Synthesis of chlorophyll a and β-carotene from ^{2}H- and ^{13}C-labelled mevalonates and ^{13}C-labelled glycine in cultured cells of liverworts, *Heteroscyphus planus* and *Lophocolea heterophylla*

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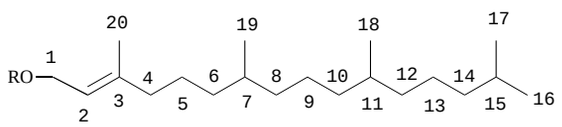
^{2}H- And ^{13}C-labelled mevalonates (MVA) are incorporated at a higher rate into the phytyl side-chain of chlorophyll a and β-carotene than into diterpenes in suspension cultures of liverworts, *Heteroscyphus planus* and *Lophocolea heterophylla*. The distribution of labels in the biosynthetically labelled chlorophyll a, phytol and β-carotene incorporating MVA determined by ^{2}H and ^{13}C NMR analyses indicates preferential labelling of the farnesyl diphosphate (FPP)-derived portion of these compounds. These findings suggest that all compounds formed from geranylgeranyl diphosphate within the chloroplasts are biosynthesized partly *via* the condensation of FPP derived from exogenous MVA and endogenous isopentenyl diphosphate, and that the pigments and normal diterpenes are biosynthesized separately at different sites within the chloroplasts. In contrast, [2-^{13}C]glycine administered to the cultured cells of *H. planus* equivalently labels both the phytyl side-chain and β-carotene, indicating that endogenously formed MVA *via* glycine is equivalently incorporated both into the phytyl moiety and β-carotene. Intense doublets due to ^{13}C–^{13}C coupling, observed in the ^{13}C-labelled phytyl side-chain and β-carotene, suggest that acetyl-CoA is mainly formed from serine derived from 5,10-methylenetetrahydrofolic acid and glycine.

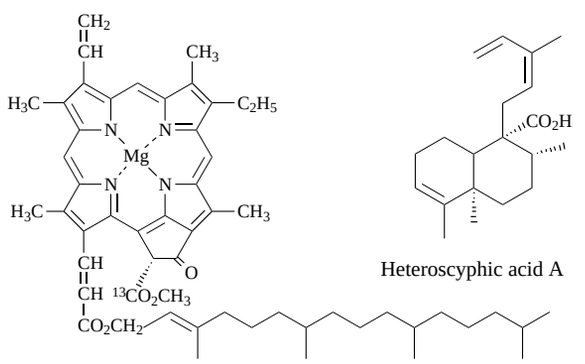
Introduction

In all vascular plants both carotenoids and chlorophylls are synthesized and accumulated in the chloroplasts of photosynthetic tissues. The reaction centre core complexes of photosystems I and II are rich in β-carotene and chlorophyll a,[1] the biosynthesis of which is an integral part of the construction of pigment–protein complexes in the thylakoids, and is closely related to the formation of other components, *e.g.* proteins and lipids.[2–4] The proteins hold the chlorophyll and carotenoid molecules in place and in close proximity for their tasks in harvesting sunlight, converting the light energy into chemical energy and protecting chloroplasts from chlorophyll-sensitized photooxidation damage. Although there is no doubt that carotenoids and the phytyl moiety of chlorophyll a are biosynthesized within the chloroplasts, there are conflicting views about whether early biosynthetic intermediates for geranylgeranyl diphosphate (GGPP) synthesis need to be imported or whether they are formed within the chloroplasts autonomously.[5,6] The formation of phytyl ester is the last step in the biosynthesis of chlorophylls and occurs in the thylakoids in the chloroplasts.[7] A considerable body of experimental evidence indicates that chlorophyllase, which catalyses esterification of chlorophyllide with GGPP, and reductase, which reduces the initial product to chlorophyll *via* chlorophyllide di- and tetra-hydrogeranylgeranyl ester, are firmly bound to the thylakoid membranes.[7,8] The location of the later stages of carotenoid biosynthesis, *e.g.* phytoene formation, desaturation and cyclization, within the chloroplasts is disputed. In spinach, phytoene synthase and desaturase activities are present in the envelope fraction,[9] whereas in radish such activities are restricted to the thylakoid fraction.[10]

Preferential labelling of the farnesyl diphosphate (FPP)-derived portion in the biosynthesis of the diterpenoic acid, heteroscyphic acid A, in cultured cells of *Heteroscyphus planus* suggested that diterpenes are biosynthesized from geranylgeranyl diphosphate (GGPP). This occurs *via* the condensation of FPP derived from the exogenously supplied mevalonate (MVA) with endogenous isopentenyl diphosphate (IPP) within chloroplasts in the liverwort.[11,12] Herein, we examine whether the non-equivalent labelling observed in the formation of heteroscyphic acid A takes place in the biosynthesis of the phytyl moiety of chlorophyll a and β-carotene, and the possibility that this type of labelling occurs in the biosynthesis of all compounds formed from GGPP in the chloroplasts of liverworts.

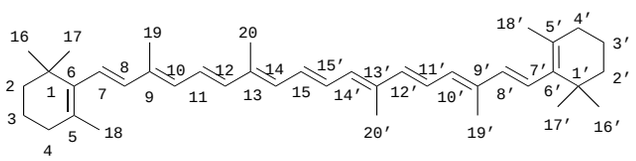


Phytol R = H
Phytyl acetate R = Ac



Heteroscyphic acid A

Chlorophyll a



β-carotene

Results and discussion

Cultures and isolation

Cell cultures of *H. planus* were grown in MSK-medium[13] (8 × 75 ml), to which were fed 1.0 mmol of potassium MVA or

Table 1 ^{13}C Enrichments of carbons in β-carotene incorporating ^{13}C-labelled mevalonates and [2-^{13}C]glycine

Carbon	δ_C in β-Carotene	^{13}C Enrichment (atom% excess)[a] [2-^{13}C]MVA	[4,5-$^{13}C_2$]MVA (J_{CC}/Hz)	[2-^{13}C]glycine (atom% of doublets)
C-1,1′	34. 27			1.98 (0.93)[c]
C-2,2′	39.62		5.83 (35.4)	0.70 (0.31)
C-3,3′	19.25			0.80 (0.38)
C-4,4′	33.10	4.65		N.d.
C-5,5′	129.38			3.00 (1.42)
C-6,6′	137.90		2.60 (57.4)	0.93 (0.41)
C-7,7′	126.60			0.83 (0.39)
C-8,8′	137.75	3.96		N.d.
C-9,9′	136.46			1.31 (0.62)
C-10,10′	130.82		4.58 (58.5)	1.08 (0.48)
C-11,11′	125.01			0.67 (0.32)
C-12,12′	137.22			N.d.
C-13,13′	136.01			1.73 (0.82)
C-14,14′	132.42			0.98 (0.44)
C-15,15′	129.97			0.74 (0.35)
C-16,16′	28.97[b]	3.66		N.d.
C-17,17′				N.d.
C-18,18′	21.76			1.50 (0.66)
C-19,19′	12.80			1.23 (0.55)
C-20,20′	12.80			2.94 (1.31)
Average		4.09	4.34	1.36 (0.63)[d]

[a] ^{13}C Enrichments in β-carotene were determined with mevalonate and glycine, respectively, as substrates. [b] ^{13}C Peaks of C-16,16′ and C-17,17′ were not separable. [c] Coupling constants in β-carotene incorporating [^{13}C]glycine, $J_{^{13}C,^{13}C}$/Hz: C-1(1′)–C-17(17′) 35.4, C-2(2′)–C-3(3′) 33.0, C-5(5′)–C-18(18′) 43.9, C-6(6′)–C-7(7′) 56.1, C-9(9′)–C-19(19′) 42.7, C-10(10′)–C-11(11′) 59.8, C-13(13′)–C-20(20′) 42.8, C-14(14′)–C-15(15′) 59.8. [d] Average value except for C-4,4′, C-8,8′, C-12,12′, C-16,16′, C-17,17′. N.d. = not determined.

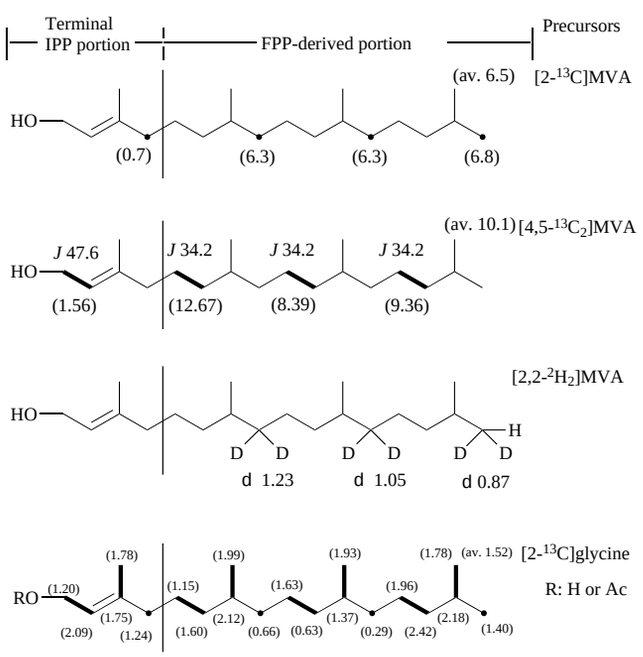


Fig. 1 Labelling pattern of the phytyl side-chain of chlorophyll a incorporating [2-^{13}C]-, [4,5-$^{13}C_2$]-, [2,2-$^{2}H_2$]-MVA and [2-^{13}C]glycine

1.0 mmol of [2-^{13}C]glycine under continuous light at 25 °C. [2,2-$^{2}H_2$]MVA was also fed to cell cultures of *Lophocolea heterophyll*.[14] Chlorophyll a and β-carotene were isolated from 21-day-old cultures by the modified method reported previously,[15] and chlorophyll a was hydrolysed by 16.7% aq. Cs_2CO_3 to afford phytol. ^{2}H{^{1}H} And ^{13}C{^{1}H} NMR spectra of biosynthetically ^{2}H- or ^{13}C-labelled chlorophyll a, phytol and β-carotene were recorded at 41.3 MHz and 67.8 MHz, respectively. Assignments of all ^{13}C atoms in phytol,[16,17] the carboxymethyl carbon[18] in chlorophyll a, and ^{1}H and ^{13}C atoms in β-carotene[19,20] were as reported previously.

MVA labelling patterns of chlorophyll a

Previously,[21] we demonstrated that exogenous MVA was predominantly incorporated into the FPP-derived portion of the phytyl moiety in chlorophyll a, while the IPP-derived portion was slightly labelled. We also reported that, as expected,[18] exogenous MVAs were not incorporated into the chlorophyllide moiety. ^{13}C Enrichment in the phytyl side-chain was determined by comparing the relative intensities of the ^{13}C-enriched carbons to the non-labelled carbon in the labelled compounds, with those of the corresponding carbons in the non-labelled compound. The ^{13}C{^{1}H} NMR spectrum of phytol incorporating [2-^{13}C]MVA showed that three ^{13}C signals corresponding to C-8, C-12 and C-16 were intensely enhanced with ^{13}C atoms (an average ^{13}C enrichment; 6.5 atom% excess, see Fig. 1), while the enrichment factor for C-4 was much less (0.7 atom%). With [4,5-$^{13}C_2$]MVA as precursor, the ^{13}C{^{1}H} NMR spectrum of phytol showed three pairs of intense ^{13}C–^{13}C coupled resonances between C-5 and C-6 (the relative peak intensity of ^{13}C–^{13}C resonance to the natural abundance peak was 12.7, $J_{C\text{-}5,C\text{-}6}$ 34.2 Hz), between C-9 and C-10 8.4 (34.2 Hz) and between C-13 and C-14 9.4 (37.8 Hz) with one weak pair between C-1 and C-2 (1.6; 47.6 Hz). These findings indeed indicate that the FPP-derived portion of the phytyl side-chain was preferentially labelled with exogenously supplied MVA. This preferential labelling was also confirmed by ^{2}H{^{1}H} NMR spectroscopy of chlorophyll a and phytol incorporating [2,2-$^{2}H_2$]MVA in cell cultures of both *H. planus* and *L. heterophylla*, which showed three broad singlets, at δ 1.23 in phytol (δ 1.17 in chlorophyll a), 1.05 (0.99) and 0.87 (0.81), corresponding to phytol labelled with ^{2}H at C-8, C-12 and C-16, but no enhanced peak in phytol at δ 2.00 corresponding to ^{2}H at C-4 (assignments of ^{1}H atoms in phytol were achieved by ^{1}H–^{13}C 2D heteronuclear chemical shift correlation NMR analysis). This also suggested that this type of non-equivalent labelling may take place in a wider range of liverworts.

MVA labelling pattern of β-carotene

The ^{13}C{^{1}H} NMR spectrum of β-carotene incorporating [2-^{13}C]MVA showed that three ^{13}C signals corresponding to the pairs C-4 and C-4′, C-8 and C-8′, and C-16 and C-16′ (carbon numbering; see formulae) were intensely enhanced with ^{13}C atoms (av. 4.1 atom% excess, see Table 1 and Fig. 2), while ^{13}C enrichment for C-12 and C-12′ was not observed. With [4,5-

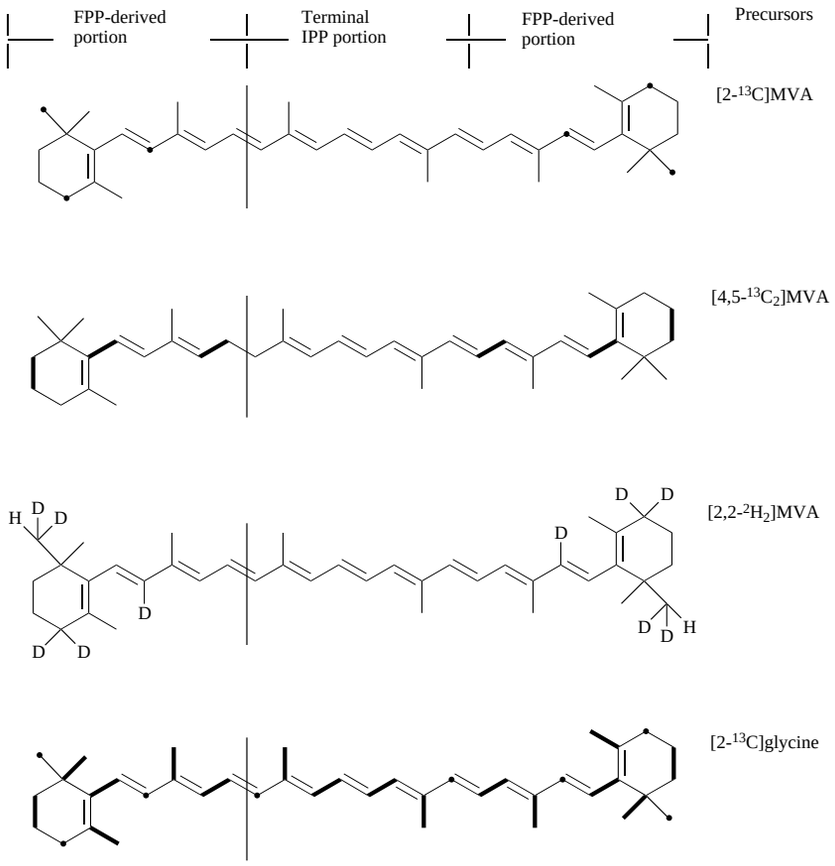


Fig. 2 Labelling pattern of β-carotene incorporating [2-^{13}C]-, [4,5-$^{13}C_2$]-, [2,2-$^{2}H_2$]-MVA and [2-^{13}C]glycine

Table 2 ^{13}C Enrichments in the phytyl side chain of chlorophyll a, β-carotene and heteroscyphic acid A derived from ^{13}C-labelled mevalonate (1.0 mmol) in the cultured cells of *H. planus*

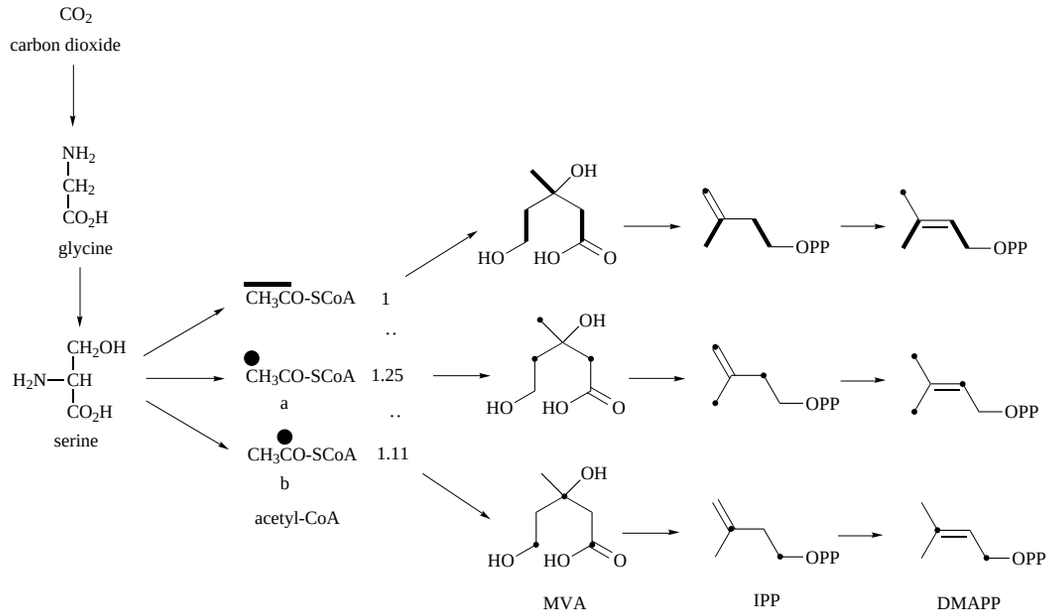
	^{13}C Enrichments (atom% excess)[a]		
Mevalonate	Phytyl side chain	β-Carotene	HAA[b]
[2-^{13}C]-	6.5	4.09	0.9
[4,5-$^{13}C_2$]-	10.1	4.34	0.3[c]

[a] Average ^{13}C enrichments in the FPP-derived portion. [b] See refs. 11 and 12. [c] ^{13}C Enrichment was estimated on the basis of peak area to natural abundant peak (2.08).

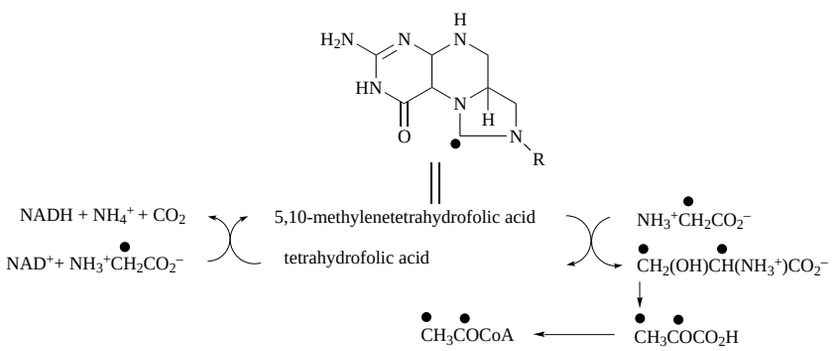
$^{13}C_2$]MVA as precursor, three pairs of intense ^{13}C–^{13}C-coupled resonances between C-2 and C-3 (and between C-2′ and C-3′, 5.9 atom% excess, *J* 35.4 Hz), between C-6 and C-7 (C-6′ and -7′, 2.6 atom% excess, 57.4 Hz) and between C-10 and C-11 (C-10′ and C-11′, 4.6 atom% excess, 58.5 Hz) were observed, but ^{13}C–^{13}C-coupled resonance between C-14 and C-15 (C-14′ and C-15′) was not observed. In addition, ^{2}H{^{1}H} NMR analysis of β-carotene incorporating [2,2-$^{2}H_2$]MVA in cell cultures of *H. planus* showed three broad singlets, at δ 1.02 (two $^{2}H_2$-16s and two $^{2}H_2$-16′s), 1.99 (two ^{2}H-4s and two ^{2}H-4′s) and 6.20 (one H-8 and one H-8′). The relative peak intensity of ^{2}H peaks at δ 1.02, 1.99 and 6.20 was ~2:2:1, suggesting that hydrogens attached to C-12 or C-12′ were not deuteriated. These findings indeed indicate that the FPP-derived portion of β-carotene was also preferentially labelled with exogenously supplied MVA as observed in the biosynthesis of chlorophyll a. Table 2 summarizes the level of ^{13}C enrichments in the phytyl side-chain of chlorophyll a, β-carotene and heteroscyphic acid A, the main diterpene in *H. planus*,[22] which were biosynthesized from the labelled MVAs in cultured cells of *H. planus*. The Table conclusively indicates that the biosynthesis of chlorophyll a and β-carotene can utilize exogenous MVA much more efficiently than that of heteroscyphic acid A (0.9 atom% excess), suggesting that heteroscyphic acid A may be formed at a different site, such as an envelope in the chloroplasts.

Glycine-labelling patterns of chlorophyll a and β-carotene

The ^{13}C{^{1}H} NMR spectrum of chlorophyll a conducted in the presence of [2-^{13}C]glycine indicated an increase in the ^{13}C signal at δ_C 51.5, corresponding to the ester Me at C-13 (4.4 atom% excess), but little or no enrichment in the chlorophyll macrocycles. This methylation process at C-13 has been previously shown to involve *S*-adenosylmethionine[23] in which the methyl group originates from the C-2 carbon of glycine.[18] The ^{13}C{^{1}H} NMR spectrum of the acetylated phytol showed 16 ^{13}C-enriched peaks with doublets due to ^{13}C–^{13}C couplings (C-1–C-2, C-3–C-20, C-5–C-6, C-7–C-19, C-9–C-10, C-11–C-18, C-13–C-14 and C-15–C-17, see Fig. 1) and four intense singlet peaks, without a doublet, at δ_C 39.9 (C-4), 37.3 (C-8), 37.4 (C-12) and 22.7 (C-16). The ^{13}C–^{13}C-coupled resonances indicate that doubly labelled acetyl-CoA, an obligatory intermediate in chloroplast terpene biosynthesis, was formed from two C-2 carbons of exogenous [2-^{13}C]glycine together with a singly labelled species (see Scheme 1) *via* known metabolic routes for formation of acetyl-CoA from a carbon dioxide-fixation pathway in chloroplasts,[24,25] and that it was further converted into MVA as indicated in Scheme 1. The intense singlet peaks arise from breaking of the C-1–C-2 bond in the resulting MVA during the conversion of MVA into IPP. The intensities of ^{13}C-enriched peaks with doublets (an average ^{13}C enrichment of 0.79 atom% excess), the centre peaks of carbons originating from methyl carbons in acetyl-CoA (C-2, C-6, C-10, C-14, C-17, C-18, C-19 and C-20 in phytol: 1.01 atom% excess), those originating from carboxy carbons in acetyl-CoA (C-1, C-3, C-5, C-7, C-9, C-11, C-13 and C-15: 0.88 atom% excess) and intense singlet peaks without doublets indicate that all carbon atoms of the



Scheme 1 Biosynthesis of isopentenyl diphosphate and dimethylallyl diphosphate from [2-^{13}C]glycine



Scheme 2 Biosynthesis of doubly labelled acetyl-CoA from [2-^{13}C]glycine (● represents ^{13}C atom)

phytyl moiety were equivalently labelled with a methylene carbon of glycine (an average ^{13}C enrichment: 1.52 atom% excess) and that the doubly labelled acetyl-CoA was formed with singly labelled acetyl-CoAs (a and b) in the proportions 1:1.28:1.11. Interestingly, observed ^{13}C enrichment (0.79 atom% excess) of doubly labelled acetyl-CoA was much higher (× 12.3) than that estimated on the basis of the natural abundance of ^{13}C and the average ^{13}C enrichment, *i.e.* ^{13}C enrichment (%) = {1.01 × 10^{-2} (natural abundance) + 1.52 × 10^{-2} (the average ^{13}C enrichment)}^{2} × 100 (%) = 2.53^2 × 10^{-2} (%) = 0.064(%), indicating that the label of [2-^{13}C]glycine preferentially gets into both positions of the acetyl-CoA in the same molecule rather than into single positions. The implication must be that degradation of glycine with tetrahydrofolic acid to form 5,10-methylenetetrahydrofolic acid and the formation of serine, which is further transformed into acetyl-CoA *via* pyruvic acid, from a second glycine and 5,10-methylenetetrahydrofolic acid must be almost coupled as indicated in Scheme 2 so that both atoms of acetyl-CoA are labelled.

Since it is difficult to convert a small quantity of β-carotene into adequate derivatives (such as an acetate in the case of phytol) for determining the extent of ^{13}C labelling in β-carotene, the ^{13}C enrichment of peaks with doublets due to ^{13}C–^{13}C coupling was tentatively determined by ^{13}C NMR analysis. This was done using the peak areas of the intense centre peaks (PA*a*, see Fig. 3), the ^{13}C–^{13}C coupling peaks (PA*b* + PA*c*) and the relative amounts of the singly labelled acetyl-CoA to the doubly labelled species (RA*s*/*d*: 1.11 for carbons derived from the carbonyl carbon of acetyl-CoA and 1.28 for those from the methyl carbon), which were determined on the basis of ^{13}C enrichments of phytol incorporating [2-^{13}C]glycine as mentioned above. The ^{13}C enrichments were estimated, equation (1), where 1.08 is the natural abundance of ^{13}C and PA_{nat}

$$\text{Enrichment} = 1.08 \times \{(PAa - PA_{nat}) + (PAb + PAc)\}/PA_{nat} \quad (1)$$

is the peak area due to the natural abundance of ^{13}C [given by equation (2)]. Thus the ^{13}C enrichment (%) is given by equation

$$PA_{nat} = PAa - RAs/d(PAb + PAc) \quad (2)$$

(3). The ^{13}C enrichments from doubly labelled acetyl-CoA were estimated using equation (4).

$$\text{Enrichment} = 1.08 + (1 + RAs/d)(PAb + PAc)/\{PAa - RAs/d\,(PAb + PAc)\} \quad (3)$$

$$^{13}\text{C enrichment of doublet (atom\% excess)} = 1.08 \times (PAb + PAc)/\{PAa - RAs/d\,(PAb + PAc)\} \quad (4)$$

For example, in the case of C-1 and C-1′, which originate from the carbonyl carbon (RA*s*/*d* = 1.11) of acetyl-CoA, PA*a*, PA*b* and PA*c* were estimated to be 13.60, 2.62 and 3.40. Thus the total ^{13}C enrichment and the ^{13}C enrichment of the doublet

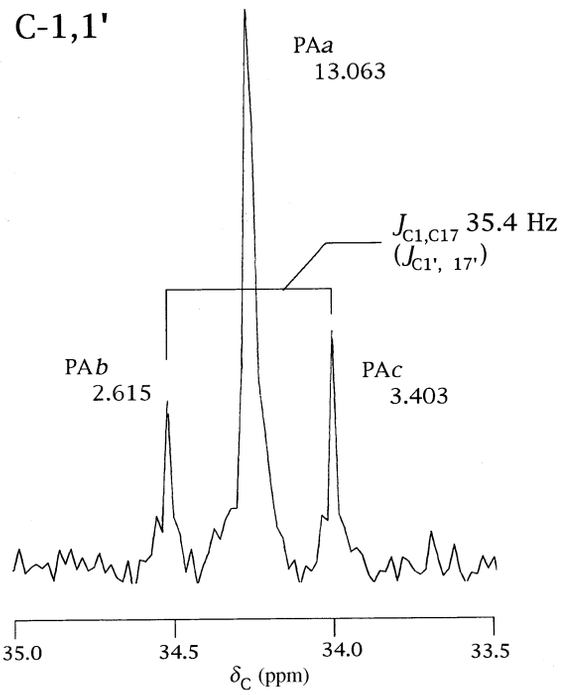


Fig. 3 Segment of ^{13}C NMR peaks (C-1 and C-1′) of β-carotene incorporating [2-^{13}C]glycine

in C-1 and C-1′ were calculated to be 1.98 and 0.93 atom%, respectively. Using such a method, all ^{13}C enrichments of carbons with doublets were calculated as shown in Table 1, while ^{13}C enrichments of singlets due to C-4 and C-4′, C-8 and C-8′, C-12 and C-12′ and C-16 and C-16′ could not be determined in this manner. All carbon atoms of β-carotene were equivalently labelled with a methylene carbon of glycine at almost an identical level (an average ^{13}C enrichment: 1.40 atom% excess) to those observed in the formation of the phytyl moiety of chlorophyll a. Thus it is clear that the endogenously formed MVA *via* glycine is equivalently incorporated into both the phytyl moiety and β-carotene.

The biosynthesis of lower terpenes from exogenous ^{14}C-labelled MVA in tissues from vascular plants yielded preferentially labelled terpenes in the isopentenyl diphosphate (IPP)-derived portion of molecules, suggesting that preferential labelling results from the condensation of IPP derived from exogenous ^{14}C-labelled MVA with dimethylallyl diphosphate (DMAPP) that is mainly present in a metabolic pool.[26] However, the present study shows that diterpenes, carotenoids and the phytyl moiety of chlorophyll, all of which are formed from GGPP in the chloroplast and related plastids, are preferentially labelled in the FPP-derived portion of molecules in liverworts. The observed non-equivalent labelling may be rationalized by two different explanations; GGPP is synthesized by the condensation of FPP formed at a certain subfraction(s) within the chloroplast, where exogenous MVA is easily accessible and rapidly converted into FPP (see A in Scheme 3) or is formed extraplastidally (B) with endogenous IPP in a metabolic pool. The equivalent incorporation of glycine into carotenoids and the phytyl moiety suggests that FPP arises from two different sources, *i.e.* from exogenously supplied mevalonate and from photosynthetically fixed carbon dioxide. Additionally, effective utilization of exogenous MVA in the biosynthesis of both the phytyl side-chain and β-carotene, but not of normal diterpenes, suggests that the site(s), *e.g.* thylakoid, of GGPP synthesis for these pigments is different from those for normal diterpenes, *e.g.* envelope. Studies with isolated chloroplasts and isolated photosynthetic membrane from liverworts may help us understand the regulation of synthesis of chlorophylls and carotenoids, the assembly of pigment–protein complexes and the molecular mechanisms of photosynthesis.

Experimental

General procedure and materials

All $^{13}C\{^1H\}$ NMR spectra of chlorophyll a, phytol, phytyl acetate and β-carotene incorporating ^{13}C labelled precursors were recorded on a JEOL EX-270 NMR spectrometer with $^{13}CDCl_3$ (0.40 ml) as internal standard (δ_C 77.0) using the JOEL experimental mode (BCM) with a spectral frequency of 67.80 MHz; spectral width of 20 kHz; observation frequency offset 135.0 kHz; observation frequency fine offset 5200 Hz; number of data points 32 768; acquisition time 0.819 s; pulse delay 2.181 s; pulse width 7.3 μs (90°); exponential broadening factor 1.22 Hz; number of scans 12 639–40 000 (the signal-to-noise ratios of the most intense signals of the biosynthetically ^{13}C-labelled compounds and non-labelled compound were ~30–40) and numbers of measurements ≥2. $^2H\{^1H\}$ NMR Spectra (41.3 MHz) were recorded for $CHCl_3$–CCl_4 (1:4) solutions with C^2HCl_3 (δ_H 7.26) as internal standard using the JOEL experimental mode (NON) with the following parameters: spectral width 10 kHz; observation frequency offset 127.0 kHz; observation frequency fine offset 5300 Hz; number of data points 16 384; acquisition time 0.819 s; pulse delay 3.969 s; pulse width 9.8 μs (90°); exponential broadening factor 0.16 Hz; number of scans 9426–16 384 and number of measurements ⩾2. [2-^{13}C]-(90 atom%), [4,5-$^{13}C_2$]- (99%) and [2,2-$^2H_2$]-MVA (99.3%) were prepared by the methods previously reported.[27] [2-^{13}C]Glycine (99.2%) was commercially available.

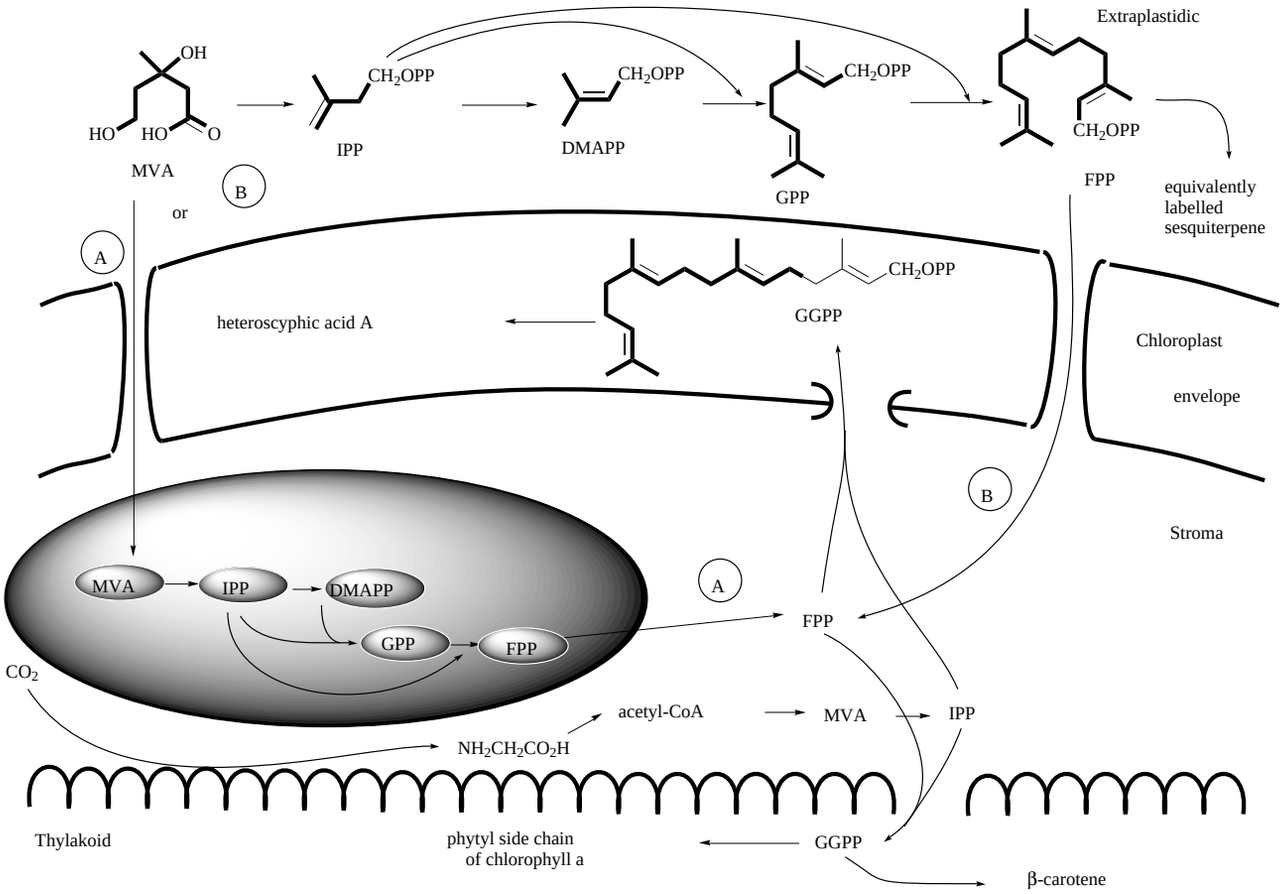
Feeding experiment

The origin of *H. planus* and *L. heterophylla*, medium and culture conditions for suspension culture have been described previously.[27] Four or eight cultures, each 2.7 g on average (fresh weight) in 75 ml MSK-4 medium were incubated with 1 mmol (130–132 mg) of isotopically labelled MVA or 1.0 mmol [2-^{13}C]glycine (75 mg). The liquid suspensions were agitated at 110 rpm at 25 ± 1 °C under continuous light of 5000 lux. The cells were harvested 21 days after inoculation.

Isolation of isotopically labelled chlorophyll a and β-carotene

Chlorophyll a was isolated according to the procedure of ref. 15. The freshly harvested cells were immersed in 5 vol. of acetone (v/w) at −30 °C and the suspension was ground for 30 min and then filtered. The cell debris was washed with 80% aq. acetone and combined with the filtrate. After one-seventh vol. of 1,4-dioxane (v/v) had been added to the acetone solution, distilled water (2.5 vol. to 1,4-dioxane) was added and the mixture was stored at −30 °C for 12 h to give a precipitate of a mixture of chlorophylls and β-carotene. The solution was filtered to obtain the crude precipitate, which was then extracted with diethyl ether. The diethyl ether solution was concentrated *in vacuo* and the residue was redissolved in acetone. The acetone solution was applied to a DEAE-Sepharose CL-6B column (10 g) and successively eluted with acetone (5 vol. of column) and then 40 ml of acetone–MeOH (10:3, v/v). β-Carotene was eluted in acetone, while chlorophylls a and b were contained in the acetone–MeOH eluate. The acetone–MeOH eluate was concentrated and the residue was chromatographed on a Sepharose CL-6B column (10 g), eluting with 48 ml of hexane–propan-2-ol (20:1) and then 45 ml of hexane–propan-2-ol (10:1). Chlorophyll a was eluted in the hexane–propan-2-ol (20:1), while chlorophyll b was eluted in the hexane–propan-2-ol (10:1) fraction. The hexane–propan-2-ol (20:1) eluate was concentrated to dryness to afford pure chlorophyll a [2.1–2.9 mg from 27–36 g of cultured cells (fresh weight)].

The acetone eluates containing β-carotene were further purified by chromatography on a silica gel column (5 g), eluting with



Scheme 3 The preferential labelling of the farnesyl diphosphate-derived portion in biosyntheses of the phytyl side-chain of chlorophyll a, β-carotene and heteroscyphic acid A. The shaded portion is a presumed site where exogenous MVA is easily accessible and rapidly converted into farnesyl diphosphate.

hexane–chloroform (9:1) and then on a silica gel column (5 g) with hexane–benzene (17:3) to give 0.3–0.6 mg of pure β-carotene from 27–48 g of cultured cells. Alternatively, β-carotene was isolated by the following procedure: cultured cells (28–48 g) were suspended in 200 ml of MeOH, ground for 1 min, diluted with 200 ml of MeOH and stored at −30 °C for 12 h. The suspension was filtered and then the residue was further extracted with 400 ml of MeOH. The MeOH extracts were combined and concentrated to 50 ml. To the concentrated MeOH solution was added 60% aq. KOH and the mixture was stirred for 3 h. The hydrolysate was extracted with 200 ml of diethyl ether. The ethereal extracts were concentrated and the residue was chromatographed on a silica gel column (5 g) eluting with hexane–chloroform (9:1) and then on a silica gel column (5 g) with hexane–benzene (17:3) to afford 4.0–4.5 mg of purified β-carotene.

Hydrolysis of chlorophyll a

The biosynthetically labelled chlorophyll a (2.1 to 2.9 mg) was dissolved in 20 ml of MeOH and 40 μl of 0.61 mmol aq. Cs_2CO_3 was added. The solution was stirred at ambient temperature for 2 h in the dark and then 100 μl of aq. Cs_2CO_3 was added. After further stirring for 1 h, the reaction solution was extracted with 50 ml of pentane (× 3). The pentane extracts were dried over dry Na_2SO_4, filtered and concentrated *in vacuo*. The residue was chromatographed on a silica gel column (10 g) eluting with hexane–ethyl acetate (7:3) to give pure phytol quantitatively.

Acetylation of phytol incorporating [2-^{13}C]glycine

Phytol incorporating [2-^{13}C]glycine was acetylated in the usual manner. Acetic anhydride and pyridine were removed by evaporation *in vacuo* at 60 °C to give the acetate.

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