therefore, unraveling the isoprenoid biosynthetic network is not only of interest for basic studies but also benefits and stimulates the construction of efficient platforms for their production [2]. Indeed, the continuously increasing number of articles published in this field illustrates how much attention it receives (for example [3–7], with totally over 30 reviews and more than 100 experimental papers, focused on isoprenoids biosynthesis, published in 2014, according to PubMed).

2. Biosynthesis of isoprenoids

Despite all the structural splendor of isoprenoids, they are derived from the common five carbon precursors: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). For many years, it was thought that the biosynthetic origin of the IPP and

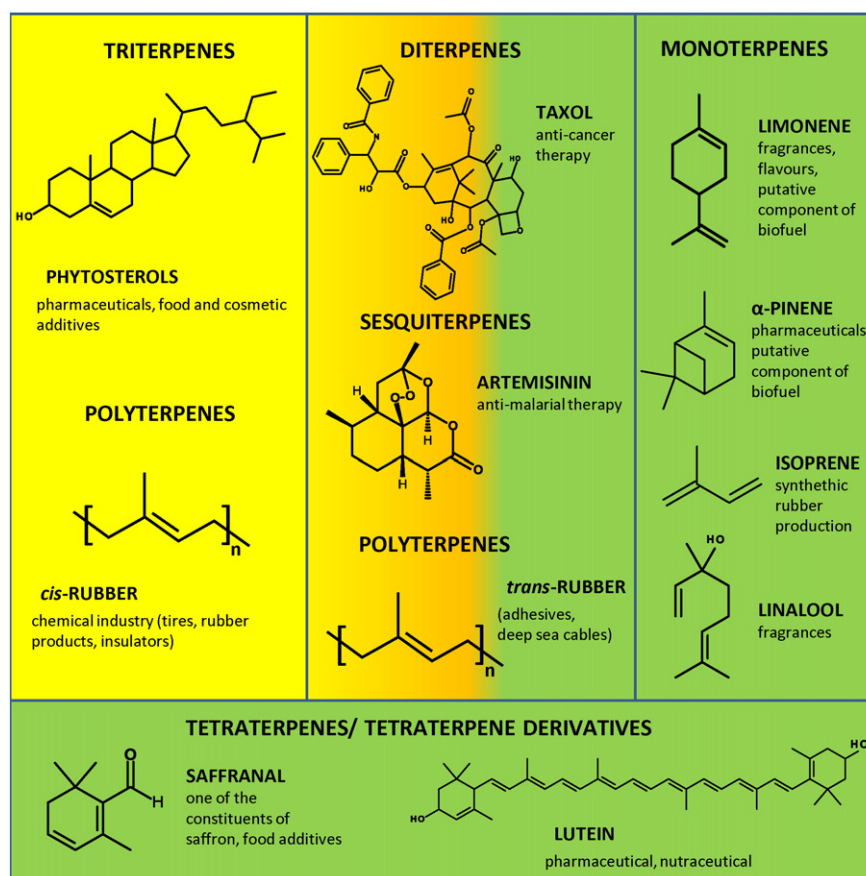


Fig. 1. Examples of commercially valuable isoprenoids. Biosynthetic origin of compounds is reflected in colouring of the background (green and yellow for the MEP and MVA pathway, respectively). Gradient background indicates substantial contribution of both pathways to biosynthetic process.

DMAPP was uniform in all kingdoms and provided by the cytoplasmic mevalonate (MVA) pathway (MVA is the first dedicated molecule of this pathway). However, in contrast to the observations made for yeast and animal cells, results obtained in the bacteria and plants questioned this assertion [8,9]. It was found, for example, that radioactive mevalonate, in contrast to CO₂, was not incorporated into β-carotene in etiolated or illuminated maize seedlings [10], similarly radioactive acetate was not incorporated into lycopene in ripening tomatoes [11]. On the other hand incorporation of CO₂ to sterols and ubiquinone was negligible in contrast to efficient labelling of plastidial lipids, i.e. phytol, carotenoids and plastoquinone in mono- and dicotyledonous plants [12]. In line with this mevinolin, a specific inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), the key enzyme of the MVA pathway, caused inhibition of accumulation of sterol, but had little effect on the chlorophyll and carotenoid in the cotyledons of radish seedlings [13]. Further on, etioplasts and etiochloroplasts of mustard seedlings were found to lack enzymatic activities for the formation of isopentenyl diphosphate via the mevalonate pathway [14]. Interestingly, though the suggestion that the mechanism of formation of plant and animal isoprenoids was different has been raised already at the time of these pioneer studies [11], it took many years until an ‘earthquake’ shocked the isoprenoid field. The existence of a novel, mevalonate-independent pathway supplying isoprenoid precursors based on the studies of side chains of bacterial hopanoids was ultimately

demonstrated in late 1980s [15,16]. This postulate fully explained the discrepancies which were previously incomprehensible in light of the MVA pathway. The existence of this new pathway was subsequently confirmed by several studies in different plant models [17,18] and the hypothesis about the alternative, non-mevalonate pathway (called sometimes the Rohmer’s or the DXP pathway) quickly became widely accepted.

Nowadays, after almost thirty years of extensive studies on the alternative pathway (presently called after its first committed precursor methylerythritol-4-phosphate, the MEP pathway) is well established as a single source of isoprenoids in the majority of eubacteria, while the ‘classical’, MVA pathway is utilized in archaeobacteria, fungi and animals. However, a substantial number of exceptions should be kept in mind. For example, bacteria from the same group use variable pathways, in other cases unrelated bacteria use the same pathway [19]. Still, in this context, the plant kingdom is unique, as majority of its representatives utilize both pathways in parallel (green algae are the exception since they depend exclusively on the MEP pathway). A schematic up-to-date representation of the MEP and MVA pathways is shown in (Fig. 2). Decades of observations of the co-existence/cooperation of the two alternative isoprenoid-generating pathways in plants has been summarized recently in an excellent and comprehensive review by Hemmerlin et al. [20].

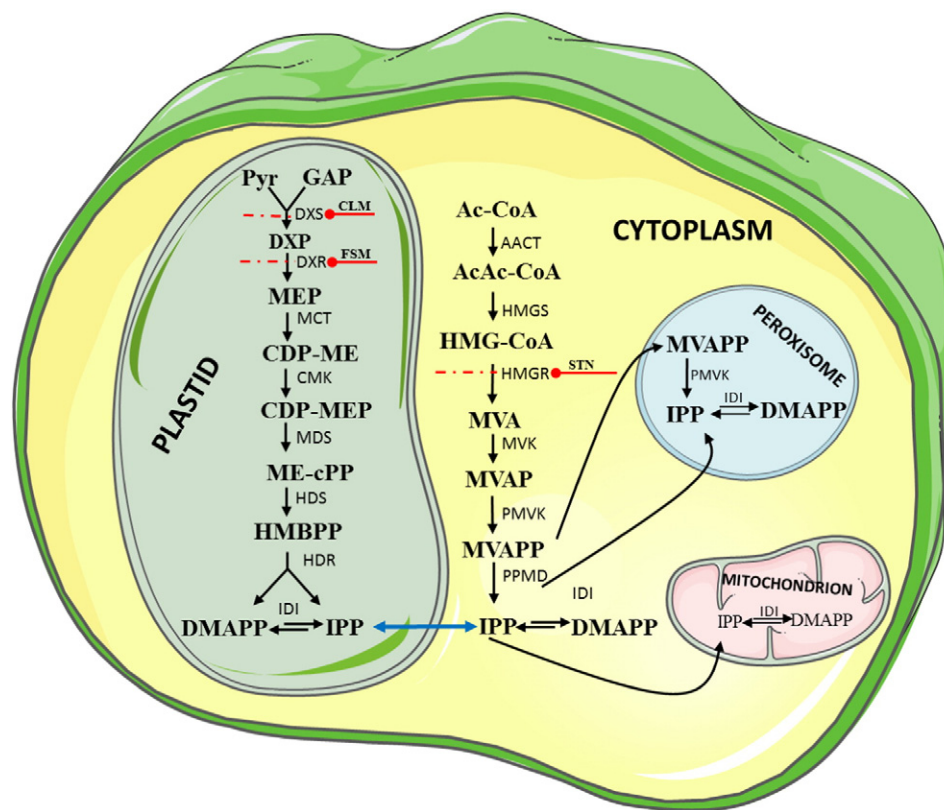


Fig. 2. Synthesis of isopentenyl diphosphate in the plant cell via the MVA and MEP pathway. MVA pathway. Enzymes: AACT (EC 2.3.1.9), acetoacetyl-CoA thiolase; HMGS (EC 2.3.3.10), 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR (EC 2.3.3.10), hydroxymethylglutaryl-CoA reductase; MVK (EC 2.7.1.36), mevalonate kinase; PMVK (EC 2.7.4.2), phosphomevalonate kinase; PPMD (EC 4.1.1.33), diphosphomevalonate decarboxylase; IDI (EC 5.3.3.2), isopentenyl diphosphate isomerase. Intermediates: Ac-CoA, acetyl-coenzyme A; AcAc, CoA acetylacetyl-coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; MVA, mevalonic acid; MVAP, mevalonate-5-phosphate; MVAPP, mevalonate-5-diphosphate; IPP, isopentenyl diphosphate isomerase; DMAPP, dimethylallyl pyrophosphate. MEP pathway. Enzymes: DXS (EC 4.1.3.37), 1-deoxy-D-xylulose-5-phosphate; DXR (EC 1.1.1.267), 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MCT (EC 2.7.7.60), 2C-methyl-D-erythritol 4-phosphate cytidyl transferase; MDS (EC 4.6.1.12), 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS (EC 1.17.4.3), 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase; HDR (EC 1.17.1.2), 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase; IDI (EC 5.3.3.2), isopentenyl diphosphate isomerase. Intermediates: Pyr, pyruvate; GAP, D-glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; MEP, 2C-methyl-D-erythritol 4-phosphate; CDP-ME, 4-diphosphocytidyl-2C-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate; ME-cPP, 2C-methyl-D-erythritol 2,4-cyclodiphosphate; HMBPP, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl pyrophosphate. Red lines indicate enzymes inhibited by specific inhibitors of the MEP or MVA pathway: clomazone (CLM), fosmidomycin (FSM), statins (STN). Blue arrow symbolises bidirectional cross-talk between the pathways.

3. Cross-talk between the MVA and MEP pathway

Possessing two active, alternative isoprenoid biosynthetic pathways provides plant cell with the possibility of producing of a large number of specialized compounds, which enable its efficient and quick adaptation to the constantly changing environment. It requires sophisticated control mechanisms to ensure an optimal energy balance and precise metabolic carbon channeling. One level of this control is physical separation. According to the current model, MVA pathway enzymes are localized in the cytoplasm, ER and peroxisomes [21], whereas the MEP pathway route enzymes are sequestered inside the plastids by their double membrane envelopes. Both these pathways give rise to and administer two separate IPP/DMAPP pools (Fig. 2). Interestingly, mitochondria do not operate either the MVA or the MEP pathway and depend on the cytoplasmic supply of the IPP and/or farnesyl diphosphate (FPP) [22,23].

Because of this compartmentalization isoprenoid end products were initially thought to be either of MVA (triterpenoids, sesquiterpenoids) or MEP (monoterpenoids, diterpenoids, tetraterpenoids) origin. However, a growing body of the *in vivo* and *in vitro* results describing the formation of compounds of mixed origin, i.e. comprised of isoprenoid units derived from both pathways, proves that an exchange of the intermediates between both pathways is possible and examples of such 'mosaic' isoprenoids identified in different plant species have been listed [20]. Hitherto, observations suggest that a bilateral mode of exchange is possible but the flow from plastids to cytoplasm seems to be more effective [24]. The best candidate for such an "exchangeable" intermediate seem to be IPP and FPP [25,26]. Despite the fact that FPP synthase (FPPS) is considered a cytosolic and mitochondrial enzyme the presence of the small plastidic FPP pool was reported in Arabidopsis plants, expressing linalool/nerolidol synthase (*FaNES1*) in the plastids. Leaves of these transgenic plants, besides linalool, produced also trace amounts of FPP-derived sesquiterpene — nerolidol. However, whether FPP is produced in plastids by yet unidentified FPPS or if it is rather transported from cytoplasm, remains elusive [27]. Export of plastidial geranyl diphosphate (GPP) to cytoplasm was proven in transgenic tomato fruits [28]. Besides oligoprenyl intermediates longer chain oligoprenyl diphosphates have also been postulated to get exported from plastids towards the cytoplasm [29]. Interestingly, enhanced cross-flow of the exchangeable intermediates from the parallel pathway is possible upon blockage or downregulation of one pathway [30] and on early developmental stages (for example to synthesize carotenoids, before the MEP pathway is fully operational [31]). Apparently, the extent of the cross-talk between the isoprenoid pathways strongly depends on internal and external stimuli and reflects the plasticity of the biosynthetic routes. It justifies the complex multilevel regulation of the enzymes involved [32]. Molecular mechanisms responsible for the exchange of isoprenoid intermediates between the cellular compartments remain elusive. Transporter-assisted mode of interchange was suggested [25], but so far none of the tested plastid membrane components was shown to mediate transport of prenyl diphosphates [33].

Here we summarize an array of methods used to unravel and quantify contribution of the MVA and MEP pathways to the biosynthesis of the isoprenoid of interest. These biochemical and genomic approaches have been applied by various research groups, over decades. Many excellent reviews have been published on the different aspects of isoprenoid biosynthesis in microbes especially interesting in terms of their application in metabolic engineering of MVA and MEP pathways [34–37]. Similarly, decades of research in the field of plant isoprenoid biosynthesis have been summarized in a number of comprehensive review articles [20,31,38].

In this review we will highlight specific features of the currently applied experimental methods and critically discuss the limitation which should be considered in experimental design and certainly when interpreting the results.

4. Biochemical tools to elucidate the origin of plant isoprenoids and cross-talk between the MVA and MEP pathway

4.1. Metabolic labelling — general comments

The term 'metabolic labelling' refers to the method in which the organism is grown in presence of a traceable compound, which is utilized as a substrate by the endogenous metabolic machinery and incorporated in a newly synthesized product(s) of interest. By this means, a particular natural building block is replaced with its chemically tagged analogue, and conclusions regarding the pathway involved in its biosynthetic route can be drawn based on identified number and/or location of the labelled atoms in the molecules of the product of interest. This is performed usually simply by culturing organisms, tissue cultures or cell cultures with the substrate of choice, and its entering the cellular metabolism depends on the uptake by cells from the surrounding medium. After a specified "feeding" period, the compound under study is isolated and the structure of the labelled product and/or the amount of the incorporated label is determined.

Radioactive (^{14}C , ^{32}P , ^3H) or stable (^{13}C , ^{31}P , ^2H) isotope analogues of metabolic precursors are utilized in metabolic labelling studies. Their usage is based on the assumption that the presence of these labelled atoms, in fact natural isotopes, does not markedly change the chemical properties of molecules, so they follow the 'native' cellular tracks and allow monitoring of biochemical processes in a 'native' milieu. Isotopic labelling as a powerful tool to elucidate metabolic flux in the context of lipid metabolism has recently been summarized in the comprehensive review [39].

4.1.1. Radioactive isotope labelled precursors

As the radioactivity of natural isotopes of carbon and hydrogen is minimal (natural abundance of ^{14}C is 0.0000000001% and even lower for ^3H) the background of the labelling experiment is negligible. Although ^{14}C or ^3H labelled end-products are easily detected by radiometric techniques, only application of a combination of adequate purification methods (e.g. chromatography, electrophoresis) ensures the satisfactory level of certainty, and that the detected radioactivity is derived from the molecule under study and not from contamination with another radioactive compound, the labelling of which is an unavoidable consequence of the isoprenoid metabolic precursor application. Depending on the analyzed molecule, radio-gas chromatography (radio-GC), radio-high performance liquid chromatography (radio-HPLC), radio-thin layer (radio-TLC) or liquid scintillation counting (LSC) are used. However, because of the safety hazards, assays are laborious and time-consuming, not to mention the cost of the labelled precursors most of which have to be prepared by custom synthesis. Additionally, identification of the position of the radio-labelled atom in the molecule is difficult (as it requires complicated chemical degradation procedures). Despite this drawback, radiolabelling with early intermediates of both pathways is still used in isoprenoid research, for instance for investigation of the origin of prenyl diphosphates (FPP and GGPP) used for prenylation of proteins, and in this case combination with autoradiography or fluorography is applied [30]. Moreover, application of the radiolabeled precursors seems to be an excellent method to determine experimental conditions to be used in further experiments with stable isotope-enriched precursors.

4.1.2. Stable isotope labelled precursors

At present metabolic labelling with stable isotopes (^{13}C , ^2H , ^{31}P) is frequently used to elucidate the contribution of the MVA, MEP or both pathways to biosynthesis of particular isoprenoid. Incorporation of these isotopes is detected as the increase in mass to charge, m/z , value of the reporter ion observed after labelling (followed by Mass Spectrometry techniques) or as the increase of the relative abundance of the ^{13}C atom at certain positions in the molecule (followed by $1\text{D } ^2\text{H}$, ^{13}C and

Table 1
Examples of employment of metabolic labelling to elucidate regulation of isoprenoid biosynthesis. Selected plant models together with the applied concentration of general or specific precursors as well as summary of the obtained results are presented.

Precursor concentration time of treatment	Plant model/stage of development	Compound of interest	Method of analysis	Observations	Reference
General precursors					
Glucose/sucrose					
[1- ¹³ C]glucose					
0.3% w/v 12 days	<i>Lemna gibba</i> (duckweed); cell culture	Phytosterols: stigmasterol, sitosterol		• Phytosterols: ¹³ C labelling pattern in accordance with the MVA pathway; weak labelling on carbon atoms derived from C-1 of IPP explained by incorporation of ¹³ CO ₂ (e.g. from oxidative pentose phosphate pathway) although incorporation of IPP from the MEP pathway was not excluded	
[1- ¹³ C]glucose	<i>Daucus carota</i> (carrot), green tissue cultures	Plastidic isoprenoids: phytol, β-carotene, lutein plastoquinone-9	¹³ C NMR	• Plastidic isoprenoids: ¹³ C labelling pattern distinctive from phytosterols, corresponding to the one observed in bacteria and green algae, in accordance with synthesis via the MEP pathway	[46]
[1- ¹³ C]glucose	<i>Hordeum vulgare</i> (barley) embryos				
1% w/v 5 days					
[1- ¹³ C]glucose	<i>Croton sublyratus</i> /20 day old callus culture	Phytosterols (β-sitosterol, stigmasterol)	¹ H and ¹³ C NMR	• All the carbon atoms labelled equally indicating both the MVA and MEP pathway contribution	[47]
0.4% w/v 3 days					
[1- ¹³ C]glucose	<i>Piper aduncum</i> young leaves	Chromenes: methyl 2,2-Dimethyl-2H-1- chromene-6-carboxylate and methyl 2,2-Dimethyl-8-(3'- methyl-2'-butenyl)-2H- 1-chromene-6-carboxylate	¹³ C NMR	• Contribution of both the MVA and MEP pathways into studied chromenes	[48]
0.1% w/v 72h		Polyisoprenoids (dolichols)			
[1- ¹³ C] glucose, [1,6- ¹³ C ₂]glucose	<i>Coluria geoides</i> hairy root cultures		¹ H and ¹³ C NMR (for [1- ¹³ C]glucose labelling)	• Labelling pattern indicates dual pathway origin of dolichols, with ω-terminal part of the molecule containing both MEP- and MVA-derived isoprene units and the α-terminal part exclusively derived from the MVA pathway	[29]
			HPLC/ESI-MS quantitative analysis	• No ¹³ C scrambling was observed • The calculated average number of isoprenoid units derived from the MEP per dolichol molecule was between 6 and 8 • Proposed spatial model of dolichol biosynthesis: oligoprenyl chain (up to 14 isoprenoid units) is synthesized in plastids from IPP pool of mixed origin, transported to cytoplasm and elongated with the IPP to the final length	
¹⁴ C sucrose	<i>Hevea brasiliensis</i> latex (cell-free system)	Latex (long chain isoprenoid polymer)	Gas flow proportional counter	• Sucrose was not incorporated into the latex structure	[49]
Carbon dioxide 440 ppm ¹³ CO ₂ Up to 200 min	<i>Quercus agrifolia</i> coast live oak, <i>Populus deltoids</i> cottonwood leaves of 5-year-old trees	Isoprene	Proton Transfer Reaction – MS GC–MS	• Rapid labeling of isoprene (80% of ¹² C in isoprene pool was replaced by ¹³ C • Labelling under conditions inhibiting photorespiration did not remove the unlabeled pool of isoprene contribution of cytosolic pool of carbon into isoprene biosynthesis is suggested	[50]
700 ppm ¹³ CO ₂ 100 min	<i>Artemisia annua</i> (high artemisin yielding line) young plants	Artemisinin	¹³ C NMR	• Mixed origin of isoprenoid units in artemisinin structure: artemisin precursor (FPP) is built of one IPP from the MEP pathway and DMAPP and IPP derived from the MVA pathway	[51]
Acetate [2- ¹⁴ C]acetate 7 days	<i>Marrubium vulgare</i> white horehound etiolate shoot culture	Marrubiin (labdane diterpene) sterols	Liquid-scintillation counting	• High incorporation rate into sterols (approx. 0.83%) and low into marrubin (approx. 0.058%) which indicates different biosynthetic routes for these compounds	[52]
[2- ¹⁴ C]acetate 24 h	<i>Zea mays</i> etiolated seedlings, grown for 6 days under weak red light and illuminated for 24 h	β-Carotene (crystals)	Radioactivity proportional counter	• No significant uptake of labelled precursor into intact seedlings in contrast to excised ones • Considerable ¹⁴ C incorporation into unsaponifiable lipid fraction (i.e. sterols), no labeling of β-carotene	[9,10]
Pyruvate					

[2- ¹³ C]pyruvate 0.1% or 0.5% w/v 30 days Amino acids [U- ¹⁴ C]-L-leucine, [¹⁴ C]-L-valine [2- ¹⁴ C]-DL-MVA	<i>Eucommia ulmoides</i> seedlings	Trans rubber-like isoprenoids	¹³ C NMR	- Pyruvate was incorporated as intermediate of the MVA pathway [53] - High-molecular weight isoprenoid was synthesized not only from the mevalonate pathway but also the MEP pathway
	<i>Cinnamomum camphora</i> Sieb. var. <i>linalooliferum</i> (camphor tree)	Linalool	Liquid scintillation counting	- Labelled leucine was incorporated in the DMAPP-derived moieties of linalool at 4-fold higher rate than [2-¹⁴C]MVA indicating the MVA-independent route of monoterpene biosynthesis [54,55] - The labelling pattern of DMAPP-derived moieties of linalool after feeding with a ¹⁴C valine confirmed contribution of non-mevalonate pathway
L-leucine/0.35% L-valine/0.2% L-asparagine/0.676% (non-labelled) Up to 14 days [2- ¹³ C]glycine 1 mM 21 days	<i>Phycomyces blakesleeana</i> (mold)	β-Carotene	Spectrophotometry	- Stimulation of the β-carotene accumulation in medium supplemented with L-valine and L-leucine [56] - Addition of amino acid did not increase lipid production - Stimulatory effect was not observed, either in medium supplemented with the excess or devoid of glucose
	<i>Heteroscyphus planus</i> (liverwort) cell suspension cultures	Chlorophyll β-carotene	² H and ¹³ C NMR	- Labelling patterns indicate that both MVA and MEP pathways contribute to chlorophyll and β-carotene synthesis [57] - The phytol side-chain and β-carotene were equivalently labelled, indicating similar mechanism of the biosynthesis
[1- ¹³ C]-DL-serine, [3- ¹³ C]-DL-serine 0.38 mmol 21 days	<i>Heteroscyphus planus</i> cell cultures	Chlorophyll Stigmasterol	¹³ C NMR	Labelling pattern in agreement with synthesis via the MVA pathway [58]
Pathway-specific precursors Mevalonic acid (MVA)/- mevalonolactone (MVL) [2- ¹⁴ C]MVA 24 h	<i>Zea mays</i> etiolated seedlings, grown for 6 days under weak red light and illuminated for 24 h	β-Carotene	Radioactivity proportional counter	- No significant uptake of labelled precursor into intact seedlings, in contrast to excised ones [9,10] - Considerable ¹⁴C incorporation into unsaponifiable lipid fraction of (i.e. sterols), no labeling of β-carotene
[2- ¹³ C]MVL [4,5- ¹³ C ₂]MVL 1 mM 21 days	<i>Lophocolea heterophyll</i> <i>Heteroscyphus planus</i> cell cultures	Chlorophyll β-Carotene Heteroscyphic acid A (diterpene)	² H and ¹³ C NMR	- MVA was incorporated into the FPP-derived portion of the β-carotene and phytol moiety in chlorophyll a, while the IPP-derived portion was only slightly labelled. [57] - Biosynthesis of chlorophyll a and β-carotene utilized exogenous MVA much more efficiently than that of heteroscyphic acid A - Different sites for biosynthesis of diterpenes and photosynthetic pigments within chloroplasts were proposed
[2- ¹³ C]MVL 3 mM 9 days in the medium supplemented with 10 μM of mevinolin (MVA pathway inhibitor)	<i>Arabidopsis thaliana</i> seedlings/liquid culture	Cytokinins (CKs): <i>cis</i> zeatin (cZ) and <i>trans</i> zeatin (tZ), isopentenyladenine (iP)	HPLC-MS	- Approx. 75% of the prenyl moiety of cZ-type CKs was labeled, which was nearly as efficient as labelling of campesterol (90%) [59] - Incorporation rates indicate the MVA pathway as the predominant source of DMAPP for cZ type CKs - tZ- and iP-type were labelled only at a low rate by [2-¹³C] MVL
[² H ₂]MVL 0.3% w/v 60 h	<i>Antirrhinum majus</i> (snapdragon) flowers	Monoterpenes: myrcene, (E)-β-ocimene and linalool; sesquiterpene-nerolidol	GC-MS	- Increased levels of emitted nerolidol, but not monoterpenes [60] - Monoterpenes remained unlabeled - High rates of incorporation into nerolidol, diurnal fluctuations in the label incorporation rates, highest during the dark period - Fosmidomycin resulted in lack of rhythmicity in nerolidol synthesis and full labelling of nerolidol - Mevinolin did not affect labelling of studied volatiles - Endogenous MVA seemed not to be formed (mevinolin had no effect on labelling efficiency), however downstream enzymes remain active
[5- ² H]MVL 0.0039%	<i>Colouria geoides</i> hairy roots	Polyisoprenoids (dolichol)	HPLC-ESI/MS	Intensities of all but the monoisotopic signals were enhanced indicating involvement of MVA pathway into dolichols biosynthesis [29]

(continued on next page)

Table 1 (continued)

Precursor concentration time of treatment	Plant model/stage of development	Compound of interest	Method of analysis	Observations	Reference
21 days				- Low incorporation rates due to low concentration of precursor in medium - No incorporation into monoterpenes and diterpene, high rates of incorporation into sesquiterpenes; - Conclusion: IPP is not transported from cytosol to plastids	[27]
[5,5- ² H ₂]MVL 0.0001% w/v 48 h	<i>Vitis vinifera</i> (grape wine) fruits at full fruit ripeness; exocarp (ex) and/or mesocarp (me)	Monoterpenes (me, ex), diterpene- 13-epi-manoyl oxide, various volatile sesquiterpenes (ex)	GC-EI/MS; enantioselective GC-EI/MS		
[2- ¹³ C]MVA 0.2% w/v 24 h of continuous feeding; pulse-chase feeding for 4, 8, 20 and 44 h	<i>Gossypium hirsutum</i> (cotton) two-week old plantlets at the developmental stage of two true leaves	Monoterpenes, sesquiterpenes, terpenoid aldehydes, chlorophylls, carotenoids, phytosterols	GC-EI/MS HPLC-APCI/MS	- The general pattern of incorporation in accordance with the classical biosynthetic model with high rate of incorporation of ²H₂ MVA into the sesquiterpenes, terpenoid aldehydes and steroids - Considerable rate of intermediate exchange between pathways as high percentage of carotenoids (up to 34%) and phytol side chains of chlorophylls (up to 44%) were labelled; majority of ¹³C isoprenoid units derived from labeled MVA recovered in sitosterol, chlorophyll a and β-carotene - Comparing organs, the highest accumulation of ¹³C sitosterol was found in roots, whereas of other isoprenoids - in the first true leaf - Enhanced accumulation of intermediates of sterol biosynthesis	[62]
1-Deoxy-D-xylulose (DX) [1- ¹³ C]DX, [2,3,4,5- ¹³ C ₄]DX 0.3 mM 7 days	<i>Catharanthus roseus</i> photomixotrophic suspension cultures	Carotenoids (β-carotene, lutein) phytol, sitosterol	¹³ C NMR	- Precursor was incorporated with the equally high efficacy into the β-carotene, lutein and phytol - DX was also incorporated into sitosterol but with 15-fold lower efficacy than into carotenoids and phytol	[63]
[1- ¹³ C]DX 0.8–1.0 mM 15 days	<i>Arabidopsis thaliana</i> mutant <i>cla1-1</i> seedlings grown in liquid culture	Cytokinins (CKs)-: <i>cis</i> zeatin (cZ) and <i>trans</i> zeatin (tZ), isopentenyladenine (iP)	HPLC-MS	- tZ- and iP-type CKs was labeled at a high rate, similarly to that observed in ABA, synthesized mainly from the MEP pathway - Labelling pattern indicates predominant MEP pathway contribution of DMAPP used for prenylation of tZ and iP type CKs ¹³C incorporation rate into cZ was significantly lower	[58]
[² H ₂]DX 2.94 or 36.25 mM up to 6 h	<i>Eucalyptus globulus</i> detached leaves	Isoprene	Photoacoustic spectroscopy	2.94 mM DX-labelled isoprene emitted from 10 to 15 min after the DX feeding onset and reached constant level after 2 h; total emission rate of isoprene remained fairly constant, photosynthesis rates were unaffected, around 50% of total isoprene emitted was labelled 36.25 mM DX-isoprene completely labelled within 2 h, total isoprene emission rate increased, photosynthesis rates decreased, the rate of isoprene emission from [²H₂] DX was around 35% of total isoprene pool	[64]
[² H ₂]DX 0.2% w/v 60 h	<i>Antirrhinum majus</i> snapdragon cut flowers, petals	Monoterpenes: myrcene, (<i>E</i>)-β-ocimene and linalool; sesquiterpene-nerolidol	GC-MS	- No influence on levels of studied volatiles - Labelling detected in all analyzed compounds, diurnal fluctuations in label incorporation rates with highest rates during dark period, - Diminished incorporation rates upon fosmidomycin treatment - Conclusion: exclusively MEP derived IPP contribute to biosynthesis of isoprenoid volatiles in snapdragon flowers	[60]
[5,5- ² H ₂]DX 0.46% w/v 21 days	<i>Colouria geoides</i> hairy roots	Polyisoprenoids (dolichols)	HPLC-ESI/MS	Increased intensities of [M + 2]⁺ as well as the following [M + 3]⁺ and [M + 4]⁺ isotopic signals indicated the MEP pathway involvement into dolichol synthesis	[30]
[5,5- ² H ₂]DX 0.2% w/v 24 h	<i>Gossypium hirsutum</i> cotton two-week old plantlets at the	Monoterpenes, sesquiterpenes, terpenoid aldehydes, chlorophylls,	GC-EI/MS LC-APCI/MS	- Low incorporation rates due to low concentration of precursor in medium - Incorporation in accordance with the classical biosynthetic model with high rate of incorporation of [²H₂]DX into monoterpenes, phytol side chains and carotenoids	[61]

	developmental stage of two true leaves	carotenoids, phytosterols		- Substantial incorporation of ^2H into compounds generally considered as the MVA derived (72–93% of squalene labelled in the leaves) - Differences in the incorporation values among the plant organs: labelling of monoterpenes, sesquiterpenes, terpenoid aldehydes, carotenoids and chlorophylls occurred preferentially in the youngest leaves, labelling of squalene appeared only in leaves but not in roots - The majority of ^2H isoprenoid units were found in chlorophyll a and β-carotene - The highest accumulation of ^2H isoprenoid units was in the youngest leaves - Both the MVA and MEP pathway contributed to sesquiterpene biosynthesis in glandular trichomes as incorporation rates of DX and MVL were similar - Incorporation rates indicated that mono- and diterpenes, in particular steviol glycosides, are almost exclusively formed by the MEP pathway.	[62]
[5,5- $^2\text{H}_2$]DX 0.27% w/v 5 days	<i>Stevia rebaudiana</i> Bertonii leaves and stems of shoot tips with four leaves	Trichome volatiles: monoterpenes, diterpenes (steviol glycosides), sesquiterpenes	GC-MS LC-ESI-MS/MS		
Other intermediates					
[1- ^3H]MEP, 400 μM [2- ^{14}C]MEcPP 10 μM , 80 μM , 0.68 mM, 6.68 mM	<i>Capsicum annum</i> , <i>Narcissus pseudo-narcissus</i> isolated chromoplast	MEcPP phytoene	Scintillation counting	[1-^3H]MEP was diverted efficiently to the MEcPP; both precursors were efficiently converted into lipid-soluble material - Incorporation of radioactivity from [1-^{14}C]MEcPP into phytoene (the main labeled component of the lipid-soluble fraction) was systematically diminished by the addition of increasing amounts of [1-^3H]MEP - In phytoene a partial scrambling of label between (Z)- and (E)-methyl groups of DMAPP derived units and for the corresponding IPP-derived internal units was observed and ascribed to a lack of fidelity of the isomerase that interconverts IPP and DMAPP.	[65]
[2,2- $^{13}\text{C}_2$]MEcPP			NMR		
[^{14}C]MEcPP 300 μM 3–14 h	Isolated chromoplasts and chloroplasts of: <i>Capsicum annum</i> (red, yellow, green pepper) fruits; <i>Citrus sinensis</i> (lemon) fruits; <i>Citrus paradise</i> (grapefruit) fruits; <i>Musa paradisiaca</i> (banana) fruits; <i>Daucus carota</i> L (carrot) root; <i>Narcissus pseudo-narcissus</i> (narcissus) flowers; <i>Chenopodium album</i> (pigweed) leaves; <i>Chelidonium majus</i> (celandine) flowers; <i>Taraxacum officinale</i> (dandelion) flowers and leaves isolated leucoplasts; <i>Solanum tuberosum</i> (potato) tubers	Unpolar lipids (hexane soluble), carotenoids	Liquid scintillation counter	- Precursor was incorporated into unpolar lipids of all studied plants, however with varying efficacy; the highest rate was observed for chromoplast preparations isolated from the petals of narcissus and celandine flowers and red pepper fruits (62% and) 25% of the substrate, respectively) - Incorporation rates into chloroplasts were considerably lower (3–4.5%) than into chromoplasts - Precursor was not incorporated in the leucoplasts isolated from potato - Incorporation into carotenoids of plant plastids was the most efficient for flower petals of narcissus and dandelion	[66]
[5- ^{13}C]ME-4Ac 0.1% or 0.5% w/v 30 days	<i>Eucommia ulmoides</i> seedlings 40- to 50-day-old	<i>Trans</i> rubber-like isoprenoids	^{13}C NMR	Precursor was not incorporated into polyisoprenoids even in the presence of the MEP pathway inhibitor	[53]

2D NMR techniques). Both these analytical methods have their advantages and limitations.

Mass spectrometry requires minute sample amounts for successful analysis and it can be combined with chromatographic separation (HPLC, GC), which gives an opportunity of analyzing complicated matrixes and makes the analytical workup less laborious by using non-purified or only partially purified samples. However, assigning the label to the certain atom in the analyte molecule is possible only after favourable and well-defined fragmentation of the molecule. Moreover, MS methods are destructive for the sample.

NMR, a technique not destructive for the analyte, provides information about the structure of the investigated molecule and defines specific positions of the incorporated isotopes even when the incorporation rates are relatively small (2% abundance at the labelled position is reported to be the threshold for ^{13}C detection) [40]. The drawbacks of the NMR technique are due to the fact that because of relatively low sensitivity the amount of the sample required for the analysis has to be rather high, which sometimes is difficult to achieve and can be costly because of the price for the precursors. Prolonged time of data collection (sometimes several days) is often necessary to increase the signal/noise ratio. Moreover, the analyzed sample needs to be purified to 'chemical homogeneity'. Even low levels of impurities in the sample, e.g. traces of organic solvents or grease, might negatively affect the results by generating signals of high intensity. Moreover, NMR is less applicable for mixtures of compounds and if a mixture of homologs is to be analyzed, their relative ratio has to be known to interpret the NMR spectra. Last but not least, NMR data are easily quantified, thus incorporation rate optimization is necessary to avoid misinterpretation of results.

4.1.3. Methods of labelling

Metabolic labelling experiments are in fact analyses of the metabolic fluxes, as the consecutive chemical modifications occurring along the flow of the labelled molecule through the successive steps of the metabolic network is analyzed. Thus, there may be two methods of performing the experiment: steady state metabolic flux analysis or pulse-chase analysis. In the first case, the experiment is carried out long enough, presuming the label enrichment on each atom reaches equilibrium and remains constant and that the whole system is metabolically stable, which is determined by the measurement of changes in substrate/product levels. This approach requires extended labelling periods (days, weeks) [41]. In contrast, pulse and pulse-chase labelling experiments are designed to investigate the dynamics of the metabolic pathway by tracking the changes of the molecule labelling status over time. It can be analyzed shortly after first exposing the organism to a labelled precursor, and usually a short period of time ("pulse" phase) is followed by exposition to the same unlabelled precursor, usually at much higher concentration ("chase" phase). The "pulse-chase" type of metabolic labelling seems to be more applicable to secondary metabolism, to track label allocation in real time under specific conditions or after elicitor treatment [42].

4.1.4. Biochemical type of precursors

The labelled biomolecule used in the metabolic labelling experiments is a precursor, either established or putative, of the end product under study. The precursor is named here 'specific', if used by only one, either the MVA or MEP metabolic pathway, to form the final metabolite of interest, or 'general' if it serves as a substrate for multiple pathways.

A general precursor in plant cells, glucose, is utilized for the synthesis of e.g. cell wall components, starch and various lipids. Via glycolysis and the pentose phosphate pathway glucose is further converted to pyruvate, which serves as a central cellular node, connecting carbohydrate, fatty acid and amino acid biosynthesis. Metabolic labelling with a general precursor is a feasible tool to determine changes in metabolic network and interconnection between the two pathways under specific conditions (environmental stress, overexpression of the enzymes of the

metabolic pathway). Studies on the cross-talk between the MVA and MEP pathways with application of the general precursor are based on the assumption that different labelling pattern of the compound of interest is observed as a result of their involvement in its production. Therefore, it is crucial that the insightful *in silico* analysis of the predicted labelling pattern in the compounds of interest precedes the selection of the precursor [41].

Theoretically, application of the pathway specific precursor should be advantageous over the general precursor in terms of incorporation rates in the end products, as it prevents splitting of the valuable labelled precursor towards metabolites from different routes. However, practically, precursor availability depends on a number of factors such as transport of the precursor to the compartment of destination (membrane permeability and transporters might constitute critical limitations) or possible degradation of the precursor in each compartment under experimental conditions. As the isoprenoid pathways intermediate levels are maintained in trace concentrations, accumulation of the specific precursor might trigger detoxification mechanisms. Therefore, it is not possible to predict the real 'starting point' concentration of the precursor entering the pathway and consequently modulation of activity of some enzymes by accumulation of substrate/product and/or allosteric regulation is likely to happen. Furthermore, concentrations of the specific precursor higher than native may cause over-accumulation of downstream intermediates and trigger activation of enzyme activity within the pathway [43]. It should be especially taken into consideration in the case of the isoprenoid network as both MVA and MEP pathway are multilevel-regulated, among others by the feedback mechanisms, and these aspects remain largely undiscovered and currently are under intensive investigation [32].

Additionally, in order to ensure a reliable result the most optimal approach would be to compare the results of parallel experiments with differently labelled precursors (leading to different final patterns of the labelled atoms) and to use more than one labelled substrate. Such experimental design would eventually leave less space for speculation. Moreover, it is recommended to apply, whenever possible, both MS and NMR techniques – combination of these two approaches provides deeper insight into the analyzed process.

4.1.5. Metabolic labelling of plant isoprenoids – case studies

Metabolic labelling deserved a good reputation in the field of isoprenoid research as repeating disproportion in the labelling patterns between the diverse isoprenoid end products eventually led to discovery of the MEP pathway. Presently it is still used to distinguish the contribution of both pathways to the isoprenoid end product as well as for metabolic flux analysis to identify rate limiting enzymes in the pathways and factors influencing their kinetics [44,45]. Selected experiments employing metabolic labelling approach are exemplified in Table 1.

4.1.5.1. Metabolic labelling with general precursors. As the general precursor is diverted to numerous metabolites in different compartments in plant cells its application in metabolic labelling might be very informative due to concomitant labelling of a number of metabolites, which might serve as positive or negative controls. Labelled glucose (mono-, poly-, and uniformly labelled), CO_2 , acetate and pyruvate have been successfully applied as general precursors in the investigation of the isoprenoids biosynthetic pathway [22,29,51,67].

4.1.5.1.1. Glucose. Carbohydrates serve as a predominant form of carbon translocation in cells, hence they are widely used as the general precursor. Many cell cultures and *in vitro* cultivation of plants are routinely performed in the presence of sucrose as the main carbon source. Nevertheless, as sucrose enters isoprenoid biosynthetic pathways after being broken down to hexoses, it holds no clear advantage to be used in labelling experiments due to its high price and consequently it is hardly ever used. In terms of stoichiometry, labelled glucose is highly applicable for the studies of the biosynthetic origin of isoprenoids, as if properly designed, it results in a highly distinctive labelling pattern of the IPP and

DMAPP molecules derived from the each particular pathway) (Fig. 3). Although $[1-^{13}\text{C}]$ glucose is most frequently used in such labelling experiments other $[^{13}\text{C}]$ glucose isotopologues might also be used. These experiments are performed in axenic conditions and usually applied to plant models with heterotrophic or mixotrophic metabolism (tissue cultures, cell cultures) to prevent dilution of the valuable label with native glucose. Labelled glucose incorporation in photosynthetically active systems was previously unsatisfactory [51]. The results obtained in experiments designed in that way might not provide reliable information on metabolic fluxes in a genuine plant, especially when the MEP pathway, being so tightly connected with natural source of carbon-photosynthesis is investigated. Indeed, exogenous sugar was shown to modulate metabolic flow through the MEP pathway [68,69].

Additionally, what has to be taken into consideration is that carbohydrates, besides their nutrition function, play a pivotal role as signaling molecules influencing for example the gene expression pattern or the activity of enzymes/pathways not directly connected with sugar metabolism [70]. This is especially true in case of isoprenoid biosynthetic pathway as it was reported that sucrose indirectly inactivates HMGR (3-hydroxy-3-methyl-glutaryl-CoA reductase), the first enzyme of the MVA pathway via influencing the activity of SnRK-1 (sucrose non-fermenting-1-related protein) type kinases [71,72]. Thus, in such experimental conditions the contribution of both pathways to the synthesis of isoprenoids, especially of mixed origin, can be modified and the experiment might lead to partially false conclusions.

An important, and sometimes neglected, aspect of glucose application is the fact that it might undergo several catabolic processes within the cell, therefore it is important to ensure that the conditions of the designed experiments favour glycolysis. To verify that ^{13}C scrambling is really minimal, supporting experimental arguments should be provided, e.g. for labelling performed with $[1-^{13}\text{C}]$ glucose – clearly low ^{13}C abundance should be found for C-3 atoms in the isoprenoid unit and synchronized (i.e. simultaneous and equal) labelling of specific atoms, i.e. C-2, C-4 and C-5 for the MVA pathway and C-1 and C-5 for MEP (see Fig. 3). If any significant scrambling occurs, equilibrated distribution of the ^{13}C label is observed. It should be kept in mind, however, that application of mono-labelled glucose, e.g. $[1-^{13}\text{C}]$ or $[6-^{13}\text{C}]$ glucose leads to the unavoidable decrease of ^{13}C abundance in the molecule of isoprenoid of interest (50% of the initial one in the precursor) due to the metabolic equivalence of C-1 and C-6 carbon atoms in glucose molecule during glycolysis. It might lead to low labelling rates and consequently result in signals of low intensity which are difficult to interpret in thus obtained spectra (NMR, MS). This problem is usually avoided by application of highly ^{13}C -enriched precursor, and especially by the usage of $[1,6-^{13}\text{C}_2]$ glucose. The labelling pattern of selected isoprenoids obtained upon feeding by the latter precursor is presented

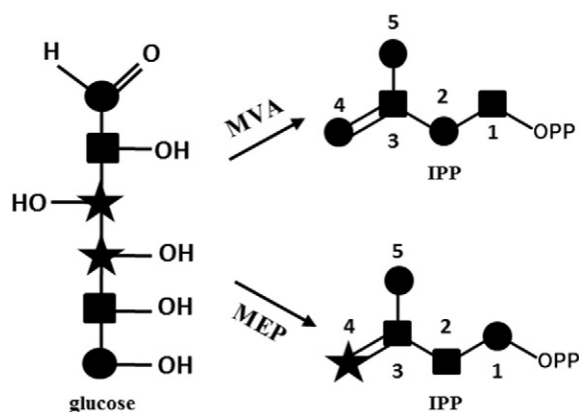


Fig. 3. MVA and MEP pathway-specific labelling pattern of isoprene residues resulting from glucose catabolism via glycolysis. Fate of the respective carbon atoms is depicted by the graphical symbols, metabolically equivalent carbon atoms of glucose molecule are indicated.

in Fig. 4. Despite those obvious obstacles, conclusions obtained so far on the basis of metabolic labelling with glucose have never been questioned when confronted with the results of experiments based on other methodological approaches. Nevertheless, it should be pointed out that in such experimental conditions the contribution of both pathways to the synthesis of isoprenoids of mixed origin might be remodeled and might lead to incorrect conclusions. A quantitative approach, aimed at estimation of the extent and the mode of exchange of intermediates between the MVA and MEP pathways, requires sophisticated and laborious statistical interpretation of the experimental results, e.g. [29].

4.1.5.1.2. Carbon dioxide. Metabolic labelling with another general precursor, CO_2 , has also been used for a long time during isoprenoid biosynthesis investigations starting from the earliest studies in 1950s and including studies on the alternative, at that time putative, non-mevalonate pathway in plant plastids. In these experiments $^{14}\text{CO}_2$ has been efficiently incorporated into chloroplastic isoprenoids (phytol, carotenoids, plastquinone) but at the same time – poorly into sterols and ubiquinone, which indicated the presence of the separate pathways in different compartments [73].

Steady-state metabolic labelling with CO_2 cannot be used to differentiate between the MEP and MVA pathways as in the stationary state all carbon atoms would be labelled. Thus, in this case isotopically non-stationary state metabolic flux analysis (INST-MFA) is usually applied. During this analysis the labelled compound is supplied to the metabolically stable system (for plants – stage of development with the maximum biomass, before the entering reproductive stage), followed by analysis of the label distributions at several time points before the isotopic equilibrium is reached [74,75]. This method has several advantages. Firstly, it is designed for phototrophic tissues and can be easily applied to the whole plants under in vivo conditions. Secondly, introduction of the labelled CO_2 does not interfere with the metabolic dynamics of the pathway (the same concentration of labelled CO_2 is used as in the atmosphere), presuming that label distribution reflects the native metabolic flow. The practical aspects of the INST-MFA method in plant systems has been recently covered by Jazmin et al. [75] and the design of a device for a single *Arabidopsis thaliana* leaf labelling with CO_2 was presented by Kölling et al. [76]. Analysis of metabolic flux based on $^{13}\text{CO}_2$ labelling does not constitute a direct method of investigation of the cross-talk between MVA and MEP pathways, but it may give some indications on the involvement of intermediates from different compartments into plastidic isoprenoids. If the compound is labelled to a lesser extent than predicted for the MEP pathway (based on metabolic flux analysis or MEP derived intermediate incorporation) its MEP origin is less plausible. Such analysis should include tracking of labelling of the isoprenoid under investigation together with other isoprenoids known to be derived from the MVA and MEP pathway in the course of time.

CO_2 pulse labelling has also been applied to identify the MEP origin of artemisinin from *Artemisia annua*. To achieve this, a systematic analysis of ^{13}C NMR spectra by spectra deconvolution affording abundances for individual isotopologues is used. This approach is based on the earlier observations that intensities of ^{13}C NMR signals, and notably those parts of the signals arising by ^{13}C – ^{13}C spin coupling, are proportional to the sum of the abundances (in mol%) of certain sets of isotopologues, and the abundances of individual isotopologues can be extracted by linear deconvolution [77].

Although not fully decisive and relatively difficult to execute, application of $^{13}\text{CO}_2$ is useful in the experiments with intact mature plants as the metabolic labelling with glucose might result in this case in unsatisfactory incorporation because of photosynthesis. In photoautotrophic experimental models high dilution of the label with native intermediates may hinder label incorporation and mask the actual biosynthetic mechanism.

Recently performed feeding experiments with $^{13}\text{CO}_2$ deserve special attention in the context of regulation of the flux through the MEP pathway. Ghirardo et al. [78] used this tool in an elegant experiment

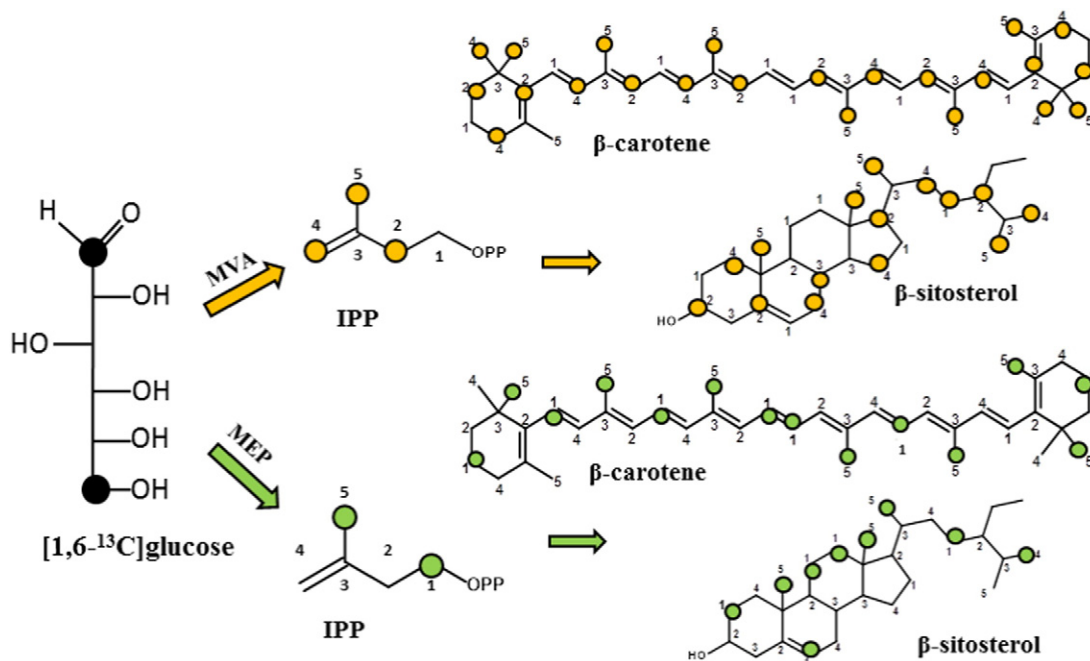


Fig. 4. Glucose in metabolic labelling of isoprenoids. Shown is the theoretically predicted labelling pattern of β-sitosterol and β-carotene expected for biosynthesis of these isoprenoids via either the MVA (yellow circles) or MEP (green circles) pathways using [1,6-¹³C]glucose as biosynthetic precursor. Black circles denote position of ¹³C atoms in the glucose molecule. Carbon numbers in β-sitosterol and β-carotene indicate their origin from IPP, common precursor for all isoprenoids. Note that C-5 carbon atoms of IPP and respective atoms in β-sitosterol and β-carotene molecules are labelled from both pathways. (IPP, isopentenyl diphosphate).

designed to demonstrate differences in the regulation of carbon flux within the MEP pathway between the isoprene emitting (IE) and genetically transformed, non-emitting (NE) poplar leaves towards volatile (isoprene, monoterpene) as well as nonvolatile isoprenoids. In IE lines major flux of photosynthetic carbon was allocated towards isoprene synthesis. Interestingly, NE lines did not present redirection of the acquired carbon towards other MEP pathway products (photosynthetic pigments) but, instead, a drastic reduction of the flux through the pathway. This tight control, adjusted to the demand for isoprenoid compounds, was to great extent achieved by the reported earlier allosteric inhibition of deoxy-D-xylulose 5-phosphate synthase (DXS) activity by DMAPP and IPP [79]. In line with this, the rate-limiting character of DXS was confirmed in another study with ¹³CO₂ labelling of *A. thaliana* lines with altered activity of this enzyme [45]. Surprisingly, labelling patterns of the MEP pathway intermediates indicated the presence of the additional pool of 2C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP) remaining unlabelled under applied experimental conditions, in lines with upregulated DXS. It was suggested that the excess of MEcPP might be translocated from plastid to the other cellular compartment. In the subsequent study a metabolic route leading to the formation of glucosylated ME tetraol 2-C-methyl-D-erythritol (ME-glcs) from the accumulated MEcPP was identified [80]. Interestingly, the small rate of formation of ME-glcs was also identified in plant lines without the MEP pathway intermediate imbalance. ME-glcs presumably serve as the inactivated form of the MEcPP, compound with the stress inducing potential [81]. However, short term ¹³CO₂ labelling orchestrated with MEP pathway inhibitors treatment of the MEP pathway proved that ME-glcs are synthesized from the preexisting rather than newly synthesized cellular pool of precursors. These very recent discoveries seem extremely interesting in the light of the current intensive efforts to increase economically desired isoprenoids but also point to the direction of the future studies.

4.1.5.1.3. Acetyl CoA and pyruvate/D-glyceraldehyde 3-phosphate. Acetyl CoA (Ac-CoA) and pyruvate/D-glyceraldehyde 3-phosphate (Pyr/GAP) are the direct substrates of the first dedicated enzymes of the

MVA and MEP pathway, respectively (Fig. 2). Experiments with labelled acetate and pyruvate were the cornerstone of the discovery of the plastidic pathway of plant isoprenoid biosynthesis [82]. Currently, they are rarely used in the investigation of isoprenoid biosynthesis. As besides isoprenoid biosynthesis these precursors are channeled to other pathways (e.g. biosynthesis of fatty acids and amino acids, tricarboxylic acid cycle) they are not specific precursors for the MVA/MEP pathways. Moreover, since neither Ac-CoA nor Pyr/GAP may serve as the sole carbon source for plant cultures they are also not used as general precursors. For these reasons application of glucose as the general precursor or pathway specific precursors described below seems a far more convenient and informative option in metabolic labelling experiments.

4.1.5.2. Metabolic labelling with pathway-specific precursors. Mevalonic acid (MVA) and 1-deoxy-D-xylulose (DX) are predominantly used as the specific precursors (Figs. 5 and 6) of the MVA and MEP pathway, respectively, in the metabolic labelling experiments (e.g., [20,30,62]). Both these compounds are produced by the key enzymes of the respective pathway (Fig. 2). HMGR synthesizes mevalonic acid through NADPH-dependent two-fold reduction of the HMG-CoA and DXS catalyses condensation of pyruvate and D-glyceraldehyde 3-phosphate to yield 1-deoxy-D-xylulose phosphate (DXP). While mevalonic acid is engaged exclusively in the isoprenoid biosynthesis, DXP was assumed to serve in a plant cell, similarly to *E. coli*, also as the precursor in the biosynthetic routes for vitamins B₁ (thiamine) and B₆ (pyridoxal) [83]. Competition for DXP might thus influence the rate of label incorporation into the isoprenoids. However, more recently a novel, DXP-independent route of vitamin B₆ biosynthesis was demonstrated for *Arabidopsis*, utilizing intermediates from pentose phosphate pathway rather than the MEP pathway [84,85]. As the MVA and MEP pathways are compartmentalised, labelling with pathway specific precursors should lead to similar isotopic enrichment in the molecules of isoprenoid compounds built up of isoprenoid units synthesized in the same compartment. Also the labelling pattern on the isoprenoids of “mixed origin” should be easy distinguishable.

In metabolic labelling experiments plants or plant cell cultures are fed with DX or MVL (mevalonolactone) as those compounds are well absorbed (however, in the latter case open acid form can be more advantageous in some experimental models i.e. BY-2 cells [30]). In order to enter the respective metabolic pathway they have to undergo some chemical modifications. DX is efficiently phosphorylated by cytoplasmic D-xylulose kinase (DXK) [86], and in the phosphorylated form is transported across the chloroplast membrane, most likely by xylulose 5-phosphate translocator [25]. Although in *A. thaliana* also a plastidic DXK has been identified, it clearly cannot compensate for the lack of cytosolic DXK either functionally complement *E. coli* DXK knockout mutants [86]. These observations suggest that cytosolic and plastidic DXK play distinct functions in a plant cell.

MVL requires conversion to the 'open' acid form (mevalonic acid) to be phosphorylated by mevalonate kinase (MVK), and hydrolysis of lactone apparently takes place within the cell. In the classical research on isolated rat hepatocytes Edwards et al. [87] found that although mevalonic acid is phosphorylated by MVK more rapidly than its lactone, after metabolic feeding to cells higher incorporation rates into sterols were obtained with mevalonolactone [87]. This was probably due to the more efficient uptake of the less polar lactone than acid by cells, as observed also for plant model – feeding of snapdragon flowers [60]. Presently, both MVA and MVL are successfully used in metabolic labelling studies in plants. Protocols for organic synthesis of labelled DX and MVL are available [88,89]. Moreover, highly enriched deuterated MVL (98% ^2H) may be synthesized by using an isotope exchange method [60].

Unlike some others intermediates of the isoprenoid biosynthetic pathways, neither DX nor MVA exert toxic or inhibitory effects per se unless used in high concentrations, multiple fold exceeding estimated cellular concentration – for DX pmol/mg of dry leaf tissue, e.g., DX concentration higher than 3mM affected photosynthesis in the leaves of *Eucalyptus globulus* [64]. These observations prove the ability of the isoprenoid biosynthetic network to rapidly adjust to the conditions interfering with carbon flux, although species-dependent variation cannot be excluded. Keeping these results in mind, it seems reasonable to measure the intermediate concentration in the course of metabolic labelling experiments in order to monitor the flow of the labelled precursors. For both, the MVA and MEP, pathways protocols for such a complex metabolite profiling have been developed [90,91].

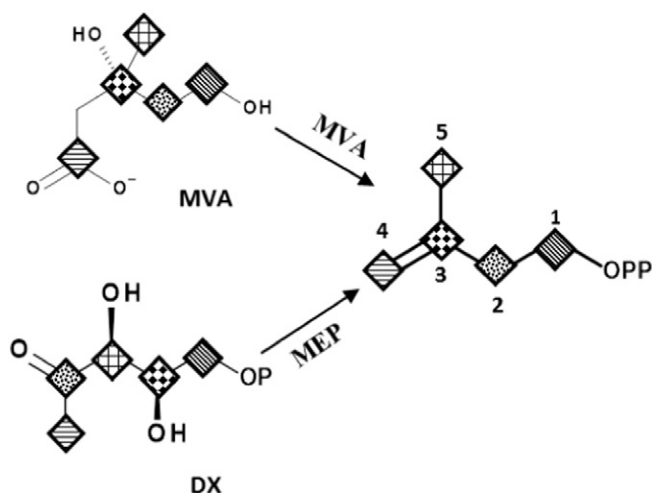


Fig. 5. Labelling pattern of isoprene residue resulting from application of the MVA or MEP pathway-specific precursors, mevalonate (MVA) and 1-deoxy-D-xylulose (DX) labelled at different positions. Fate of each carbon atom is depicted by the graphical symbols. Please note that C-1 of mevalonic acid is not incorporated into isoprenoids due to the decarboxylation of mevalonate-5-diphosphate in the course of the IPP formation.

As either DX or MVA are not accumulated by cells, their supplementation might also affect the level of various up- and downstream intermediates and in turn modulate the activity of particular enzymes and consequently perturb the activity of the corresponding pathway. It has been shown, for example, that HMGR activity was inhibited by HMG-CoA and free CoA in *Pisum sativum* and by NADPH and NADP⁺ in radish but not by mevalonate [92,93]. MVK, the next enzyme of the pathway, seems to be highly regulated by several downstream metabolites (mainly FPP, but also IPP, DMAPP, GPP or even phytyl diphosphate derived from the MEP pathway) [94,95]. Similarly, the plastidic pool of DMAPP negatively regulates activity DXS, efficiently maintaining constant carbon flux through the pathway [64,79]. It might be crucial in the light of the fact that MeCPP acts as a retrograde signalling molecule, influencing the expression of nuclear stress responsive genes and thereby seems to be a master regulator modulating whole plant metabolism [81].

It should be also noted that feeding by specific precursors might result in perturbations in enzymatic steps downstream the synthesis of IPP/DMAPP and lead to potential pitfalls in quantitative metabolic elucidations. For example in pulse chase experiments, metabolic labelling of various isoprenoids in cotton seedlings with labelled MVA resulted in accumulation of sterol biosynthesis intermediates (squalene, cycloartenol and 24-methylene cycloartenol), which caused decreased label flux towards the end products, and Δ^{24} -sterol methyltransferase turned out to be a rate limiting enzyme in the presence of exogenously supplied MVL [61].

Intermediates from the MVA and MEP pathways other than MVL/MVA and DX are not routinely used in metabolic labelling. Worth remembering was the observation that labelled ME – the dephosphorylated form of the MEP pathway intermediate – was not incorporated into isoprenoids in *Catharanthus roseus* [96] most probably due to the lack of the corresponding kinase in plant cells. In turn, exogenous MEcPP was incorporated into the lipid fraction of chromoplasts, leucoplasts, amyloplasts and chloroplasts of different plant species. Some variable labelling efficiency depending on plant species and type of plastid [65,66] was noted, very low in case of chloroplasts [66]. Taking into consideration that the uptake of compounds containing phosphate groups by plant cells and tissues is not efficient, DX and MVA/MVL are constantly the most reliable candidates for metabolic labelling experiments.

4.1.5.3. Isoprenoids as precursors. Labelling with isoprenoids does not permit to directly address the question of the MVA/MEP origin of the compound of interest although various aspects of metabolic pathways have been elucidated with this approach. IPP, GPP and, with lower efficiency, DMAPP and FPP were found to be transported across the plastidial envelope in experiments with intact chloroplasts and proteoliposomes containing reconstituted inner plastidial proteins [26,97]. Moreover, both MVA and MEP pathways give rise to separate pools of geranylgeranyl diphosphate (GGPP) and conceivably farnesyl diphosphate (FPP) [3,38] and in line with this, GGPP synthases accepting both GPP and FPP as the substrates might be localized in plastids, ER and mitochondria in *A. thaliana* [98]. In fact some GGPPs are recently called isoprenoid diphosphate synthases due to their promiscuous product specificity [99].

Labelled *trans*-farnesol (FOH) has been efficiently incorporated into the phytosterols, prenyl side chain of ubiquinone [100] and sesquiterpenoids upon treatment with the elicitor when incubated with *N. tabacum* cell cultures [23], however, in higher concentrations it caused inhibition of cell division [101]. Later, incubation of *N. tabacum* cells with fluorescent analog of farnesol proved the severe cytotoxic effect above a critical concentration (approx. 50 μM) leading to plasma membrane disruption and appearance of vesicle-like structures [102]. This effect seems to be specifically farnesol-induced as for other isoprenoid alcohols like geraniol, geranylgeraniol (GOH, GGOH), toxic concentration threshold was much higher or this effect was not observed at all.

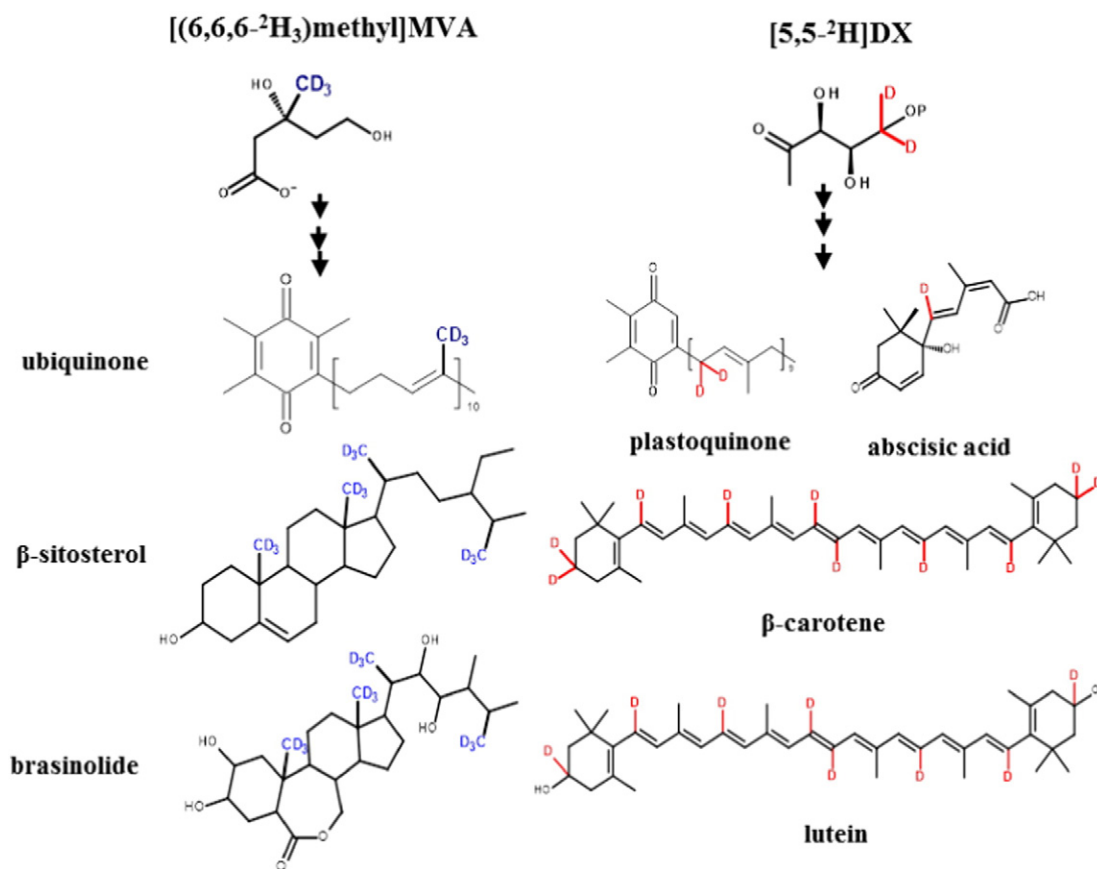


Fig. 6. Metabolic labelling of isoprenoids with deuterium labelled specific precursors of the MVA or MEP pathway, $[(^2\text{H}_3)\text{methyl}]\text{MVA}$ or $[5,5\text{-}^2\text{H}_2]\text{DX}$. Expected labelling pattern of selected isoprenoids – end products of both pathways are shown. Position and number of incorporated deuterium atoms is depicted. Please note that some deuterium atoms of $[5,5\text{-}^2\text{H}_2]\text{DXP}$ are eliminated upon formation of abscisic acid or carotenoids.

Cross-talk of the MEP and MVA pathway is also mirrored in post-translational modification of proteins – geranylgeranylation and farnesylation. Protein prenylation is crucial for functioning of numerous proteins and consequently for a number of metabolic processes such as phytohormone signalling, meristem development and stress responses [103–106] and references therein. This modification is predicted to affect hundreds of proteins in plant cell and is thought to increase protein affinity towards membranes. Prenylated proteins usually locate outside of plastids (plasma membrane, nuclear and mitochondrial fractions). The classical protocol for investigating farnesylation/geranylgeranylation comprises incubation with a radiolabelled isoprenoid anchor precursor: FOH, GGOH as well as MVL or DX, separation of proteins by one dimensional SDS or two dimensional high resolution 2D electrophoresis and subjecting gels to radiography/fluorography for days/weeks [30,107]. More recently, a faster and more sensitive method using scanner of radioactivity to trace the labelled prenyl anchor of the targeted protein has been described [108]. Incorporation of the radioactivity after incubation of *N. tabacum* cells with $[2\text{-}^{14}\text{C}]\text{DX}$ into the low molecular weight proteins strongly suggested the MEP but not MVA biosynthetic origin of prenyl anchor [109]. The same researchers designed an elegant reporter system for investigation of protein geranylgeranylation and identified the factors influencing this process [110]. In this model, BY-2 cells were transformed to express green fluorescent protein (GFP) fused with a short peptide bearing canonical sequence for geranylgeranylation. This model allowed to prove the existence of the cross-talk between the MEP and MVA pathways in this process since upon combination of inhibitors of both pathways prenylation of the fusion protein was completely abolished. Furthermore, very recent comprehensive study has shown that production of

MVA-derived sesquiterpenoid – capsidiol in *N. tabacum* requires MEP driven geranylgeranylation of a yet unidentified protein [111]. Interestingly, a monoterpene – *S*-carvone was also identified as the inhibitor of protein prenyltransferase (PPT).

4.1.5.4. Interpretation of the MS data. It should be emphasized that results of metabolic labelling, either with general or specific precursors, require careful considerations. Moreover, whenever possible quantitative analysis of NMR and especially MS data should be performed. An interesting method for quantification of MS data has been established for mono- (myrcene, ocimene) and sesquiterpenes (nerolidol) emitted by snapdragon flowers fed with deuterated DX or MVL. The percentage of labelling was determined as the intensity of the shifted representative fragment ion, divided by the sum of intensities for unshifted and shifted representative fragment ions [60]. In more recent publication, labelling incorporation from the $[^{13}\text{C}]\text{MVL}$ and $[^2\text{H}_2]\text{DX}$ specific precursors into a broad scope of isoprenoids was quantified likewise. Additionally, natural abundance of ^{13}C as well as variation in the number of C5 units and biosynthetic rates of selected isoprenoid compounds was taken into account [61]. This approach allowed the accurate evaluation of the relative contribution of both, the MVA and MEP pathway, into biosynthesis of investigated isoprenoids and showed high level of the cross-talk, even in the group of compounds classically ascribed exclusively to the one or another pathway (sterols, carotenoids). Therefore, critical evaluation of the raw incorporation data in the context of a knowledge of the MVA and MEP pathway dynamics, e.g. circadian clock driven flux through the MEP pathway [60] should be performed to achieve meaningful conclusions.

4.2. Non-labelling approach – $^{13}\text{C}/^{12}\text{C}$ and $^2\text{H}/^1\text{H}$ ratio as indicator of the biosynthetic pathway

Methods of investigation of the biosynthetic origin of isoprenoids described above require, to greater or lesser extent, artificial i.e. external ‘intervention’ into the native cellular metabolic process and certainly are not neutral for a highly responsive plant metabolism. Thus, the results and conclusions are vitiated by unavoidable errors and always raise doubts concerning their relevance. The ideal tool should be ‘metabolically transparent’, i.e. not interfering with plant metabolism and not involving any exogenous substance.

These objectives appear to be met by carbon isotopic composition ($^{13}\text{C}/^{12}\text{C}$) assessment in the molecules of the isoprenoids of interest. This method takes advantage of the variable abundances of naturally occurring $^{13}\text{C}/^{12}\text{C}$ carbon isotopes in the isoprenoid molecule under investigation, which is dependent on its MVA or MEP pathway biosynthetic origin. This is possible due to the kinetic isotope fractionation, which takes place when one of the isotopes present in the substrate is preferred to the other by the enzyme [112]. A pronounced discrimination of isotopes is exhibited by pyruvate dehydrogenase, which catalyzes decarboxylation of pyruvate to produce acetyl-CoA with preference toward “lighter” carbon (^{12}C) isotopes. Three molecules of acetyl-CoA yield ^{13}C depleted mevalonic acid, a specific precursor of the MVA pathway. Generation of the MEP pathway precursor, DXP, requires only one decarboxylation of pyruvate. Intact glyceraldehyde 3-phosphate (GAP) of natural ^{13}C abundance is incorporated in the final molecule of the ‘heavier’ precursor DXP. Consequently, these reactions result in a clear difference between carbon $^{12}\text{C}/^{13}\text{C}$ isotopic ratio of precursors of mevalonic acid vs. deoxyxylulose phosphate and in the end products produced via one or the other pathway [112].

The isotopic ratio in the molecules of the compounds of interest is analyzed using Isotope Ratio Mass Spectrometry (IRMS) comprising gas chromatography, with online combustion or pyrolysis of the eluted molecules to CO_2 , coupled to the mass spectrometer [112–114]. By this means (δ) ^{13}C values are calculated where (δ) ^{13}C [‰] refers to ^{13}C isotope ratio compared to a standard: (δ) ^{13}C [‰] = $(R_{\text{sam}} / R_{\text{std}} - 1) \times 1000$ where the R_{sam} and R_{std} are absolute ratios $^{13}\text{C}/^{12}\text{C}$ ratios of a sample and standard, respectively. Standard here corresponds to limestone (Pee Dee Belemnite) or another carbonate with a known, stable $^{13}\text{C}/^{12}\text{C}$ ratio. This tool was applied to prove the mixed origin of the homoterpene (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT), in lima bean upon treatment with elicitor. Indeed, (δ) ^{13}C values calculated for DMNT after incubation of lima beans with cerivastatin, a specific inhibitor of the MVA pathway, were similar to those displayed by the typical MEP derived isoprenoid-ocimene, whereas while after inhibition with fosmidomycin, the DMNT (δ) ^{13}C value was substantially lower, indicating its biosynthesis via the MVA pathway. These results were confirmed by treatment with elicitors channelling intermediates toward DMNT production either via the MVA and MEP pathway (herbivore feeding or fungal elicitor, respectively). Similarly to inhibitor treatment also in this case compensation from the alternative pathway was reflected by shifts in the isotopic ratio in DMNT.

(δ) ^2H values might also indicate the biosynthetic origin of isoprenoids, as products of the MEP pathway seem to be depleted in ^2H in comparison to those of the MVA pathway [115]. It is proposed that this depletion is a consequence of distinct cytoplasmic and plastidial pools of NADPH being separately engaged in isoprenoid biosynthesis in respective compartments [116]. This approach was applied to elucidate the biosynthetic origin of the sesquiterpene (–)- α bisabolol in different plant species. Analyses revealed that in contrast to *Vanillosmopsis*, α bisabolol isolated from chamomile shows a ^2H distribution pattern similar to the one expected for the MEP pathway suggesting its involvement in the biosynthesis of (–)- α bisabolol in some, but not all, plant species [117]. Both IRMS and ^2H NMR SNIF-NMR (site-specific natural isotope fractionation) methods permit to evaluate the contribution of the MEP and MVA pathways to the biosynthesis of isoprenoids of

mixed origin. While IRMS analysis gives an insight into global $^{12}\text{C}/^{13}\text{C}$ ratio in the molecule SNIF-NMR is a quantitative method which delivers information on local ^{13}C or ^2H enrichment hereby creating possibility of elucidation of exchanged intermediate(s) [118,119]. Moreover, ^{13}C SNIF-NMR methods of analysis of ^{13}C distribution are currently being optimized [120]. Although (δ) ^{13}C and also (δ) ^2H values are distinctive for particular isoprenoid compound and depend on plant class, photosynthesis mode (isoprenoids of C4 plants like maize are more strongly depleted of ^2H and ^{13}C than of C3 plants), growth conditions and environmental cues, substantial differences of these values described above led to the identification of the relative contribution of the MVA and MEP pathway to their formation [121,122]. Substantial differences of these values between the MVA and MEP pathway-derived isoprenoids allow to identify relative contribution of each pathway to their formation.

In conclusion, analysis of natural abundance of stable isotopes seems advantageous since it provides relevant information on the biosynthetic origin of natural products without the interventions into the metabolic flux. In contrast, isotopic enrichment followed after metabolic labelling might potentially disturb intracellular metabolic flux and intermediate allocation within the pathway during the experiments [123]. However, limitations of this method (low sensitivity, insufficiently resolved spectra) make the determination of the $^1\text{H}/^2\text{H}$ ratio in each position of the molecule very complicated if not virtually impossible. Additional restriction of IRMS comes from the fact that the gas chromatography method applied in this methodology is suitable exclusively for volatile compounds. Though it is unlikely that the measurement of natural distribution of stable isotopes will replace metabolic labelling experiments, it seems a highly valuable entry on the list of tools used in the investigation of isoprenoid biosynthesis.

4.3. MVA and MEP pathway inhibitors and investigation of the cross-talk

Application of specific, efficient inhibitors of crucial enzymes of the pathway affects accumulation of the end products of this route. In case of ‘mosaic’ isoprenoid compounds synthesized due to contribution of both pathways, inhibitors of both of them should interfere with its synthesis (e.g., [29,111]). Application of a labelled analogue of the product of a blocked enzyme in combination with this inhibitor may increase incorporation rate in the end products by minimizing dilution by the unlabelled native compound. For both the MVA and MEP pathways inhibitors are available, and their application is well documented in the literature. These inhibitors are of great importance not only for investigations on isoprenoid synthesis but also for the pharmacological industry.

Inhibitors of the MVA pathway, statins, competitively inhibiting HMGR – the rate limiting enzyme of this pathway – lower cholesterol levels, and as anti-cardiovascular disease agents are number one among the most prescribed drugs in the world [124]. Statins comprise metabolites of fungal origin (mevastatin, lovastatin, pravastatin and simvastatin) and statin-like synthetic compounds (atorvastatin, fluvastatin). All statins contain a HMG-like moiety, either in an inactive lactone form which in vivo is hydrolysed to the active hydroxyl acid (lovastatin, simvastatin), or in the already active ‘open’ form (atorvastatin, rosuvastatin). Despite structural differences, all statins act as competitive inhibitors of HMGR, however, they exhibit the tendency to tightly bind to the enzyme [93]. Access of the native substrate HMG-CoA to the enzyme is blocked while statins are tightly bound to the substrate binding pocket [125]. In plant studies lovastatin/mevinolin is the most often used inhibitor of HMGR [126] and atorvastatin is used far less frequently [127]. Inhibitors in lactone form are poorly soluble in aqueous solutions. To maximize their solubility in plant growth media they should be applied as an ethanolic solution [128]. Otherwise, conversion of the lactone form into the water soluble sodium salt is recommended [129]. Influence of both mevinolin and atorvastatin on isoprenoid metabolism was investigated in the aquatic plant *Lemna gibba* [130]. Besides

biochemical results, this article provides a number of valuable methodological and experimental suggestions such as statin solubility and exposure concentration.

MEP pathway inhibitors are tested as potential therapeutics against many human pathogens (*Plasmodium falciparum*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*) since the MEP pathway is the only source of indispensable isoprenoids in these pathogens, and on the other hand the MEP pathway is absent from mammalian cells [131,132]. Moreover, some herbicides are based on interference with the MEP pathway and at present it is the field of very extensive studies [133,134]. Clomazone (CLM, 2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidinone) and fosmidomycin (FSM) are applied as specific inhibitors of the MEP pathway. Clomazone has been also used in agriculture as a bleaching herbicide. Later on, it was discovered that it blocks production of plastidic isoprenoids by inhibiting the first enzyme of the MEP pathway, DXS [135]. The inhibitory effect is caused not by clomazone itself but by its metabolite, oxoclomazone. Some plant species (tobacco, soybean) are resistant or highly tolerant to CLM due to the lack of a pathway responsible for its bioconversion to the active compounds or due to detoxification [135], and the tolerance levels may differ between varieties of the same species [136]. Currently, many novel inhibitors of the MEP pathway, candidates for antimalarial drugs and new generation antibiotics are FSM analogues [137,138]. FSM, originally isolated from *Streptomyces lavendulae*, is a competitive inhibitor of the DXR [139]. Although not approved as a drug because of the unfavourable pharmacokinetics, it is commonly used in plant research. Similarly to CLM, it causes bleaching and decrease of the content of photosynthetic pigments. Interestingly, there are some discrete differences between the phenotype of CLM and FSM treated plants. Both cotyledon and true leaf bleaching was observed for plants growing in medium supplemented with FSM, but only true leaves were affected when plants were grown in the presence of CLM [140]. Treatment with both inhibitors results in strong bleaching of young expanding leaves, while only a slight effect is noted for mature leaves which may indicate that photosynthetic pigment turnover in mature leaves is slow [20,140]. MEP pathway inhibitors cause growth inhibition in tobacco BY-2 cells, callus as well as *S. miltiorhiza* hairy roots, but it was less severe than upon statin treatment. Most probably deprivation of sterols, main component of cellular membranes causes more severe perturbations in plant growth than lowering of the levels of MEP pathway end products [30,129,139]. Specific inhibitors shut down the corresponding pathway and inhibit synthesis of their end products in the micromolar (μM) concentration range (Table 2). Blocking of either of the pathways with higher concentrations of inhibitor became eventually lethal, despite the flow of the precursors from the alternative pathway ([20] and references therein). Obviously, one route is not able to meet the requirements of plant cells for isoprenoids. Additionally, the phytotoxic effect of the inhibitor not connected with decreased isoprenoid production cannot be excluded. On the other hand, increased resistance of plants to the mentioned inhibitors [147] is usually attributed to the supplementation with intermediates from the alternative pathway or bioconversion of the inhibitor, which results in modulation of its properties in plant cells. The optimal sublethal concentration of the inhibitor should be carefully established for the particular experimental model (e.g., for cell cultures lower than for the plants), developmental stages (for seedlings lower than for mature plants), growth conditions (light intensity, temperature), plant species and duration of the treatment (available data are summarized in Table 2).

Inevitably, applying exogenous substances to block the biosynthesis of such vital compounds as isoprenoids triggers modification of the activity of the targeted as well as other enzymes of the MVA and MEP pathways at various levels of regulation (transcriptional, post-transcriptional, translational, post-translational), and this seems to be species- and tissue-dependent. For example, in inhibitor-treated *A. thaliana* seedlings, changes of the isoprenoid levels were not reflected in the transcription profile of the genes encoding enzymes either of the

MEP or MVA pathway [128]. In contrast, fosmidomycin caused accumulation of DXS and DXR transcripts in *Salvia miltiorhiza* hairy roots and in tomato fruit [129,148]. Additionally, during incubation with inhibitors, lovastatin or fosmidomycin, the transcription pattern of hundreds of genes not directly involved in the MVA and MEP pathways was altered [128] indicating the profound influence of these chemicals on the plant metabolic network. Combination of the inhibitor treatment with metabolic labelling by specific precursors of the pathway led to increased incorporation of the labelled precursors in pathway end products (e.g. [44]). Besides the typical inhibitors of the MVA/MEP enzymes, inhibitors of other enzymes/processes appeared useful for investigation of the cross-talk between MVA and MEP pathway. DL-Glyceraldehyde — an inhibitor of carbon assimilation during photosynthesis and sodium pyrophosphate (NaPP) — were both used to interfere with IPP translocation across the plastid membrane [129,149]. Although these chemicals inhibited production of particular isoprenoids in a concentration-dependent manner and strengthened the inhibitory effect of fosmidomycin and mevinolin, the mechanism of their activity remains unclear.

Taken together, some precautions have to be kept in mind when interpreting results obtained from inhibitor treatment. In order to achieve reliable results, a broader experimental scheme employing other tools mentioned in this review (e.g. metabolic labelling) is strongly advised. Further analysis may reveal the actual character of the MVA and MEP pathway cooperation (exchanging of precursors or engagement of the regulatory mechanisms employing e.g. protein prenylation) [111]. For example, inhibitor treatment and, in parallel, other tools were used to describe the contribution of both the MVA and MEP pathways to biosynthesis of several isoprenoids, e.g. dolichols in *C. geoides* [29], tanshinones in *S. miltiorhiza* [129] ginsenosides in ginseng [142] and artemisinin in *A. annua* [143].

5. Genetic tools to elucidate the origin of plant isoprenoids and cross-talk between the MVA and MEP pathway

Studies on the biosynthesis of isoprenoids described above were based on chemical intervention in plant cell metabolism. Besides, targeted genetic modification of the particular gene(s) appeared useful to elucidate the origin of the isoprenoid of interest and contribution of the MVA and MEP pathways to its formation. Keeping in mind reservations described for application of precursors or inhibitors, this genetic block overcomes some serious drawbacks of the chemical treatments. This goal is achieved by generation of mutant lines with altered level of accumulation of the investigated isoprenoid. Numerous mutants have been described and are available, e.g. T-DNA insertion mutants for *Arabidopsis* [150].

5.1. Mutants in genes encoding enzymes of the MVA/MEP pathway

Using null mutant plants deprived of functional enzymes or mutants with substantially decreased activity of the enzymes of either the MVA or MEP pathway seems a powerful tool to investigate cross-talk of these pathways. Selected examples are listed in Table 3 to illustrate the applicability of this approach.

In the well characterized plant model *A. thaliana* six enzymatic steps constituting the MVA pathway are encoded by nine genes including three isozyme pairs: AACT (AcAC-CoA thiolase), HMGR (3-hydroxy-3-methylglutaryl-CoA reductase) and diphospho-mevalonate decarboxylase [177]. Surprisingly, despite the long-lasting studies on the MVA pathway, limited data on functional analysis of enzymes encoded by the MVA pathway genes are available, and this topic is currently still under extensive investigation in various plant species. Nevertheless, analysis of accessible knockout mutants has shown that isoforms of enzymes within the MVA pathway are not fully redundant [154]. In the majority of plant species the MEP pathway enzymes are encoded by single copy genes with the well-known exception of DXS, as several DXS-

Table 2
Effect of pathway-specific inhibitors on plant metabolism. Selected plant models together with the applied concentration of inhibitor are presented. MEV – mevinolin, FSM – fosmidomycin, CLM – clomazone, DLG – D,L-glyceraldehyde.

Plant experimental model/stage of development	Inhibitor concentration time of treatment	Compound of interest	Observations	Reference
<i>Taxus baccata</i> suspension culture elicited with methyl jasmonate	MEV (1 μ M) 22 days FSM (200 μ M) 22 days	Diterpenoid paclitaxel and precursor of paclitaxel – baccatin III	Slight inhibition of paclitaxel accumulation, no influence on baccatin III accumulation Inhibition of paclitaxel and baccatin III accumulation	[141]
<i>Salvia miltiorrhiza</i> hairy roots 18 days old	MEV (10 μ M) 6 days FSM (150 μ M) 6 days DLG (1 mM) 6 days	Diterpenoid tanshinones (cryptotanshinones, tanshinone I and II, dihydrotanshinone I)	Root growth inhibition (to 66% of control), reduction of tanshinones accumulation Root growth inhibition (to 75% of control); reduction of tanshinones accumulation No influence on root growth or tanshinones biosynthesis but strengthen inhibitory effect of MEV and FSM	[128]
<i>Panax ginseng</i> hairy root culture	MEV (50 μ M) 7 days FSM (50 μ M) 7 days	Ginsenosides	Root growth inhibition, reduction of ginsenoside accumulation (42% of control) Root growth inhibition, reduction of ginsenoside accumulation (58% of control)	[142]
<i>Artemisia annua</i> seedlings 7 or 14 days post cotyledon	MEV (100 μ M) 7 or 14 days FSM (100 μ M) 7 or 14 days MEV + FSM 14 days	Artemisinin	Inhibition of shoots and roots elongation, biomass not affected, inhibition of artemisin accumulation 7 days: chlorosis (younger plants more affected); 14 days: chlorosis, root inhibition, inhibition of artemisinin accumulation Artemisin production abolished in 7 days old plants	[142]
<i>A. thaliana</i> hydroponically grown plants 6 weeks old	MEV (10 μ M) 8 days FSM (50 μ M) 6 days		Growth inhibition (lack of inflorescence shoots formation), numerous phenotypic abnormalities	[20]
<i>A. thaliana</i> seedlings	MEV (100 μ M) Up to 96 h FSM (400 μ M) Up to 96 h	Main MEP pathway end products (carotenoids and chlorophylls) and MVA pathway end products (sterols)	Downregulation of photosynthetic pigments (bleaching of leaves, albino phenotype of de novo formed inflorescence shoots) Changes in lipid content are presented as percent of the control; 3 h: sterols – 87%, carotenoids – 121%, chlorophylls – 132%; 48 h: carotenoids – 113%, chlorophylls – 122%, sterols 106%; 96 h: carotenoids – 116%, chlorophyll – 137%, sterols – 106%; no changes in expression of main MVA and MEP pathway genes	[127]
<i>Cannabis sativa</i> 2 month-old plant	MEV (0.1, 1, 10 μ M)	Chlorophyll, carotenoids, tocopherol, tetrahydrocannabinol (THC), cannabidiol (CBD)	Accumulation of carotenoids and chlorophyll (highest with 0.1 μ M MEV); accumulation of α -tocopherol (highest with 10 μ M MEV); decrease of the THC and increase of the CBD levels	[144]
<i>Rubia cordifolia</i> cell culture	MEV(5 μ M) Up to 27 days CLM (10 μ M) Up to 27 days	Anthraquinone purpurin and naphthoquinones mollugin	Accumulation of purpurin and mollugin Decrease of purpurin and mollugin yield	[145]
<i>Satureja khuzistanica</i> Jamzad plantlets – micro shoots	MEV (10, 25, 50, 75, 100 μ M) FSM (10, 25, 50, 75, 100 μ M)	Monoterpene carvacol	Reduction in the fresh weight, no influence on carotenoids and chlorophyll level, increased density of non-glandular trichomes, increased accumulation of carvacol (at concentrations of 75 and 100 μ M, increased DXR activity and expression of DXR gene at concentrations of 75 and 100 μ M) Reduction in the fresh weight, changed morphology of newly grown leaves (albino, slimmer, longer), decrease of carotenoids and the chlorophyll level, reduced density of glandular and non-glandular trichomes, decreased accumulation of carvacol, concentration dependent decrease of DXR activity, increase of DXR gene expression (at concentrations of 50,75 and 100 μ M)	[146]

like genes have been identified, among others, in *A. thaliana*, *Zea mays*, *Medicago truncatula*, *Oryza sativa*, *Picea abies* ([178] and references therein). However, similarly to the MVA pathway, these isoforms display specific, species-dependent expression patterns and mutant lines analysis has indicated the lack of their functional redundancy [179].

5.1.1. Loss-of-function mutants

Numerous available *A. thaliana* T-DNA mutant lines as well as mutants of other species, obtained mostly via gene silencing techniques, together with their phenotypes are listed in a landmark review by Hemmerlin (Table 1, page 107 in [20]), while more recent studies are summarized in Table 3. As the isoprenoid biosynthesis routes are essential in the early stages of development, knockout lines of genes of both pathways are severely affected showing embryo lethality (*aact2*), or sporophytic/gamethophytic male sterility (MVA pathway mutants) and albino lethality (chloroplast biogenesis disruption in MEP pathway mutants) [154,180]. Some of these lines have been successfully used to demonstrate bidirectional exchange of intermediates between pathways after feeding with specific precursors [59]. However, embryo-lethality of knock-out lines precludes the possibility of their direct application in investigations on cross-talk. Thus, gene silencing techniques such as RNA interference (RNAi) and Virus Induced Gene Silencing (VIGS) have been applied to reduce or eliminate the expression of those essential genes in later stages of plant development; such mutants have been obtained for different plant species. Lately, RNAi has been used to silence HMGR and DXR encoding genes in *Withania somnifera* in order to investigate the contribution of the MVA and MEP pathway to withanolides [134]. The effect of the *aact2* mutation in *A. thaliana* was also elucidated by using this technique [154].

Plants with reduced flux through the MVA or MEP pathway, caused by partial disruption of the activity of enzymes from early steps of isoprenoid biosynthesis, seem to be a valuable model for metabolic labelling; although a specific precursor entering the corresponding pathway downstream of the mutation should be used. Then, a rate of label incorporation is expected to be high and, as the applied precursor improves or even restores the flux through the pathway, the MVA/MEP cross-talk may be investigated. Exogenous supplementation with specific precursor, DX, partially restores WT phenotype in temperature-sensitive *dxs* mutant [181], whereas supplementation with MVL attenuates phenotypic and biochemical effects of AACT2 downregulation [154].

5.1.2. Mutants overexpressing the genes of the MVA or MEP pathway

It might be expected that overexpression of one or several enzymes within one of the pathways should result in the increased total yield of the end product of this pathway and might be also useful to investigate the contribution of this pathway to biosynthesis of particular compounds. This method was successful in several cases [159,167,182]. However, because of the dominant role of post-transcriptional mechanisms in the regulation of enzymes of the MVA and MEP pathway, accumulation of corresponding transcripts may not always result in a higher level of upregulated enzyme activity. Furthermore, even in the case of positively correlated overexpression of a gene and its respective enzyme activity, downstream enzymes do not necessarily display enhanced activity since it might cause accumulation of intermediates or trigger feedback mechanisms. This seems to hold true especially for the MEP pathway. In transgenic *A. thaliana* overexpressing *DXS* increased *DXS* activity apparently correlated with activation of the next enzyme of the pathway, *DXR*, as the levels of the product of this enzyme, MeCPP increased in the same manner. However, it did not result in higher levels of plastidic isoprenoids. Interestingly, it is probably not caused by the feedback regulation, but unexpectedly by the efflux of part of MeCPP from plastids to the cytoplasm [45]. Therefore, although such approach might reveal new interesting results, it might appear disappointing in the context of the initial goal. For more examples see Table 3.

5.2. Mutant lines with affected flux through either the MVA or MEP pathway

Because of the essential role of isoprenoids in plant overall fitness, their biosynthetic network is under tight regulation by external and internal cues. Thus many external factors as well as disturbances in other pathways may contribute to modifications of the metabolic flux through the MVA or/and MEP pathways [31,44]. During the past decades a huge number of mutated genes were found to influence accumulation of isoprenoids, thereby unravelling the complexity of the regulatory mechanisms but also potentially providing some hints about the biosynthetic origin of particular isoprenoids. Applicable mutant lines might be identified after chemical mutagenesis, for instance by the increased resistance to the MVA or MEP pathway inhibitors or alternatively, they might be depicted due to the higher levels of accumulation of respective isoprenoids.

In regard to the MVA pathway, an example of such a mutant is *rcn1-1*, with reduced activity of Protein Phosphatase 2A (PP2A) acting as a posttranslational negative regulator of HMGR. Mutant *rcn1* plants exhibited higher than WT resistance to mevinolin resulting from increased activity of the HMGR [169]. Although the levels of the MVA pathway end products were not investigated in the study, accumulation of sterols following upregulation of HMGR might be predicted as it is well documented in the literature [167,183]. Increased resistance to MVA pathway blockage resulting from upregulation of HMGR activity during seedling development has also been noted for mutants in the genes encoding main photoreceptors (phytochromes and cryptochromes) with impaired light signalling pathways [174]. Interestingly, while in phytochrome mutants (*phyA*, *phyB*) resistance to mevinolin is accompanied by the increased resistance to the MEP pathway inhibitor, fosmidomycin, this has not been the case for cryptochrome mutants. Even more strikingly, increased fosmidomycin resistance in *phyB* mutants is not correlated either with the enhanced activity of key MEP pathway enzymes (*DXS*, *DXR*) or accumulation of photosynthetic pigments and thus suggests intensification of the intermediate flow from the MVA pathway. It should be kept in mind however, that these experiments were performed in the early stage of seedling development. Different regulation of the isoprenoid biosynthetic pathways in mature plants cannot be ruled out, especially considering cross-talk rate which strongly depends on the developmental stage, e.g. of plastids.

Higher tolerance to fosmidomycin and clomazone, MEP pathway inhibitors, along with enhanced accumulation of chlorophylls and carotenoids has been observed in *prl1* (*rif18*) [69]. In parallel *prl1* plants display downregulated activity of HMGR yet without affecting sterol accumulation. PRL1 itself has been identified earlier as a regulator of sugar and hormone responses in *Arabidopsis* [184]. Further analysis of the *prl1* mutant has led to the conclusion that increased sugar (sucrose) availability is the factor responsible for the observed phenotype as it results in an increased pool of the MEP pathway intermediates [69]. This explains herbicide resistance (increased availability of substrates of the inhibited enzymes) and accumulation of the photosynthetic pigments without alteration of the activity of key MEP pathway enzymes. Additionally, lack of differences in labelling rates after metabolic labelling with specific precursors has shown that the cross-talk between the MVA and MEP pathways is not altered [69]. Nevertheless, the mechanism underlying modulation of sucrose availability by PRL1 protein is not clear yet. Selected examples of mutants affecting the formation of isoprenoids in plants are listed in Table 3.

Identification of mutations resulting in an enhanced resistance to specific MVA or MEP pathway inhibitors points towards the existence of extra- and intracellular stimuli involved in the regulation of the isoprenoid network (e.g. light, exogenous sugar). Thus, the mutant lines described above as well as identified factors modifying final output of the MVA and MEP pathway and levels of corresponding end products might be attractive, yet unexploited, tools to investigate contribution of both pathways to the biosynthesis of particular

Table 3

Application of transgenic plants in isoprenoid research. Three types of mutants are presented — loss-of-function mutants, mutants overexpressing genes of the isoprenoid-generating pathway and mutants with impaired regulation of MVA and/or MEP pathway resulting in altered metabolic flux. A comprehensive list of knock-out and knock-down mutants in the MEP and MVA pathway encoding genes has been presented by Hemmerlin et al., 2012.

Loss of function mutants					
Gene (mutant allele)	Function	Species	Mutagen	Phenotype	References
MEP-pathway encoding genes					
<i>HDS</i> (<i>ceh1</i>)	4-Hydroxy-3-methylbut-2-enyl diphosphate synthase	<i>A. thaliana</i>	EMS	Stunted growth, altered responses to biotic and abiotic stress (elevated levels of stress responsive genes, accumulation of free salicylic acid, increased resistance to <i>P. syringae</i>) caused by accumulation of MEP pathway intermediate MEcPP; increased transcript and protein levels for a subset of the core unfolded protein response (UPR) genes	[80,81,151]
<i>DXS2</i> (<i>sldxs2</i>)	1-Deoxy-D-xylulose 5-phosphate synthase	<i>S. lycopersicum</i>	RNAi	No growth phenotype, mutant viable, increased trichome density contributing to elevated resistance to caterpillar feeding (<i>S. littoralis</i>); lower accumulation of trichome monoterpenes and higher sesquiterpens levels, elevated incorporation of MVA-derived IPP into monoterpene and sesquiterpenes in transgenic plants	[123]
<i>DXL1</i> homologous to <i>DXS2</i>	1-Deoxy-D-xylulose 5-phosphate synthase	<i>A. thaliana</i>	T-DNA	No phenotype, not essential for growth and development	[152]
<i>HDR/IDS</i> (<i>zb7</i>)	4-Hydroxy-3-methylbut-2-enyl diphosphate reductase/isopentenyl diphosphate synthase	<i>Z. mays</i>	RNAi	Transverse light green/yellow striped leaves in young plants, albino sections enlarged under high temperatures and alternate light/dark cycles, phenotype suppressed by low temperatures, stripes gradually disappears after 10 leaf stage	[153]
MVA-pathway encoding genes					
<i>AACT2</i> (<i>aact2</i>)	Acetoacetyl-CoA thiolase	<i>A. thaliana</i>	T-DNA RNAi	Embryo lethality, impaired male gametogenesis (reduced pollen germination and pollen tube germination) Growth reduction, extended life span and flowering duration, sterility, decrease of phytosterol level, altered composition of phytosterols in the roots (increase of campesterol to sitosterol plus stigmasterol ratio), differential effects on expression of MVA pathway genes downstream of <i>AACT2</i> (i.e. <i>FPS1</i> , <i>FPS2</i> and <i>SMT2</i>) expression is reduced, <i>HMGR1</i> expression unaltered	[154]
<i>HMGR1</i> (<i>hmgr1</i>)	3-Hydroxy-3-methylglutaryl-CoA reductase	<i>M. truncatula</i>	RNAi	No effect on root growth, reduced ability of mutant to produce root nodule when inoculated with rhizobium	[155]
<i>HMGR1</i> (<i>mad3</i>)	3-Hydroxy-3-methylglutaryl-CoA reductase	<i>A. thaliana</i>	EMS	Stunted growth and narrow, undeveloped true leaves, sterol reduction by 40% in leaves and 60% in flowers, impaired miRNA activity	[156]
<i>IPK</i> (<i>ipk</i> , <i>ipk2</i>)	Isopentenyl phosphate kinase	<i>A. thaliana</i>	T-DNA	Significant decrease in campesterol and sitosterol, but not stigmasterol content, reduced emission of β -caryophyllene (sesquiterpene)	[157]
<i>MDD</i> (<i>mdd1</i> , <i>mdd2</i>)	Mevalonate diphosphate decarboxylase	<i>A. thaliana</i>	T-DNA	Decreased campesterol and sitosterol content, relative decreases in β -caryophyllene (sesquiterpene) emission	[157]
Mutants overexpressing genes of the isoprenoid-generating pathway					
Overexpressed gene	Transformed organism	Gene(s) Source(s)	Phenotype		Reference
MEP pathway encoding genes					
<i>DXS</i>	<i>A. thaliana</i>	<i>A. thaliana</i>	Increased CO ₂ assimilation rates, slightly higher accumulation of isoprenoid end products, no significant changes in abundance of transcripts of other MEP pathway genes, accumulation of MEcPP, identification of second non-chloroplastidic pool of MEcPP		[45]
<i>DXS</i>	<i>Lavandula latifolia</i>	<i>A. thaliana</i>	No growth phenotype, increased essential oils accumulation (mainly monoterpenes), chlorophyll and carotenoid content not affected		[158]
<i>DXS</i>	<i>S. sclarea</i> hairy roots	<i>A. thaliana</i>	Inhibition of root growth in case of excessive <i>DXS</i> transcript level (4-fold increase), accumulation of diterpenoids (preferentially aethiopinone, an abietane-quinone-type diterpene)		[159]
<i>DXR</i>	<i>A. thaliana</i>	<i>A. thaliana</i>	No phenotype in majority of plants, resistance to FSM, but not to CLM, no influence on <i>DXS</i> transcript level and activity, enhanced accumulation of chlorophyll and carotenoids (by 25%)		[160]
<i>DXR</i>	<i>L. latifolia</i>	<i>A. thaliana</i>	No obvious phenotype, no significant increase in essential oils accumulation, chlorophyll content was age dependent with slightly higher content of chlorophyll and carotenoids in young leaves and similar levels to control in mature leaves		[161]
<i>DXR</i>	<i>Mentha X piperita</i> L	<i>Mentha X piperita</i> L	No obvious phenotype, 50% increase of essential oils yield, no difference in essential oils composition		[162]
<i>DXR</i>	<i>S. sclarea</i> hairy roots	<i>A. thaliana</i>	No growth phenotype, accumulation of abietane type diterpenoids, levels higher than in case of <i>AtDXS</i> transgenic hairy roots		[159]
<i>HDR</i>	<i>A. thaliana</i>	<i>L. esculentum</i>	Delayed germination of seeds; 50% higher levels of the major chloroplast carotenoids (β -carotene, lutein, and violaxanthin) in seedlings,		[163]
<i>HDR; TXS</i> (taxadiene synthase)	<i>A. thaliana</i>	<i>L. esculentum</i> <i>T. baccata</i>	50% higher levels of carotenoids and 13-fold higher accumulation of taxadiene comparing with seedlings expressing only <i>TXS</i> transgene		[163]
MVA pathway encoding genes					
<i>HMGS</i> WT and mutant (H188N, S359A and H188N/S359A)	<i>A. thaliana</i>	<i>Brassica juncea</i>	Earlier germination of seeds, induced expression of <i>HMGR</i> and range of genes encoding enzymes involved in MVA-dependent steroid biosynthesis; significant increase of sterol accumulation (most prominent in OE S359A), unaltered levels of		[164]

(continued on next page)

Table 3 (continued)

Mutants overexpressing genes of the isoprenoid-generating pathway					
Overexpressed gene	Transformed organism	Gene(s) Source(s)	Phenotype		Reference
<i>HMGR</i>	<i>A. thaliana</i>	<i>A. thaliana</i>	chlorophyll and carotenoids; induced PR gene expression and enhanced resistance to <i>B. cinerea</i>		
<i>HMGR</i> (N-terminal truncated)	<i>N. tabacum</i>	<i>C. griseus</i> (Chinese hamster)	No growth phenotype, no significant increase of MVA and MEP derived isoprenoids (sterols and carotenoids respectively)		[165]
<i>HMGR</i>	<i>S. lycopersicum</i>	<i>A. thaliana</i>	3 to 8-fold increased sterol content in transgenic lines, tissue and age dependant sterol composition accumulation of cycloartenol (intermediate of sterol biosynthesis), unaltered levels of MEP derived isoprenoids and sesquiterpenes		[166]
<i>HMGR</i> (truncated)	<i>Physcomitrella patens</i> (moss, transgenic, expressing patchoulol synthase; <i>PTS</i>)	<i>P. patens</i> <i>S. cerevisiae</i>	No growth phenotype, elevated phytosterols in leaves and ripe fruit (β -sitosterol, stigmasterol and campesterol), unaltered level of plastid derived isoprenoids		[167]
			Expression of both, moss and yeast <i>HMGR</i> resulted in comparable increase of patchoulol (sesquiterpene) and stigmasterol (2.2- and 1.5-fold, respectively)		[168]
Mutants with affected metabolic flux					
<i>RCN1</i> (<i>PP2A</i>)/protein phosphatase 2A	Posttranslational negative regulator of <i>HMGR</i>	<i>A. thaliana</i>	T-DNA	Increased levels of <i>HMGR</i> transcript, protein and activity; increased resistance to mevinolin	[72]
<i>LOI1</i> (<i>loi1</i>)/PPR1: penta-tricopeptide repeat protein	RNA editing of three mitochondrial genes of respiratory chain (NAD4, CCB203 and COX3)	<i>A. thaliana</i>	T-DNA	Longer than WT roots under LOV treatment, resistance to LOV (increased activity of <i>HMGR</i> , transcript accumulation not affected) and CLM, involvement of respiratory chain components in regulation of MVA and MEP pathways	[170,171]
<i>PRL1</i> (<i>rif18</i>)/pleiotropic regulatory locus 1	Global regulator of sugar, stress, and hormone responses by inhibition of SnRK1, sugar-mediated control of isoprenoid homeostasis	<i>A. thaliana</i>	T-DNA	Dark-green colour of plants, inhibition of root growth, flat leaves with serrated margins, early flowering, elevated levels of carotenoids and chlorophylls, resistance to FSM and CLM unaltered transcription and activity of MEP pathway genes, decreased activity of <i>HMGR</i> , unaffected level of sterols, the same phenotype obtained when plants were grown on medium supplemented with sucrose	[69]
<i>NOA1</i> (<i>rif1</i>)/nitric oxide-associated 1	Correct ribosome assembly in plastids	<i>A. thaliana</i>	T-DNA	Reduced growth, pale cotyledons, clearly delayed development and greening of true leaves, pale young but green mature leaves, resistance to FSM, increased levels of <i>DXS</i> (1.5-fold) and <i>DXR</i> transcripts (almost 2-fold), decreased production of plastome-encoded proteins, inhibited degradation of MEP pathway enzymes	[172]
<i>PNPT1</i> (<i>rif10</i>)/polyribonucleotide phosphorylase	Chloroplast targeted exoribonuclease, RNA processing	<i>A. thaliana</i>	T-DNA	Pale and small young plants, increased resistance to FSM, accumulation of <i>DXS</i> , <i>DXR</i> , <i>HDS</i> and <i>HDR</i> transcripts, decreased levels of plastome-encoded proteins, lower levels of chlorophylls and carotenoids	[173]
<i>PHY B</i> (<i>rim-1</i>)/phytochrome B	Photoreceptor-mediated responses to blue light	<i>A. thaliana</i>	T-DNA	Pale green leaves early flowering, significantly longer hypocotyl under long-day conditions but not in the dark, elongated leaf petioles and inflorescences, resistance to MEV (upregulation of <i>HMGR</i> gene expression and enzyme activity) and to FSM (unaltered activity of MEP pathway enzymes, resistance to FSM might be derived from increased cross-talk), impaired light perception	[174]
SrUGT85C2	UDP glycosyltransferase, regulation of carbon flux between steviol glycoside and gibberellin biosynthesis	<i>Stevia rebaudiana</i>	RNAi (AMTS)	Decreased steviol glycoside and significantly increased gibberellin GA3 content	[175]
SrUGT85C2	UDP glycosyltransferase	<i>A. thaliana</i>	Over-expression	Decreased gibberellin GA3 content (approx. 20% of control), stunted hypocotyl length, reduced shoot growth and relative water content, reduced chlorophyll a and b content, decreased biomass, downregulated expression of geranylgeranyl diphosphate synthase (GGDPS), copalyl diphosphate synthase (CDPS), kaurenic acid oxidase (KAO), chlorophyll synthetase and chlorophyll a oxygenase	[176]

isoprenoids. Additionally, these data shed light on how various metabolic changes resulting from mutations in the genes not belonging to the isoprenoid generating systems might add new clues that must be taken into consideration when the MEP/MVA cross-talk is discussed.

6. Concluding remarks

Plants are unique in terms of harboring two isoprenoid generating pathways operating and co-operating in the cell. These pathways produce myriads of structurally and functionally diverse compounds. Numerous of such are targets for bioengineering due to their

applications, e.g., in pharmaceutical industry (the sesquiterpenoid artemisinin or the diterpenoid tanshinones) or agronomic (phytosterols, accumulation of which contributes to increased plant growth) [185–187]. Thus, the presence of two tightly regulated and interconnected pathways poses genuine challenges in terms of overproduction of desired isoprenoids in microorganisms or plants. To achieve this, elucidation of their biosynthetic routes, especially contribution of the MVA and MEP pathways is mandatory. Undoubtedly, this demanding task requires reliable methods to elucidate the enzymatics and the dynamics of the isoprenoid producing network. To this end, application of various parallel experimental approaches permits to draw reliable conclusions. Moreover, careful selection of the plant model and experimental

conditions together with their appropriate adjustment (e.g., concentration of inhibitor, time of labelling) are crucial. Experiments should be also designed with awareness that the rate of the cross-talk between the MVA and MEP pathways is dependent on the plant developmental stage or the cell/tissue suspension culture age and growth conditions, as it might fluctuate in response to stress or the diurnal cycle. Results obtained for one plant species require verification for other ones to create a general biosynthetic model useful to design an efficient platform for obtaining the desired products.

The aim of this review was to present a comprehensive description of both classical, routinely used methods as well as novel approaches applied to investigate the biosynthetic origin of isoprenoids. Advantages and limitations of each method are highlighted and suitable references are provided.

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References

- Christianson DW. Unearthing the roots of the terpenome. *Curr Opin Chem Biol* 2008;12:141–50.
- Zhuang X, Chappell J. Building terpene production platforms in yeast. *Biotechnol Bioeng* 2015;112:1854–64.
- Kumari S, Priya P, Misra G, Yadav G, Structural and biochemical perspectives in plant isoprenoid biosynthesis. *Phytochem Rev* 2013;12:255–91.
- Rodríguez-Concepción M, Boronat A. Breaking new ground in the regulation of the early steps of plant isoprenoid biosynthesis. *Curr Opin Plant Biol* 2015;25:17–22.
- Tholl D. Biosynthesis and biological functions of terpenoids in plants. *Adv Biochem Eng Biotechnol* 2015;148:63–106.
- Pandian S, Saengchjan S, Raman TS. An alternative pathway for the biosynthesis of isoprenoid compounds in bacteria. *Biochem J* 1981;196:675–81.
- Goodwin TW. Incorporation of $^{14}\text{CO}_2$ [2- ^{14}C] acetate and [2- ^{14}C] mevalonic acid into β -carotene in etiolated maize seedlings. *Biochem J* 1958;68:26–7.
- Goodwin TW. Studies in carotenogenesis. 25. The incorporation of $^{14}\text{CO}_2$ [2- ^{14}C] acetate and [2- ^{14}C] mevalonate into β -carotene by illuminated etiolated maize seedlings. *Biochem J* 1958;70:612–7.
- Zabin I. The formation of radioactive lycopene in ripening tomatoes. *J Biol Chem* 1957;226:851–9.
- Treharne KJ, Mercer EI, Goodwin TW. Incorporation of ^{14}C carbon dioxide and 2- ^{14}C mevalonic acid into terpenoids of higher plants during chloroplast development. *Biochem J* 1966;99:239–45.
- Bach TJ, Lichtenthaler HK. Inhibition by mevinolin of plant growth, sterol formation and pigment accumulation. *Physiol Plant* 1983;59:50–60.
- Lütke-Brinkhaus F, Kleinig H. Formation of isopentenyl diphosphate via mevalonate does not occur within etioplasts and etiochloroplasts of mustard (*Sinapis alba* L.) seedlings. *Planta* 1987;171:406–11.
- Flesch G, Rohmer M. Prokaryotic hopanoids – the biosynthesis of the bacteriohopane skeleton – formation of isoprenic units from 2 distinct acetate pools and a novel type of carbon carbon linkage between a triterpene and D-ribose. *Eur J Biochem* 1988;175:405–11.
- Rohmer M, Knani M, Simonin P, Sutter B, Sahn H. Isoprenoid biosynthesis in bacteria – a novel pathway for the early steps leading to isopentenyl diphosphate. *Biochem J* 1993;295:517–24.
- Hano Y, Ayukawa A, Nomura T, Ueda S. Origin of the acetate units composing the hemiterpene moieties of chalconaricin in *Morus alba* cell-cultures. *J Am Chem Soc* 1994;116:4189–93.
- Eisenreich W, Schwarz M, Cartayrade A, Arigoni D, Zenk MH, Bacher A. The deoxyxylulose phosphate pathway of terpenoid biosynthesis in plants and microorganisms. *Chem Biol* 1998;5:R221–33.
- Eisenreich W, Bacher A, Arigoni D, Rohdich F. Biosynthesis of isoprenoids via the non-mevalonate pathway. *Cell Mol Life Sci* 2004;61:1401–26.
- Hemmerlin A, Harwood JL, Bach TJ. A raison d'être for two distinct pathways in the early steps of plant isoprenoid biosynthesis? *Prog Lipid Res* 2012;51:95–148.
- Sapir-Mir M, Mett A, Belasov E, Tal-Meshulam S, Frydman A, Gidoni D, et al. Peroxisomal localization of *Arabidopsis* isopentenyl diphosphate isomerases suggests that part of the plant isoprenoid mevalonic acid pathway is compartmentalized to peroxisomes. *Plant Physiol* 2008;148:1219–28.
- Disch A, Rohmer M. On the absence of the glyceraldehyde 3-phosphate pyruvate pathway for isoprenoid biosynthesis in fungi and yeasts. *FEMS Microbiol Lett* 1998;168:201–8.
- Hartmann MA, Bach TJ. Incorporation of all-*trans*-farnesol into sterols and ubiquinone in *Nicotiana tabacum* L. cv Bright Yellow-2 cell cultures. *Tetrahedron Lett* 2001;42:655–7.
- Schuh CA, Radekewicz T, Sagner S, Latzel C, Zenk MH, Arigoni D, et al. Quantitative assessment of crosstalk between the two isoprenoid biosynthesis pathways in plants by NMR spectroscopy. *Phytochem Rev* 2003;2:3–16 (2003).
- Flügge UI, Gao W. Transport of isoprenoid intermediates across chloroplast envelope membranes. *Plant Biol (Stuttg)* 2005;7:91–7.
- Bick JA, Lange BM. Metabolic cross talk between cytosolic and plastidial pathways of isoprenoid biosynthesis: unidirectional transport of intermediates across the chloroplast envelope membrane. *Arch Biochem Biophys* 2003;415:146–54.
- Aharoni A, Ashok GP, Deuerlein S, Griepink F, de Kogel WJ, Verstappen FW, et al. Terpenoid metabolism in wild-type and transgenic *Arabidopsis* plants. *Plant Cell* 2003;15:2866–84.
- Gutenson M, Orlova I, Nguyen TT, Davidovich-Rikanati R, Ferruzzi MG, Sitrit Y, et al. Cytosolic monoterpene biosynthesis is supported by plastid-generated geranyl diphosphate substrate in transgenic tomato fruits. *Plant J* 2013;75:351–63.
- Skorupinska-Tudek K, Poznanski J, Wojcik J, Bienkowski T, Szostkiewicz I, Zelman-Femiak M, et al. Contribution of the mevalonate and methylerythritol phosphate pathways to the biosynthesis of dolichols in plants. *J Biol Chem* 2008;283:21024–35.
- Hemmerlin A, Hoeffler JF, Meyer O, Tritsch D, Kagan IA, Grosdemange-Billiard C, et al. Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in tobacco bright yellow-2 cells. *J Biol Chem* 2003;278:26666–76.
- Rodríguez-Concepción M. Early steps in isoprenoid biosynthesis: multilevel regulation of the supply of common precursors in plant cells. *Phytochem Rev* 2006;5:1–15.
- Hemmerlin A. Post-translational events and modifications regulating plant enzymes involved in isoprenoid precursor biosynthesis. *Plant Sci* 2013;204:41–54.
- Pick TR, APM Weber. Unknown components of the plastidial permeome. *Front Plant Sci* 2014;5:410.
- Pérez-Gil J, Rodríguez-Concepción M. Metabolic plasticity for isoprenoid biosynthesis in bacteria. *Biochem J* 2013;452:19–25.
- Maury J, Asadollahi MA, Möller K, Clark A, Nielsen J. Microbial isoprenoid production: an example of green chemistry through metabolic engineering. *Adv Biochem Eng Biotechnol* 2005;100:19–51.
- Kitooka N, Lu X, Yang B, Peters RJ. The application of synthetic biology to elucidation of plant mono-, sesqui-, and diterpenoid metabolism. *Mol Plant* 2015;8:6–16.
- Suzuki S, Koeduka T, Sugiyama A, Yazaki K, Umezawa T. Microbial production of plant specialized metabolites. *Plant Biotechnol J* 2014;31:465–82.
- Vranová E, Coman D, Griessem W. Structure and dynamics of the isoprenoid pathway network. *Mol Plant* 2012;5:318–33.
- Allen DK, Bates PD, Tjellström H. Tracking the metabolic pulse of plant lipid production with isotopic labeling and flux analyses: past, present and future. *Prog Lipid Res* 2015;58:97–120.
- Bacher A, Rieder C, Eichinger D, Arigoni D, Fuchs G, Eisenreich W. Elucidation of novel biosynthetic pathways and metabolite flux patterns by retrobiosynthetic NMR analysis. *FEMS Microbiol Rev* 1998;22:567–98.
- Libourel IGL, Gehan JP, Shachar-Hill Y. Design of substrate label for steady state flux measurements in plant systems using the metabolic network of *Brassica napus* embryos. *Phytochemistry* 2007;68:2211–21.
- Kruger NJ, Masakapalli SK, Ratcliffe RG. Strategies for investigating the plant metabolic network with steady-state metabolic flux analysis: lessons from an *Arabidopsis* cell culture and other systems. *J Exp Bot* 2012;63:2309–23.
- Rohmer M. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Nat Prod Rep* 1999;16:565–74.
- Mongélard G, Seemann M, Boisson A-M, Rohmer M, Bligny R, Rivasseau C. Measurement of carbon flux through the MEP pathway for isoprenoid synthesis by ^{31}P -NMR spectroscopy after specific inhibition of 2-C-methyl-D-erythritol 2,4-cyclodiphosphate reductase. Effect of light and temperature. *Plant Cell Environ* 2011;34:1241–7.
- Wright LP, Rohrer JM, Ghirardo A, Hammerbacher A, Ortiz-Alcaide MR, et al. Deoxyxylulose 5-phosphate synthase controls flux through the methylerythritol 4-phosphate pathway in *Arabidopsis*. *Plant Physiol* 2014;165:1488–504.
- Lichtenthaler HK, Schwender J, Disch A, Rohmer M. Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. *FEBS Lett* 1996;400:271–4.
- De-Eknamkul W, Potduang B. Biosynthesis of β -sitosterol and stigmasterol in *Croton sublyrata* proceeds via a mixed origin of isoprene units. *Phytochemistry* 2003;62:389–98.
- Leite AC, Lopes AA, Kato MJ, Bolzani VD, Furlan M. Biosynthetic origin of the isoprene units in chromenes of *Piper aduncum* (Piperaceae). *J Braz Chem Soc* 2007;18:1500–3.
- Bandurski RS, Teas HJ. Rubber biosynthesis in latex of *Hevea brasiliensis*. *Plant Physiol* 1957;32:643–8.

- [50] Karl T, Fall R, Rosenstiel TN, Prazeller P, Larsen B, Seufert G, Lindinger W. On-line analysis of the $^{13}\text{C}_2$ labeling of leaf isoprene suggests multiple subcellular origins of isoprene precursors. *Planta* 2002;215:894–905.
- [51] Schramek N, Wang H, Römisch-Margl W, Keil B, Radykewicz T, Winzenhöerlein B, et al. Artemisinin biosynthesis in growing plants of *Artemisia annua*. A $^{13}\text{C}_2$ study. *Phytochemistry* 2010;71:179–87.
- [52] Knöss W, Reuter B, Zapp J. Biosynthesis of the labdane diterpene marrubium in *Marrubium vulgare* via a non-mevalonate pathway. *Biochem J* 1997;326:449–54.
- [53] Bamba T, Murayoshi M, Gyoksen K, Nakazawa Y, Okumoto H, Katto H, et al. Contribution of mevalonate and methylerythritol phosphate pathways to polyisoprenoid biosynthesis in the rubber-producing plant *Eucommia ulmoides* Oliver. *Z Naturforsch C* 2010;65:363–72.
- [54] Suga T, Hirata T, Shishibori T, Tange K. The first proof of the biosynthesis of isoprenoid from amino acid in higher plant the incorporation of L-leucine into linalool. *Chem Lett* 1974;3:189–92.
- [55] Suga T, Hirata T, Tange K. Biosynthesis of isoprenoid from amino acid in higher plant incorporation of L-valine into linalool. *Chem Lett* 1975;4:131–2.
- [56] Goodwin TW, Lijinsky W. Studies in carotenogenesis. 2. Carotene production by *Phycomyces blakesleeana*: the effect of different amino-acids when used in media containing low concentrations of glucose. *Biochem J* 1951;50:268–73.
- [57] Nabeta K, Kawae T, Saitoh T, Kikuchi T. Synthesis of chlorophyll *a* and β -carotene from ^2H - and ^{13}C -labeled mevalonates and ^{13}C -labelled glycine in cultured cells of liverworts, *Heteroscyphus planus* and *Lophocolea heterophylla*. *J Chem Soc, Perkin Trans 1* 1995;3:3111–5.
- [58] Itoh D, Kawano K, Nabeta K. Biosynthesis of chloroplastidic and extrachloroplastidic terpenoids in liverwort cultured cells: ^{13}C serine as a probe of terpene biosynthesis via mevalonate and non-mevalonate pathways. *J Nat Prod* 2003;66:332–6.
- [59] Kasahara H, Takei K, Ueda N, Hishiyama S, Yamaya T, Kamiya Y, et al. Distinct isoprenoid origins of *cis*- and *trans*-zeatin biosyntheses in *Arabidopsis*. *J Biol Chem* 2004;279:14049–54.
- [60] Dudareva N, Andersson S, Orlova I, Gatto N, Reichelt M, Rhodes D, et al. The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. *Proc Natl Acad Sci U S A* 2005;102:933–8.
- [61] Opitz S, Nes WD, Gershenzon J. Both methylerythritol phosphate and mevalonate pathways contribute to biosynthesis of each of the major isoprenoid classes in young cotton seedlings. *Phytochemistry* 2014;98:110–9.
- [62] Wöelwer-Rieck U, May B, Lankes C, Wüst M. Methylerythritol and mevalonate pathway contributions to biosynthesis of mono-, sesqui-, and diterpenes in glandular trichomes and leaves of *Stevia rebaudiana* Bertoni. *J Agric Food Chem* 2014;62:2428–35.
- [63] Arigoni D, Sagner S, Latzel C, Eisenreich W, Bacher A, Zenk MH. Terpenoid biosynthesis from 1-deoxy-D-xylulose in higher plants by intramolecular skeletal rearrangement. *Proc Natl Acad Sci U S A* 1997;94:10600–5.
- [64] Wolfertz M, Sharkey TD, Boland W, Kühnemann F. Rapid regulation of the methylerythritol 4-phosphate pathway during isoprene synthesis. *Plant Physiol* 2004;135:1939–45.
- [65] Fellermeier M, Raschke M, Sagner S, Wungsintaweekul J, Schuhr CA, Hecht S, et al. Studies on the nonmevalonate pathway of terpene biosynthesis – role of 2 C-methyl-D-erythritol 2,4-cyclodiphosphate in plants. *Eur J Biochem* 2001;268: 6302–10.
- [66] Mazikin KV, Ershov YV, Goncharenko AV, Ostrovskii DN. 2- ^{14}C -Methylerythritol 2,4-cyclodiphosphate incorporation into bacterial and plant isoprenoids. *Appl Biochem Microbiol* 2009;45:503–5 (Originally published in Prikl Biokhim Mikrobiol. 2009;45:561–4).
- [67] Rohmer M, Seemann M, Horbach S, Bringer-Meyer S, Sahm H. Glyceraldehyde 3-phosphate and pyruvate as precursors of isoprenic units in an alternative non-mevalonate pathway for terpenoid biosynthesis. *J Am Chem Soc* 1996;118:2564–6.
- [68] Mortain-Bertrand A, Stammitt L, Telef N, Colardelle P, Brouquisse R, Rolin D, et al. Effects of exogenous glucose on carotenoid accumulation in tomato leaves. *Physiol Plant* 2008;134:246–56.
- [69] Florés-Pérez U, Pérez-Gila J, Closa M, Wright LP, Botella-Pavía P, Phillips MA, Ferrer A, Gershenzon J, Rodríguez-Concepción M. Pleiotropic Regulatory Locus 1 (PRL1) integrates the regulation of sugar responses with isoprenoid metabolism in *Arabidopsis*. *Mol Plant* 2010;3:101–12.
- [70] Villadsen D, Smith SM. Identification of more than 200 glucose-responsive *Arabidopsis* genes none of which responds to 3-O-methylglucose or 6-deoxyglucose. *Plant Mol Biol* 2004;55:467–77.
- [71] Sugden C, Donaghy PG, Halford NG, Hardie DG. Two SNF1-related protein kinases from spinach leaf phosphorylate and inactivate 3-hydroxy-3-methylglutaryl-coenzyme A reductase, nitrate reductase, and sucrose phosphate synthase *in vitro*. *Plant Physiol* 1999;120:257–74.
- [72] Antolin-Llovera M, Leivar P, Arró M, Ferrer A, Boronat A, Campos N. Modulation of plant HMG-CoA reductase by protein phosphatase 2A: positive and negative control at a key node of metabolism. *Plant Signal Behav* 2011;6:1127–31.
- [73] Disch A, Hemmerlin A, Bach TJ, Rohmer M. Mevalonate-derived isopentenyl diphosphate is the biosynthetic precursor of ubiquinone prenyl side chain in tobacco BY-2 cells. *Biochem J* 1998;331 (615–2).
- [74] Noeh K, Wiechert W. The benefits of being transient: isotope-based metabolic flux analysis at the short time scale. *Appl Microbiol Biotechnol* 2011;91:1247–65.
- [75] Jazmin LJ, O'Grady JP, Ma F, Allen DK, Morgan JA, Young JD. Isotopically nonstationary MFA (INST-MFA) of autotrophic metabolism. *Methods Mol Biol* 2014;1090: 181–210.
- [76] Kölling K, Müllner A, Flutsch P, Zeeman SC. A device for single leaf labelling with CO_2 isotopes to study carbon allocation and partitioning in *Arabidopsis thaliana*. *Plant Methods* 2013;9:45.
- [77] Römisch-Margl W, Schramek N, Radykewicz T, Ettenhuber C, Eylert E, Huber C, et al. $^{13}\text{C}_2$ as a universal metabolic tracer in isotopologue perturbation experiments. *Phytochemistry* 2007;68:2273–89.
- [78] Ghirardo A, Wright LP, Bi Z, Rosenkranz M, Pulido P, Rodríguez-Concepción M, et al. Metabolic flux analysis of plastidic isoprenoid biosynthesis in poplar leaves emitting and nonemitting isoprene. *Plant Physiol* 2014;165:37–51.
- [79] Banerjee A, Wu Y, Banerjee R, Li Y, Yan H, Sharkey TD. Feedback inhibition of deoxy-D-xylulose-5-phosphate synthase regulates the methylerythritol 4-phosphate pathway. *J Biol Chem* 2013;288:16926–36.
- [80] González-Cabanas D, Wright LP, Paetz C, Onkokesung N, Gershenzon J, Rodríguez-Concepción M, et al. The diversion of 2-C-methyl-D-erythritol-2,4-cyclodiphosphate from the 2-C-methyl-D-erythritol 4-phosphate pathway to hemiterpene glycosides mediates stress responses in *Arabidopsis thaliana*. *Plant J* 2015;82:122–37.
- [81] Xiao Y, Savchenko T, Baidoo EEK, Chehab WE, Hayden DM, Tolstikov V, et al. Retrograde signaling by the plastidial metabolite MEcPP regulates expression of nuclear stress-response genes. *Cell* 2012;149:1525–35.
- [82] Braithwaite GD, TW Goodwin. Studies in carotenogenesis. 27. Incorporation of 2- ^{14}C acetate, DL-2- ^{14}C mevalonate and $^{14}\text{C}_2$ into carrot-root preparations. *Biochem J* 1960;76:194–7.
- [83] Lois LM, Campos N, Putra SR, Danielsen K, Rohmer M, Boronat A. Cloning and characterization of a gene from *Escherichia coli* encoding a transketolase-like enzyme that catalyzes the synthesis of D-1-deoxyxylulose 5-phosphate, a common precursor for isoprenoid, thiamin, and pyridoxol biosynthesis. *Proc Natl Acad Sci U S A* 1998;95:2105–10.
- [84] Tambasco-Studart M, Titiz O, Raschle T, Forster G, Amrhein N, Fitzpatrick TB. Vitamin B6 biosynthesis in higher plants. *Proc Natl Acad Sci U S A* 2005;102: 13687–92.
- [85] Fitzpatrick TB, Amrhein N, Kappes B, Macheroux P, Tews I, Raschle T. Two independent routes of de novo vitamin B6 biosynthesis: not that different after all. *Biochem J* 2007;407:1–13.
- [86] Hemmerlin A, Tritsch D, Hartmann M, Pacaud K, Hoeffler J-F, van Dorsselaer A, et al. A cytosolic arabidopsis D-xylulose kinase catalyzes the phosphorylation of 1-deoxy-D-xylulose into a precursor of the plastidial isoprenoid pathway. *Plant Physiol* 2006;142:441–57.
- [87] Edwards PA, Edmond J, Fogelman AM, Popják G. Preferential uptake and utilization of mevalonolactone over mevalonate for sterol biosynthesis in isolated rat hepatocytes. *Biochim Biophys Acta* 1977;488:493–501.
- [88] Meyer O, Hoeffler JF, Grosdemange-Billiard C, Rohmer M. Practical synthesis of 1-deoxy-D-xylulose and 1-deoxy-D-xylulose 5-phosphate allowing deuterium labeling. *Tetrahedron* 2004;60:12153–62.
- [89] Dickschat JS, Citron CA, Brock NL, Riclea R, Kuhz H. Synthesis of deuterated mevalonolactone isotopomers. *Eur J Org Chem* 2011;3339–46.
- [90] Henneman L, van Cruchten AG, Kulik W, Waterham HR. Inhibition of the isoprenoid biosynthesis pathway; detection of intermediates by UPLC-MS/MS. *Biochim Biophys Acta* 1811;2011:227–33.
- [91] Baidoo EEK, Xiao Y, Dehesh K, Keasling JD. Metabolite profiling of plastidial deoxyxylulose-5-phosphate pathway intermediates by liquid chromatography and mass spectrometry. *Methods Mol Biol* 2014;1153:57–76.
- [92] Brooker JD, Russell DW. Properties of microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase from *Pisum sativum* seedlings. *Arch Biochem Biophys* 1975; 167:723–9.
- [93] Bach TJ, Rogers DH, Rudney H. Detergent-solubilization, purification, and characterization of membrane-bound 3-hydroxy-3-methylglutaryl-coenzyme-A reductase from radish seedlings. *Eur J Biochem* 1986;154:103–11.
- [94] Dorsey JK, Porter JW. Inhibition of mevalonic kinase by geranyl and farnesyl pyrophosphates. *J Biol Chem* 1968;243:4667–70.
- [95] Gray JC, Kekwick RG. Inhibition of plant mevalonate kinase preparations by prenyl pyrophosphates. *Biochim Biophys Acta* 1972;279:290–6.
- [96] Sagner S, Eisenreich W, Fellermeier M, Latzel C, Bacher A, Zenk MH. Biosynthesis of 2-C-methyl-D-erythritol in plants by rearrangement of the terpenoid precursor, 1-deoxy-D-xylulose 5-phosphate. *Tetrahedron Lett* 1998;39:2091–4.
- [97] Bartram S, Jux A, Gleixner G, Boland W. Dynamic pathway allocation in early terpenoid biosynthesis of stress-induced lima bean leaves. *Phytochemistry* 2006;67: 1661–72.
- [98] Okada K, Saito T, Nakagawa T, Kawamukai M, Kamiya Y. Five geranylgeranyl diphosphate synthases expressed in different organs are localized into three subcellular compartments in *Arabidopsis*. *Plant Physiol* 2000;122:1045–56.
- [99] Nagel R, Bernholz C, Vranová E, Košuth J, Bergau N, Ludwig S, et al. *Arabidopsis thaliana* isoprenyl diphosphate synthases produce the C25 intermediate, geranylgeranyl diphosphate. *Plant J* 2015;84:847–59.
- [100] Thai L, Rush JS, Maul JE, Devarenne T, Rodgers DL, Chappell J, et al. Farnesol is utilized for isoprenoid biosynthesis in plant cells via farnesyl pyrophosphate formed by successive monophosphorylation reactions. *Proc Natl Acad Sci U S A* 1999;96: 13080–5.
- [101] Hemmerlin A, Bach TJ. Farnesol-induced cell death and stimulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity in tobacco cv Bright Yellow-2 cells. *Plant Physiol* 2000;123:1257–68.
- [102] Hemmerlin A, Reents R, Mutterer J, Feldtrauer JF, Waldmann H, Bach TJ. Monitoring farnesol-induced toxicity in tobacco BY-2 cells with a fluorescent analog. *Arch Biochem Biophys* 2006;448:93–103.
- [103] Swiezewska E, Thelin A, Dallner G, Andersson B, Ernster L. Occurrence of prenylated proteins in plant cells. *Biochem Biophys Res Commun* 1993;192:161–6.
- [104] Yalovsky S, Kulukian A, Rodríguez-Concepción M, Young CA, Gruissem W. Functional requirement of plant farnesyltransferase during development in *Arabidopsis*. *Plant Cell* 2000;12:1267–78.

- [105] Crowell DN. Functional implications of protein isoprenylation in plants. *Prog Lipid Res* 2000;39:393–408.
- [106] Hemsley PA. The importance of lipid modified proteins in plants. *New Phytol* 2015; 205:476–89.
- [107] Hála M, Soukupova H, Synek L, Žarský V. *Arabidopsis* RAB geranylgeranyl transferase β -subunit mutant is constitutively photomorphogenic, and has shoot growth and gravitropic defects. *Plant J* 2010;62:615–27.
- [108] Benetka W, Koranda M, Maurer-Stroh S, Pittner F, Eisenhaber F. Farnesylation or geranylgeranylation? Efficient assays for testing protein prenylation *in vitro* and *in vivo*. *BMC Biochem* 2006;7:6.
- [109] Gerber E, Hemmerlin A, Hartmann M, Heintz D, Hartmann M-A, Mutterer J, et al. The plastidial 2-C-methyl-D-erythritol 4-phosphate pathway provides the isoprenyl moiety for protein geranylgeranylation in tobacco BY-2 cells. *Plant Cell* 2009;21:285–300.
- [110] Hartmann M, Hemmerlin A, Gas-Pascual E, Gerber E, Tritsch D, Rohmer M, et al. The effect of MEP pathway and other inhibitors on the intracellular localization of a plasma membrane-targeted, isoprenylable GFP reporter protein in tobacco BY-2 cells. *F1000Res*, 2. ; 2013. p. 170.
- [111] Huchelmann A, Gastaldo C, Veinante M, Zeng Y, Heintz D, Tritsch D, Schaller H, Rohmer M, Bach TJ, Hemmerlin A. S-carvone suppresses cellulase-induced capsidiol production in *Nicotiana tabacum* by interfering with protein isoprenylation. *Plant Physiol* 2014;164:935–50.
- [112] Cui Y, Ma J, Sun W. Application of stable isotope techniques to the study of soil salinization. *J Arid Land* 2011;3:285–91.
- [113] Jux A, Gleixner G, Boland W. Classification of terpenoids according to the methylerythritolphosphate or the mevalonate pathway with natural C-12/C-13 isotope ratios: dynamic allocation of resources in induced plants. *Angew Chem Int Ed Eng* 2001;40:2091–4.
- [114] Chikaraishi Y, Naraoka H, Poulson SR. Carbon and hydrogen isotopic fractionation during lipid biosynthesis in a higher plant (*Cryptomeria japonica*). *Phytochemistry* 2004;65:323–30.
- [115] Schmidt HL, Werner RA, Eisenreich W, Fuganti C, Fronza G, Remaud G, Robins RJ. The prediction of isotopic patterns in phenylpropanoids from their precursors and the mechanism of the NIH-shift: basis of the isotopic characteristics of natural aromatic compounds. *Phytochemistry* 2006;67:1094–103.
- [116] Sessions AL, Burgoyne TW, Schimmelmann A, Hayes JM. Fractionation of hydrogen isotopes in lipid biosynthesis. *Org Geochem* 1999;30:1193–200.
- [117] Carle R, Beyer J, Cheminat A, Krempp E. Studies on the origin of (–)- α -bisabolol in chamomile preparations. 2. H-2 NMR determination of site-specific natural isotope fractionation in (–)- α -bisabolols. *Phytochemistry* 1992;31:171–4.
- [118] Schneider B. Nuclear magnetic resonance spectroscopy in biosynthetic studies. *Prog Nucl Magn Reson Spectrosc* 2007;51:155–98.
- [119] Martin GJ, Lavoine-Hanneguelle S, Mabon F, Martin ML. The fellowship of natural abundance H-2 isotopomers of monoterpenes. *Phytochemistry* 2004;65:2815–31.
- [120] Clendinen CS, Lee-McMullen B, Williams CM, Stupp GS, Vandenborne K, Hahn DA, et al. ^{13}C -NMR metabolomics: applications at natural abundance. *Anal Chem* 2014; 86:9242–50.
- [121] Chikaraishi Y, Naraoka H, Poulson SR. Hydrogen and carbon isotopic fractionations of lipid biosynthesis among terrestrial (C3, C4 and CAM) and aquatic plants. *Phytochemistry* 2004;65:1369–81.
- [122] Zhou Y, Stuart-Williams H, Grice K, Kayler ZE, Zavadlav S, Vogts A, et al. Allocate carbon for a reason: priorities are reflected in the $^{13}\text{C}/^{12}\text{C}$ ratios of plant lipids synthesized via three independent biosynthetic pathways. *Phytochemistry* 2015;111: 14–20.
- [123] Paetzold H, Garms S, Bartram S, Wiczorek J, Urós-Gracia E-M, Rodríguez-Concepción M, et al. The isogene 1-deoxy-D-xylulose 5-phosphate synthase 2 controls isoprenoid profiles, precursor pathway allocation, and density of tomato trichomes. *Mol Plant* 2010;3:904–16.
- [124] Alberts AW, Chen J, Kuron G, Hunt V, Huff J, Hoffman C, et al. Mevinolin – a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme-A reductase and a cholesterol-lowering agent. *Proc Natl Acad Sci U S A* 1980;77:3957–61.
- [125] Istvan ES. Structural mechanism for statin inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Am Heart J* 2002;144:27–32.
- [126] Bach TJ, Lichtenthaler HK. Mevinolin – a highly specific inhibitor of microsomal 3-hydroxy-3-methylglutaryl-coenzyme-A reductase of radish plants. *Z Naturforsch C* 1982;37:46–50.
- [127] Doblas VG, Amorim-Silva V, Posé D, Rosado A, Esteban A, Arró M, et al. The SUD1 gene encodes a putative E3 ubiquitin ligase and is a positive regulator of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in *Arabidopsis*. *Plant Cell* 2013;25:728–43.
- [128] Laule O, Fürholz A, Chang HS, Zhu T, Wang X, Heifetz PB, et al. Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* 2003;100:6866–71.
- [129] Yang D, Du X, Liang X, Han R, Liang Z, Liu Y, Liu F, Zhao J. Different roles of the mevalonate and methylerythritol phosphate pathways in cell growth and tanshinone production of *Salvia miltiorrhiza* hairy roots. *PLoS One* 2012;7:e46797.
- [130] Brain RA, Reitsma TS, Lissimore LJ, Bestari K, Sibley PK, Solomon KR. Herbicidal effects of statin pharmaceuticals in *Lemna gibba*. *Environ Sci Technol* 2006;40: 5116–23.
- [131] Masini T, Hirsch AKH. Development of inhibitors of the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway enzymes as potential anti-infective agents. *J Med Chem* 2014;57:9740–63.
- [132] Humnabadkar V, Jha RK, Ghatnekar N, De Sousa SM. A high-throughput screening assay for simultaneous selection of inhibitors of *Mycobacterium tuberculosis* 1-deoxy-D-xylulose-5-phosphate synthase (DXS) or 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR). *J Biomol Screen* 2011;16:303–12.
- [133] Corniani N, Velini ED, Silva FML, Nanayakkara NPD, Witschel M, Dayan FE. Novel bioassay for the discovery of inhibitors of the 2-C-methyl-D-erythritol 4-phosphate (MEP) and terpenoid pathways leading to carotenoid biosynthesis. *PLoS One* 2014;9:e103704.
- [134] Singh S, Pal S, Shanker K, Chanotiya CS, Gupta MM, Dwivedi UN, Shasany AK. Sterol partitioning by HMGR and DXR for routing intermediates toward withanolide biosynthesis. *Physiol Plant* 2014;152:617–33.
- [135] Ferhatoglu Y, Barrett M. Studies of clomazone mode of action. *Pestic Biochem Physiol* 2006;85:7–14.
- [136] Darwish M, Vidal V, Lopez-Lauri F, Alnaser O, Junglee S, El Maataoui M, et al. Tolerance to clomazone herbicide is linked to the state of LHC, PQ-pool and ROS detoxification in tobacco (*Nicotiana tabacum* L.). *J Plant Physiol* 2015;175:122–30.
- [137] Kuzuyama T, Shimizu T, Takahashi S, Seto H. Fosmidomycin, a specific inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase in the nonmevalonate pathway for terpenoid biosynthesis. *Tetrahedron Lett* 1998;39:7913–6.
- [138] Faísca Phillips AM, Nogueira F, Murtinheira F, Barros MT. Synthesis and antimalarial evaluation of prodrugs of novel fosmidomycin analogues. *Bioorg Med Chem Lett* 2015;25:2112–6.
- [139] Han M, Heppel SC, Su T, Bogs J, Zu Y, An Z, Rausch T. Enzyme inhibitor studies reveal complex control of methyl-D-erythritol 4-phosphate (MEP) pathway enzyme expression in *Catharanthus roseus*. *PLoS One* 2013;8:e62467.
- [140] Camper ND, McDonald SK, PM Burrows. Light and clomazone effects on tobacco (*Nicotiana tabacum*) callus and leaf discs. *J Environ Sci Health B* 2003;38:747–55.
- [141] Palazón J, Cusidó RM, Bonfill M, Morales C, Piñol MT. Inhibition of paclitaxel and baccatin III accumulation by mevinolin and fosmidomycin in suspension cultures of *Taxus baccata*. *J Biotechnol* 2003;101:157–63.
- [142] Zhao S, Wang L, Liu L, Liang Y, Sun Y, Wu J. Both the mevalonate and the non-mevalonate pathways are involved in ginsenoside biosynthesis. *Plant Cell Rep* 2014;33:393–400.
- [143] Towler MJ, Weathers PJ. Evidence of artemisinin production from IPP stemming from both the mevalonate and the nonmevalonate pathways. *Plant Cell Rep* 2007;26:2129–36.
- [144] Mansouri H, Salari F. Influence of mevinolin on chloroplast terpenoids in *Cannabis sativa*. *Physiol Mol Biol Plants* 2014;20:273–7.
- [145] Fan X, Hu G-S, Li N, Han Z-F, Jia J-M. Effects of lovastatin, clomazone and methyl jasmonate treatment on the accumulation of purpurin and mullugin in cell suspension cultures of *Rubia cordifolia*. *Chin J Nat Med* 2013;11:396–400.
- [146] Ramak P, Osaloo SK, Ebrahimzadeh H, Sharifi M, Behmanesh M. Inhibition of the mevalonate pathway enhances carvacrol biosynthesis and DXR gene expression in shoot cultures of *Satureja khuzistanica* Jamzad. *J Plant Physiol* 2013;170:1187–93.
- [147] Perello C, Rodríguez-Concepción M, Pulido P. Quantification of plant resistance to isoprenoid biosynthesis inhibitors. *Methods Mol Biol* 2014;1153:273–83.
- [148] Rodríguez-Concepción M, Ahumada I, Díez-Juez E, Saurer-Güeto S, Lois LM, Gallego F, et al. 1-Deoxy-D-xylulose 5-phosphate reductoisomerase and plastid isoprenoid biosynthesis during tomato fruit ripening. *Plant J* 2001;27:213–22.
- [149] Wang YD, Yuan YJ, Lu M, Wu JC, Jiang JL. Inhibitor studies of isopentenyl pyrophosphate biosynthesis in suspension cultures of the yew *Taxus chinensis* var. mairei. *Biotechnol Appl Biochem* 2003;37:39–43.
- [150] Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen HM, Shinn P, et al. Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* 2003;301:653–7.
- [151] Walley J, Xiao Y, Wang J, Baidoo EE, Keasling JD, Shen Z, et al. Plastid-produced interorganelle stress signal MecPP potentiates induction of the unfolded protein response in endoplasmic reticulum. *Proc Natl Acad Sci U S A* 2015;112: 6212–7.
- [152] Carretero-Paulet L, Cairó A, Talavera D, Saura A, Imperial S, Rodríguez-Concepción M, et al. Functional and evolutionary analysis of *DXL1*, a non-essential gene encoding a 1-deoxy-D-xylulose 5-phosphate synthase like protein in *Arabidopsis thaliana*. *Gene* 2013;524:40–53.
- [153] Lu XM, Hu XJ, Zhao YZ, Song WB, Zhang M, Chen ZL, et al. Map-based cloning of *zb7* encoding an IPP and DMAPP synthase in the MEP pathway of maize. *Mol Plant* 2012;5:1100–12.
- [154] Jin H, Song Z, Nikolau BJ. Reverse genetic characterization of two paralogous acetoacetyl CoA thiolase genes in *Arabidopsis* reveals their importance in plant growth and development. *Plant J* 2012;70:1015–32.
- [155] Venkateshwaran M, Jayaraman D, Chabaud M, Genre A, Balloon AJ, Maeda J, et al. A role for the mevalonate pathway in early plant symbiotic signaling. *Proc Natl Acad Sci U S A* 2015;112:9781–6.
- [156] Brodersen P, Sakvarelidze-Achard L, Schaller H, Khafif M, Schott G, et al. Isoprenoid biosynthesis is required for miRNA function and affects membrane association of ARGONAUTE 1 in *Arabidopsis*. *Proc Natl Acad Sci U S A* 2012;109:1778–83.
- [157] Henry LK, Gutensohn M, Thomas ST, Noel JP, Dudareva N. Orthologs of the archaeal isopentenyl phosphate kinase regulate terpenoid production in plants. *Proc Natl Acad Sci U S A* 2015;112:10050–5.
- [158] Muñoz-Bertomeu J, Arrillaga I, Ros R, Segura J. Up-regulation of 1-deoxy-D-xylulose-5-phosphate synthase enhances production of essential oils in transgenic spike lavender. *Plant Physiol* 2006;142:890–900.
- [159] Vaccaro M, Malafrente N, Alfieri M, De Tommasi N, Leone A. Enhanced biosynthesis of bioactive abietane diterpenes by overexpressing *AtDXS* or *AtDXR* genes in *Salvia sclarea* hairy roots. *Plant Cell Tiss Org* 2014;119:65–77.
- [160] Carretero-Paulet L, Cairó A, Botella-Pavía P, Besumbes O, Campos N, Boronat A, Rodríguez-Concepción. Enhanced flux through the methylerythritol 4-phosphate pathway in *Arabidopsis* plants overexpressing deoxyxylulose 5-phosphate reductoisomerase. *Plant Mol Biol* 2006;62:683–95.
- [161] Mendoza-Poudereux I, Muñoz-Bertomeu J, Arrillaga I, Segura J. Deoxyxylulose 5-phosphate reductoisomerase is not a rate-determining enzyme for essential oil production in spike lavender. *J Plant Physiol* 2014;171:1564–70.

- [162] Mahmoud SS, Croteau RB. Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. *Proc Natl Acad Sci U S A* 2001;15:8915–20.
- [163] Botella-Pavía P, Besumbes O, Phillips MA, Carretero-Paulet L, Boronat A, Rodríguez-Concepción M. Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors. *Plant J* 2004;40:188–99.
- [164] Wang H, Nagegowda DA, Rawat R, Bouvier-Navé P, Guo D, Bach TJ, et al. Overexpression of *Brassica juncea* wild-type and mutant HMG-CoA synthase 1 in *Arabidopsis* up-regulates genes in sterol biosynthesis and enhances sterol production and stress tolerance. *Plant Biotechnol J* 2012;10:31–42.
- [165] Re EB, Jones D, Learned RM. Co-expression of native and introduced genes reveals cryptic regulation of HMG CoA reductase expression in *Arabidopsis*. 1995;7:771–84.
- [166] Chappell J, Wolf F, Proulx J, Cuellar R, Saunders C. Is the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme-A reductase a rate-limiting step for isoprenoid biosynthesis in plants. *Plant Physiol* 1995;109:1337–43.
- [167] Enfissi EMA, Fraser PD, Lois LM, Boronat A, Schuch W, Bramley PM. Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. *Plant Biotechnol J* 2005;3:17–27.
- [168] Zhan X, Zhang YH, Chen DF, Simonsen HT. Metabolic engineering of the moss *Physcomitrella patens* to produce the sesquiterpenoids patchoulol and α/β -santalene. *Front Plant Sci* 2014;5:636.
- [169] Leivar P, Antolín-Llovera M, Ferrero S, Closa M, Arró M, Ferrer A, et al. Multilevel control of *Arabidopsis* 3-hydroxy-3-methylglutaryl coenzyme A reductase by protein phosphatase 2A. *Plant Cell* 2011;23:1494–511.
- [170] Kobayashi K, Suzuki M, Tang J, Nagata N, Ohshima K, Seki H, et al. LOVASTATIN INSENSITIVE 1, a novel pentatricopeptide repeat protein, is a potential regulatory factor of isoprenoid biosynthesis in *Arabidopsis*. *Plant Cell Physiol* 2007;48:322–31.
- [171] Tang J, Kobayashi K, Suzuki M, Matsumoto S, Muranaka T. The mitochondrial PPR protein LOVASTATIN INSENSITIVE 1 plays regulatory roles in cytosolic and plastidial isoprenoid biosynthesis through RNA editing. *Plant J* 2010;61:456–66.
- [172] Flores-Pérez U, Sauret-Güeto S, Gas E, Jarvis P, Rodríguez-Concepción M. A mutant impaired in the production of plastome-encoded proteins uncovers a mechanism for the homeostasis of isoprenoid biosynthetic enzymes in *Arabidopsis* plastids. *Plant Cell* 2008;20:1303–15.
- [173] Sauret-Güeto S, Botella-Pavía P, Flores-Pérez U, Martínez-García JF, San Román C, León P, et al. Plastid cues posttranscriptionally regulate the accumulation of key enzymes of the methylerythritol phosphate pathway in *Arabidopsis*. *Plant Physiol* 2006;141:75–84.
- [174] Rodríguez-Concepción M, Forés O, Martínez-García JF, González V, Phillips MA, Ferrer A, Boronat A. Distinct light-mediated pathways regulate the biosynthesis and exchange of isoprenoid precursors during *Arabidopsis* seedling development. *Plant Cell* 2004;16:144–56.
- [175] Guleria P, Yadav SK. *Agrobacterium* mediated transient gene silencing (AMTS) in *Stevia rebaudiana*: insights into steviol glycoside biosynthesis pathway. *PLoS One* 2013;8:e74731.
- [176] Guleria P, Yadav SK. Overexpression of a glycosyltransferase gene *SrUGT74G1* from *Stevia* improved growth and yield of transgenic *Arabidopsis* by catechin accumulation. *Mol Biol Rep* 2014;41:1741–52.
- [177] Lange BM, Ghassemian M. Genome organization in *Arabidopsis thaliana*: a survey for genes involved in isoprenoid and chlorophyll metabolism. *Plant Mol Biol* 2003;51:925–48.
- [178] Córdoba E, Porta H, Arroyo A, San Román C, Medina L, Rodríguez-Concepción, et al. Functional characterization of the three genes encoding 1-deoxy-D-xylulose 5-phosphate synthase in maize. *J Exp Bot* 2011;62:2023–38.
- [179] Budziszewski GJ, Lewis SP, Glover LW, Reineke J, Jones G, Ziemnik LS, et al. *Arabidopsis* genes essential for seedling viability: isolation of insertional mutants and molecular cloning. *Genetics* 2001;159:1765–78.
- [180] Guevara-García A, San Román C, Arroyo A, Cortés ME, Gutiérrez-Nava MD, León P. Characterization of the *Arabidopsis clb6* mutant illustrates the importance of post-transcriptional regulation of the methyl-D-erythritol 4-phosphate pathway. *Plant Cell* 2005;17:628–43.
- [181] Araki N, Kusumi K, Masamoto K, Niwa Y, Iba K. Temperature-sensitive *Arabidopsis* mutant defective in 1-deoxy-D-xylulose 5-phosphate synthase within the plastid non-mevalonate pathway of isoprenoid biosynthesis. *Physiol Plant* 2000;108:19–24.
- [182] Estévez JM, Cantero A, Reindl A, Reichler S, León P. 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. *J Biol Chem* 2001;276:22901–9.
- [183] Gondet L, Weber T, Maillot-Vernier P, Benveniste P, Bach TJ. Regulatory role of microsomal 3-hydroxy-3-methylglutaryl-coenzyme-A reductase in a tobacco mutant that overproduces sterols. *Biochem Biophys Res Commun* 1992;186:888–93.
- [184] Németh K, Salchert K, Putnoky P, Bhalerao R, Koncz-Kálmán Z, Stankovic-Stangeland B, et al. Pleiotropic control of glucose and hormone responses by PRL1, a nuclear WD protein, in *Arabidopsis*. *Genes Dev* 1998;12:3059–73.
- [185] Ye VM, Bhatia SK. Metabolic engineering for the production of clinically important molecules: omega-3 fatty acids, artemisinin, and taxol. *Biotechnol J* 2012;7:20–33.
- [186] Zhang Y, Jiang P, Ye M, Kim S-H, Jiang C, Lu J. Tanshinones: sources, pharmacokinetics and anti-cancer activities. *Int J Mol Sci* 2012;13:13621–66.
- [187] Liao P, Wang H, Wang M, Hsiao A-S, Bach TJ, Chye M-L. Transgenic tobacco overexpressing *Brassica juncea* HMG-CoA synthase 1 shows increased plant growth, pod size and seed yield. *PLoS One* 2014;9:e98264.