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**SPECIAL ISSUE REVIEW**

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# Analysis of plant secondary metabolism using stable isotope-labelled precursors

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**Abstract**

**Introduction:** Analysis of biochemical pathways typically involves feeding a labelled precursor to an organism, and then monitoring the metabolic fate of the label. Initial studies used radioisotopes as a label and then monitored radioactivity in the metabolic products. As analytical equipment improved and became more widely available, preference shifted the use stable 'heavy' isotopes like deuterium (<sup>2</sup>H)-, carbon-13 (<sup>13</sup>C)- and nitrogen-15 (<sup>15</sup>N)-atoms as labels. Incorporation of the labels could be monitored by mass spectrometry (MS), as part of a hyphenated tool kits, e.g. Liquid chromatography (LC)-MS, gas chromatography (GC)-MS, LC-MS/MS. MS offers great sensitivity but the exact location of an isotope label in a given metabolite cannot always be unambiguously established. Nuclear magnetic resonance (NMR) can also be used to pick up signals of stable isotopes, and can give information on the precise location of incorporated label in the metabolites. However, the detection limit for NMR is quite a bit higher than that for MS.

**Objectives:** A number of experiments involving feeding stable isotope-labelled precursors followed by NMR analysis of the metabolites is presented. The aim is to highlight the use of NMR analysis in identifying the precise fate of isotope labels after precursor feeding experiments. As more powerful NMR equipment becomes available, applications as described in this review may become more commonplace in pathway analysis.

**Conclusion and Prospects:** NMR is a widely accepted tool for chemical structure elucidation and is now increasingly used in metabolomic studies. In addition, NMR, combined with stable isotope feeding, should be considered as a tool for metabolic flux analyses.

**KEYWORDS**

biosynthesis, biosynthetic pathways, metabolic flux, NMR, terpenoids, tropane alkaloids

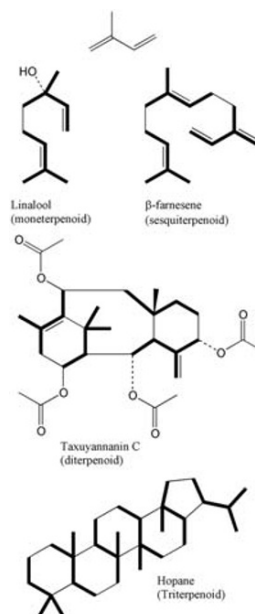
Dedicated to the memory of Professor Jack G Woolley (1940–2012)

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## 1 | INTRODUCTION

Analysis of biochemical pathways typically involves feeding a labelled precursor to an organism, and then monitoring the metabolic fate of the label. Initial studies used radioisotopes as a label and then monitored radioactivity in the metabolic products.<sup>1,2</sup> As analytical equipment improved and became more widely available, preference shifted the use stable 'heavy' isotopes like deuterium (<sup>2</sup>H)-, carbon-13 (<sup>13</sup>C)- and nitrogen-15 (<sup>15</sup>N)-atoms as labels.<sup>3,4</sup> Incorporation of the labels could be monitored by mass spectrometry (MS), as part of a hyphenated tool kits, e.g. liquid chromatography (LC)-MS, gas chromatography (GC)-MS, LC-MS/MS. MS offers great sensitivity but the exact location of an isotope label in a given metabolite cannot always be unambiguously established. Although the detection limit for nuclear magnetic resonance (NMR) is quite a bit higher than that for MS, this technique can also be used to pick up signals of stable isotopes, and give information on the precise location of incorporated label in the metabolites. As the sensitivity and spectral resolution of NMR increase, this analytical tool can be expected to play an ever-increasing important role in studying the regulation of biosynthetic pathways.



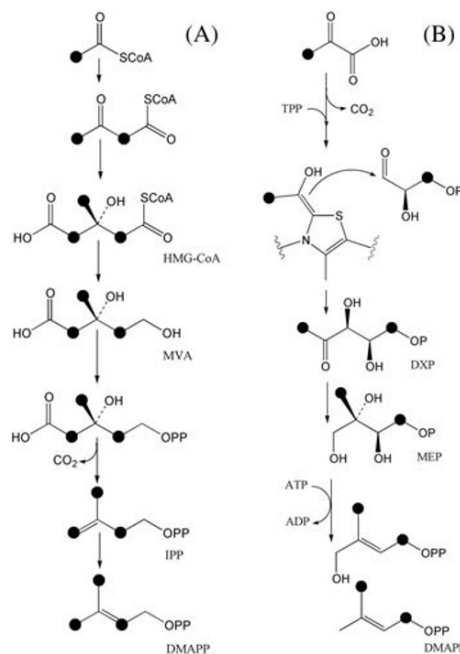
**FIGURE 1** Isoprene unit, and structures of monoterpene, sesquiterpene, diterpene and triterpene structures indicating the metabolic fate of the individual isoprene precursors

## 2 | BIOSYNTHESIS OF TERPENOIDS

The terpenoids (a.k.a. isoprenoids) are a large and diverse class of naturally occurring compounds. The common precursor for all terpenoids are isoprene units, either as isopentenyl diphosphate (IPP) or dimethylallyl diphosphate (DMAPP). Depending on the number of C<sub>5</sub> isoprene units incorporated in the final structure, terpenoids can be subdivided into hemiterpenoids (C<sub>5</sub>), monoterpenoids (C<sub>10</sub>), sesquiterpenoids (C<sub>15</sub>), diterpenoids (C<sub>20</sub>), sesterterpenoids (C<sub>25</sub>), triterpenoids (C<sub>30</sub>), etc. (Figure 1).

It was a fact universally acknowledged that the formation of IPP was via mevalonate (MVA), which in turn is synthesised by the condensation of two acetyl-coenzyme A (acetyl-CoA) units, giving acetoacetyl-CoA, and subsequent addition of a third acetyl-CoA resulting in the formation of β-hydroxy-β-methylglutaryl-CoA (Figure 2A). This pathway had been established in the 1950s based on feeding experiments with <sup>2</sup>H- and <sup>13</sup>C-labelled precursors followed by careful mass spectrometric analysis of the formed products.<sup>5</sup>

In the 1980s, the wider availability of NMR instruments allowed assigning the signals in <sup>13</sup>C-NMR spectra of hopanoids to the carbon atoms by reference to published spectra, and homonuclear <sup>1</sup>H-<sup>1</sup>H correlation (COSY) and by heteronuclear <sup>1</sup>H-<sup>13</sup>C correlation using <sup>1</sup>J coupling constants. It was then found that feeding [1-<sup>13</sup>C]acetate to various bacterial species resulted in the formation of hopanoids with <sup>13</sup>C labelling patterns that differed completely from those that would be expected if these triterpenoids were made via the classical MVA-biosynthetic route.<sup>6,7</sup> A novel hypothetical pathway was proposed (Figure 2B) in which the C<sub>5</sub> isoprenic units IPP or DMAPP were formed by direct condensation of thiamine-activated acetaldehyde arising from pyruvate decarboxylation on a C<sub>3</sub> unit derived from a



**FIGURE 2** (A) The mevalonate (MVA) pathway. (B) The deoxyxylulose phosphate (DXP) or methylerythritol phosphate (MEP) pathway. The black dots indicate the position of <sup>13</sup>C-label if the precursors were glycolytic products derived from [1,2-<sup>13</sup>C<sub>2</sub>]-glucose

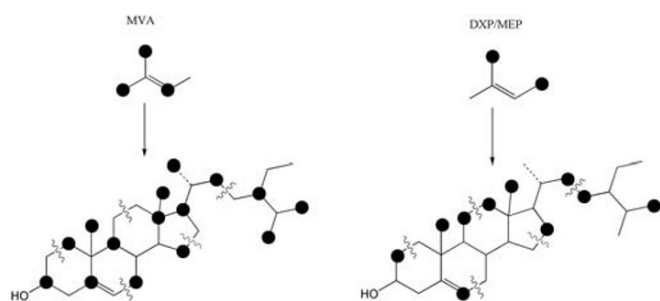
triose phosphate.<sup>8</sup> Feeding bacteria with [1-<sup>13</sup>C]-, [2-<sup>13</sup>C]-, [3-<sup>13</sup>C]-, [5-<sup>13</sup>C]- or [6-<sup>13</sup>C]-glucose allowed determination of the origin of each carbon atom of the isoprenic units from the hopanoids. Isotopic abundances were determined by <sup>13</sup>C-NMR spectroscopy. Shortly

after, the newly discovered biosynthetic pathway was also found to be active in green algae,<sup>9</sup> and in higher plants.<sup>10</sup>

It became clear that in plants two pathways are active that lead to the formation of IPP and DMAPP, and thus to all terpenoids, i.e. the MVA pathway and the deoxyxylulose phosphate (DXP) or methylerythritol phosphate (MEP) pathway. Both pathways start with precursors derived from glycolysis; the DXP/MEP pathway – which is localised in the plastids – starts with pyruvate and D-glyceraldehyde-3-phosphate, whereas the MVA pathway – localised in the cytosol – starts with acetyl-CoA units which are formed by oxidation of pyruvate in the mitochondria. Although the pathways are compartmentalised, substrate exchange across membranes does occur.<sup>11</sup> Acetyl-CoA is a common precursor for a range of biosynthetic pathways, but any acetyl-CoA units that enter the plastids are used for fatty acid synthesis rather than terpenoid biosynthesis.<sup>12</sup>

In order to assess whether a particular terpenoid is formed via the MVA route or via the DXP/MEP route, feeding experiments can be done using either [1-<sup>13</sup>C]-glucose, [6-<sup>13</sup>C]-glucose, or [1,6-<sup>13</sup>C<sub>2</sub>]-glucose.<sup>13</sup> Glycolysis of these labelled glucose molecules will result in the formation of [1-<sup>13</sup>C]-pyruvate, [3-<sup>13</sup>C]-D-glyceraldehyde phosphate and [2-<sup>13</sup>C]-acetyl-CoA, which can subsequently be used as precursors in either the DXP/MEP pathway or in the MVA pathway respectively to form terpenoids. Each of the pathways will result in a specific labelling pattern in the biosynthetic product (Figure 3) which will be indicative of the pathway followed. Alternatively, feeding experiments can be done with <sup>13</sup>C-labelled acetate; in this case the label will only appear in terpenoids that are formed via the MVA pathway. The position <sup>13</sup>C-label and the level of <sup>13</sup>C abundance can be assessed by quantitative NMR spectroscopy. Proton (<sup>1</sup>H)-decoupled <sup>13</sup>C-NMR spectra of samples from incorporation experiments and of samples with natural <sup>13</sup>C abundance (1.1% <sup>13</sup>C) should be recorded under identical conditions. Relative <sup>13</sup>C abundance of individual carbon atoms can then be calculated by comparison of <sup>13</sup>C signal integrals between <sup>13</sup>C-labelled and unlabelled products.

When [1,2-<sup>13</sup>C<sub>2</sub>]-acetate was fed to cell cultures of *Taxus chinensis*, <sup>13</sup>C label could be traced in the four acetyl groups of taxuyunnanin C, but not in the taxane ring system. Label from [1-<sup>13</sup>C]-glucose was incorporated into both the taxane ring system and the acetyl groups. These results indicate that the taxane carbon skeleton in this case was derived of the DXP/MEP pathway.<sup>14</sup> Similar



**FIGURE 3** Incorporation of <sup>13</sup>C-label in β-sitosterol, depending of their origin from the MVA pathway or the DXP/MEP pathway. In case of mixed origins, a mixed pattern of labelling will be seen<sup>13</sup>

experiments have shown that diterpenes in the liverwort *Fossombronia alaskana*, robustaquinone from *Cinchona* 'Robusta', pyrethrins from *Chrysanthemum cinerariaefolium*, plaunotol from *Croton stellatopilosus* cut shoots, Salvinorin A from *Salvia divinorum* shoot cultures, and andrographolide from *Andrographis paniculata* are all solely DXP/MEP derived.<sup>15–20</sup>

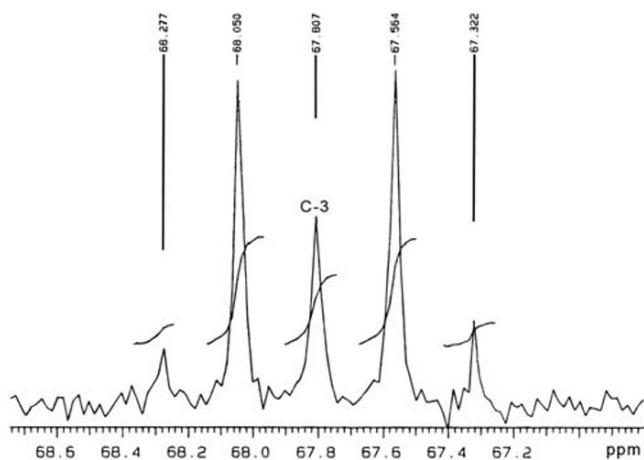
Quantitative <sup>13</sup>C-NMR spectroscopic analysis of metabolic products obtained after feeding [1-<sup>13</sup>C]-glucose to axenic cultures of *Tanacetum vulgare* L. or seedlings of *Gossypium hirsutum* L. showed that here monoterpenes diterpenes are formed via the DXP/MEP pathway, whereas sesquiterpenes and the sterols originate from the MVA pathway.<sup>21,22</sup> In a callus culture of *Croton sublyratus* Kurz, the incorporation pattern of label from [1-<sup>13</sup>C]-glucose into sitosterol and stigmasterol pointed at mixed MEV and DXP/MEP origins of sterols.<sup>23</sup> Mixed origins were also found for the formation of withanolides in *Withania somnifera* Dunal.<sup>24</sup>

In *Coluria geoides* hairy root cultures, label derived from [1-<sup>13</sup>C]-glucose or [1,6-<sup>13</sup>C<sub>2</sub>]-glucose was incorporated into dolichols, i.e. linear polyisoprenoid alcohols. Here, NMR analysis revealed that polymers up to 13 isoprene units long are synthesised in plastids with isoprenes derived from both the DXP/MEP and MVA pathways. Longer polymers are completed in the cytoplasm with several units derived solely from the MVA pathway.<sup>25</sup>

### 3 | BIOSYNTHESIS OF TROPANE ALKALOIDS

The tropane alkaloids are all derived from the amino acid ornithine. Attempts to elucidate the exact individual steps leading from ornithine to hyoscyamine date back to the early 1950s. Initially, biosynthetic studies were done by feeding <sup>14</sup>C- or <sup>3</sup>H-labelled precursors to plants followed by isolation and careful chemical degradation of the metabolic products as a means to locate the position of the radioactive label.<sup>26</sup> Although sporadic reports mention NMR spectroscopic analysis locate the position of <sup>2</sup>H, <sup>13</sup>C, or <sup>15</sup>N stable isotopes after precursor feeding before 1990, NMR spectrometers only became widely available as a tool from the 1990s onwards. Then, feeding experiments with <sup>13</sup>C-labelled precursors, followed by <sup>13</sup>C-NMR analysis, confirmed previous findings and added further detail, e.g. feeding of [1,2-<sup>13</sup>C<sub>2</sub>]-acetate resulted in a spin-spin coupling pattern of <sup>13</sup>C-signal in the hyoscyamine spectrum (Figure 4), showing symmetrical incorporation of <sup>13</sup>C-label into the C-2 and C-4 positions, which in turn indicated incorporation of an intact 4-carbon unit rather than subsequent addition of two acetate units to form the tropane moiety.<sup>27</sup> Whereas spin-spin coupling is a common occurrence in <sup>1</sup>H-NMR spectra, it is normally not seen in a <sup>13</sup>C-NMR spectrum due to the low abundance of <sup>13</sup>C atoms (1.1%); the chance that two naturally occurring <sup>13</sup>C-atoms in a molecule are contiguous is too small.

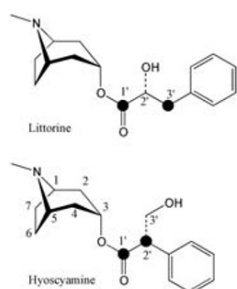
What remained a puzzle was that, though hyoscyamine is an ester of tropine and tropic acid, feeding experiments with radioisotope labelled precursors had shown that labelled phenylalanine and phenyllactic acid are efficiently incorporated into hyoscyamine, feeding



**FIGURE 4** A doublet symmetrically overlapped with a triplet in the  $^{13}\text{C}$ -NMR spectrum of  $^{13}\text{C}$ -labelled tropine formed after  $[1,2\text{-}^{13}\text{C}_2]$ -acetate feeding<sup>26</sup>

labelled tropine did not result in labelled hyoscyamine. In an elegant series of experiments in which 3-phenyl[2- $^{13}\text{C}$ , 2- $^2\text{H}$ ]-lactate, phenyl [1,3- $^{13}\text{C}_2$ ]-lactic acid, or [1',3'- $^{13}\text{C}$ , methyl- $^2\text{H}_3$ ]-littorine were fed to hairy root cultures of *Datura stramonium*, followed by  $^1\text{H}$ -,  $^2\text{H}$ -,  $^{13}\text{C}$ - and two-dimensional (2D)-NMR spectroscopic analysis, it was shown that tropine initially forms an ester with phenyllactic acid, resulting in the formation of littorine.<sup>28-31</sup> Then, through intramolecular rearrangement, littorine converts into hyoscyamine. The rearrangement could be made visible in the  $^{13}\text{C}$ -NMR spectrum since the  $^{13}\text{C}$ -1' and  $^{13}\text{C}$ -3' atoms of littorine were rearranged to form the contiguous  $^{13}\text{C}$ -1' and  $^{13}\text{C}$ -2' in hyoscyamine (Figure 5). The rearranged contiguous  $^{13}\text{C}$ -labels again resulted in the rarely seen split peak pattern in the  $^{13}\text{C}$ -NMR spectrum due to spin-spin coupling.<sup>3,31</sup>

The process by which littorine rearranges to hyoscyamine is still a matter of debate.<sup>32-34</sup> A cytochrome P450 enzyme CYP80F1, has been identified from *Hyoscyamus niger*. Expression of the gene in yeast confirmed that CYP80F1 catalyses the oxidation of (*R*)-littorine with rearrangement to form hyoscyamine aldehyde, a putative precursor to hyoscyamine.<sup>35</sup> The enzyme assays use GC-MS as a detection tool since this analytical technique has a much lower detection limit than NMR.



**FIGURE 5** Intramolecular rearrangement of littorine to form hyoscyamine. The black dots indicate the position of  $^{13}\text{C}$  label<sup>30</sup>

Another stable isotope that can be used to monitor bioconversion of alkaloids in plants is  $^{15}\text{N}$ . This stable isotope has a low natural abundance (0.37%), and it is usually necessary to enrich the sample with  $^{15}\text{N}$  before NMR analysis, e.g. by supplying plants with  $^{15}\text{N}$ -labelled nitrate or ammonium salts. Then, similar to the  $^{13}\text{C}$ -NMR experiments described earlier,  $^{15}\text{N}$ -NMR can be used to explore the redistribution of the label through the plant metabolic network.<sup>36</sup> A drawback of  $^{15}\text{N}$ -NMR for plant secondary pathway analysis is that numerous compounds of interest resonate very close together, resulting in poor resolution of peaks in the NMR spectrum. However, this problem can be overcome by using  $^{15}\text{N}$ - $^1\text{H}$  2D spectra, e.g. in heteronuclear multiple quantum coherence (HMQC) spectroscopy, or heteronuclear multiple-bond coherence (HMBC) spectroscopy.<sup>37,38</sup>

In contrast to MS, NMR is a non-destructive analytical technique, which allows spectra to be recorded *in vivo*. When living root cultures of *Datura stramonium* were monitored over a 24 h period using  $^{15}\text{N}$ -NMR it could be shown that [ $^{15}\text{N}$ ]-tropinone was metabolised to [ $^{15}\text{N}$ ]-tropine and simple [ $^{15}\text{N}$ ]-tropine esters.<sup>39</sup> However, the need to meet the physiological requirements of living cell cultures within the confines of the NMR magnet during *in vivo* measurements is a major challenge. Therefore, *in vivo* NMR approach is in practice more demanding and time consuming than the analysis of plant extracts.<sup>36</sup>

## 4 | METABOLOMICS – FLUX ANALYSIS – PATHWAY ANALYSIS

Over the past few decades, more powerful NMR spectrometers have become available and increasingly higher resolution spectra could be analysed for identification and structure elucidation purposes. The increasing resolution has also made possible progress in the field of metabolomics and allowed it to be integrated into the other OMICS technologies that rely on shared massive databases.

Metabolomic analysis, using only natural abundance of isotopes, provides us with snapshots of metabolic states. Comparing metabolomic profiles, e.g. over time, or by comparing diseased states with control healthy states, can show us changes in metabolic states that have occurred. For human metabolomic research, the human metabolome database can be used;<sup>40</sup> users can submit peak lists from  $^1\text{H}$ - or  $^{13}\text{C}$ -NMR spectra (both pure and mixtures) or 2D TOCSY or  $^{13}\text{C}$ -HSQC spectra, respectively, and compare their spectra to the NMR libraries contained in the database, thus allowing the identification of metabolites in mixtures via NMR spectroscopy. It should be noted though that most of the spectra in the database are derived from *in silico* spectral prediction rather than experimental spectral data. A plant metabolome database also exists<sup>41</sup> though as yet it has fewer functionalities than the human database.

Metabolic flux analysis, sometimes called fluxomics, adds feeding of rare isotopes ( $^{13}\text{C}$  or  $^{15}\text{N}$ ) into the equation. By selectively monitoring the fate of the fed isotopes over time, a more dynamic picture of metabolism can be provided. Stable isotope labelling experiments can be used for the quantitative determination of the flux or precursors and intermediates through a metabolic network. In addition, tracing

experiments can be applied to qualitative evaluation such as the partitioning of fluxes into diverging pathways at a branching point in a metabolic pathway.<sup>42</sup> Partitioning of metabolic flux into different pathways can also be qualitatively evaluated if the products of the alternative pathways have different labelling patterns; careful selection of appropriate precursor isotopomers ('isotopic isomers') of isotopologues ('isotopic homologues') is of key importance. The difference between MVA- versus the DXP/MEP-derived metabolites could be visualised by feeding either [1-<sup>13</sup>C]-glucose, [6-<sup>13</sup>C]-glucose, or [1,6-<sup>13</sup>C<sub>2</sub>]-glucose; feeding of [U-<sup>13</sup>C<sub>6</sub>]-glucose does not result in a different labelling pattern, since the label from the latter would be evenly spread over virtually all carbon-containing molecules in the plant.

Metabolic pathway analysis may be considered as a subset of metabolic flux analysis; the main difference is the choice of precursors. As outlined earlier for the terpenoid biosynthetic pathway, feeding [1,2-<sup>13</sup>C<sub>2</sub>]-acetate to *Taxus chinensis* left the taxane skeleton unlabelled, because no <sup>13</sup>C-label entered the DXP/MEP pathway.<sup>14</sup> Further labelled precursors that are selectively used in one pathway only have been designed, e.g. [2,3,4,5-<sup>13</sup>C<sub>4</sub>]-deoxyxylulose, [1-<sup>13</sup>C;3,4-<sup>2</sup>H<sub>2</sub>]-deoxyxylulose, and [2-<sup>13</sup>C,4-<sup>2</sup>H]-deoxyxylulose.<sup>9,17,43</sup> Similarly, feeding <sup>15</sup>N-[nitrate] and [<sup>15</sup>N]-ammonium to plants will label all nitrogen-containing molecules in a plant, whereas feeding [<sup>15</sup>N]-tropinone only labels the subset of molecules belonging to the tropane alkaloid pathway.<sup>37,39</sup> For pathway analysis, non-generic precursors should be chosen that will selectively label only a selected subset of metabolites. The major challenge is to design and make these precursors since, unlike more generic stable isotope labelled precursors as glucose and acetate, they are not usually commercially available. However, once appropriate labelled precursors are made, the powerful toolset of <sup>13</sup>C metabolic flux analysis, which is developed in the wake of the general emphasis on OMICS technology,<sup>44,45</sup> is available for analysis. Metabolic flux analysis by NMR has now developed to an extent that it has become a leading provider of data for the study of regulation of plant secondary metabolic pathways.

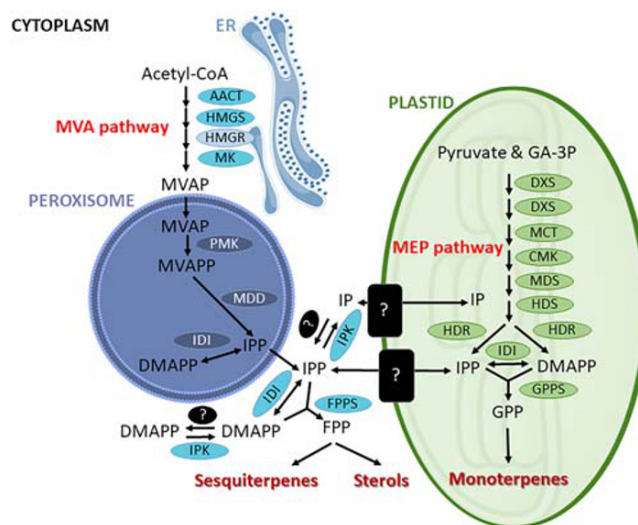
## 5 | FUTURE DEVELOPMENTS IN CONTEXT OF PLANT RESEARCH

Any attempt to modify production bioactive plant secondary metabolites requires structural identification of individual precursors and end-products, but also an in-depth analysis of the specific branches of the metabolic pathways of interest, and their integration within overall plant metabolism.<sup>46</sup> With the advent of functional genomics and metabolic engineering, analysis of secondary plant metabolism by NMR, using precursors labelled with stable isotopes becomes even more important as it can help in these analyses.

Further work is required on our yet incomplete understanding of the functioning of individual pathways and their integration within metabolic networks. NMR, coupled with the use of stable isotope-labelled precursors, can provide a very direct and practical

window on metabolism,<sup>47</sup> and may be used to explore secondary pathways and their functioning while simultaneously providing information on the integration of these metabolic pathways with primary metabolism. It also provides information on the regulation of these metabolic pathways, in particular when *in vitro* and also *in vivo* measurements are integrated. In particular, NMR *in vivo* has demonstrated its potential for direct characterisation of the physiological and metabolic state of living cells in a non-invasive manner and an ability to track metabolic changes in response to development and changes in the environment.<sup>48</sup> This approach is all the more capable of providing information on the regulation of a metabolic pathway, metabolic bottlenecks, concurrent pathways, etc., when using perturbed biological systems such as mutants, transgenic lines or elicited cells.

Whereas basic metabolomic analyses can show the presence of selected metabolites in different tissues, they do not provide information on the enzymatic activities in a metabolic pathway.<sup>49</sup> A better way to determine the role and the flux of metabolites in metabolic



**FIGURE 6** An overview of the compartmentalisation of terpenoid biosynthetic pathways in plants (adapted from Henry *et al.*<sup>54</sup>). Enzymes abbreviations: AACT: acetoacetyl-CoA thiolase; CMK: 4-(cytidine 5'- diphospho)-2-C-methyl-D-erythritol kinase; DMAP: dimethylallyl phosphate; DMAPP: dimethylallyl diphosphate; DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS: 1-deoxy-D-xylulose-5-phosphate synthase; FPP: farnesyl diphosphate; FPPS: farnesyl diphosphate synthase; GA-3P: D-glyceraldehyde-3-phosphate; GPP: geranyl diphosphate; GPPS: geranyl diphosphate synthase; HDR: (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; HDS: (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase; HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase; HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase; IDI: isopentenyl diphosphate isomerase; IP: isopentenyl phosphate; IPK: isopentyl phosphate kinase; IPP: isopentenyl diphosphate; MCT: 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase; MDD: mevalonate diphosphate decarboxylase; MDS: 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; MK: mevalonate kinase; MPD: phosphomevalonate decarboxylase; MVAP: mevalonate-5-phosphate; MVAPP: mevalonate diphosphate; PMK: phosphomevalonate kinase



pathways is to follow their fate with stable tracer isotopes. The variation in the rate of a given metabolite results from the speed of the enzymes which catalyse its conversion. Metabolic network flux analysis tools have proven themselves to be powerful aids to metabolic engineering of bacterial cultures, but have as yet found relatively little application in plant systems.<sup>50,51</sup>

Plants, as multicellular eukaryotic organisms, have a more complex spatial distribution of metabolic pathways than bacteria,<sup>52</sup> which can pose a major challenge for metabolic network flux analysis and the overall understanding of the regulation of a metabolic pathway.<sup>53</sup> In particular, plants have a high degree of compartmentalisation at the tissue and subcellular levels with pools of metabolites and enzymes located in different places and parallel pathways which can coexist within different organelles, as illustrated perfectly in the terpenoid biosynthetic pathway (Figure 6). The need for compartmental information on proteins and metabolites can be met using already established organelle purification methods. Tools for metabolic flux analysis that were developed and applied previously to microbial or mammalian cell cultures are now being adapted for use in plant metabolic studies.<sup>55</sup>

## 6 | CONCLUDING REMARKS

At present, many black boxes still exist in our understanding of the regulation of plant secondary metabolism, its integration and its links with the primary metabolism, and its subcellular localisation. However, NMR analysis, in combination with stable isotope-labelled precursor feeding can contribute to a better quantitative and qualitative understanding of the interconnection of multiple metabolic pathways and in the characterisation of metabolic phenotypes of interest. The research tools are available; however, it does require a multi-disciplinary investigating team to use these tools properly. This approach can contribute to a rational modification of the metabolic networks of plants, with a view to increase the yield of secondary metabolites by metabolic engineering.

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