Biosynthesis of phytyl side-chain of chlorophyll *a*: apparent reutilization of carbon dioxide evolved during acetate assimilation in biosynthesis of chloroplastidic isoprenoid

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Equal incorporation of either acetyl methyl or carboxy carbons into all carbon atoms of the phytyl side-chain of chlorophyll *via* doubly labelled acetyl CoA, with complete loss of methyl hydrogens, indicated that CO_2 evolved from the carboxy carbon during acetate assimilation through the TCA cycle may be reutilized *via* the reductive pentose phosphate cycle followed by the glycolic pathway, while incorporation of [2-^{13}C]glycerol and [6,6-$^{2}H_2$]glucose into the phytyl chain demonstrated the simultaneous operation of two distinct pathways, both the mevalonate pathway and the 1-deoxy-D-xylulose mediating pathways (non-mevalonate pathway) in isopentenyl diphosphate formation within liverwort chloroplasts.

The labelling pattern of the phytyl side-chain of chlorophyll was determined by incorporation studies using ^{2}H- or ^{13}C-labelled acetates, glycerol and glucose into cultured cells of liverwort, *Heteroscyphus planus*. The most unexpected feature of the labelling pattern was that either acetyl methyl or carboxy carbons were equally incorporated into all carbon atoms of the phytyl side-chain *via* doubly labelled acetyl CoA with complete loss of methyl hydrogens. The labelling pattern of the phytyl side-chain incorporating [2-^{13}C]glycerol and [6,6-$^{2}H_2$]glucose together with ^{2}H- and ^{13}C-labelled mevalonate (MVA)[1,2] revealed the simultaneous operation of two distinct pathways, the classical acetate/MVA (mevalonate) pathway[3] and the novel 1-deoxy-D-xylulose (non-mevalonate) pathway,[4] in isopentenyl diphosphate (IPP) formation within liverwort chloroplasts.

Cell cultures of *Heteroscyphus planus* were grown in MSK-4 medium[5] (6–8 × 75 ml), and fed with sodium [1-^{13}C]-, [2-^{13}C]-, [1,2-$^{13}C_2$]-, [2,2,2-$^{2}H_3$, 1-^{13}C]- and [2,2,2-$^{2}H_3$, 2-^{13}C]-acetate, (> 99 atom%, 0.5 mmol), [2-^{13}C]glycerol (60 atom%, 0.5 mmol in 1 ml of 10% aq. EtOH) and [6,6-$^{2}H_2$]glucose (20 atom%, 11.1 mmol) under continuous light at 25 °C.[6] Chlorophyll *a* was isolated and hydrolyzed, and the isolated phytol was acetylated by the procedure reported previously.

The ^{13}C enrichment [sixteen ^{13}C-enriched peaks with doublets due to ^{13}C–^{13}C coupling (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) and four intense singlet peaks (C-4, C-8, C-12 and C-16) as shown in Fig. 1 and Table 1] in the phytols incorporating [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]-acetates was identical, indicating that the doubly ^{13}C-labeled acetyl CoA was formed from either two carboxy carbons of [1-^{13}C]acetate or two methyl carbons of [2-^{13}C]acetate. Transposition of ^{13}C label between the C-4 and C-5 carbons in IPP was also observed. The average intensity of the ^{13}C–^{13}C coupled peaks relative to the intense center peak in phytol incorporating [1,2-$^{13}C_2$]acetate (observed

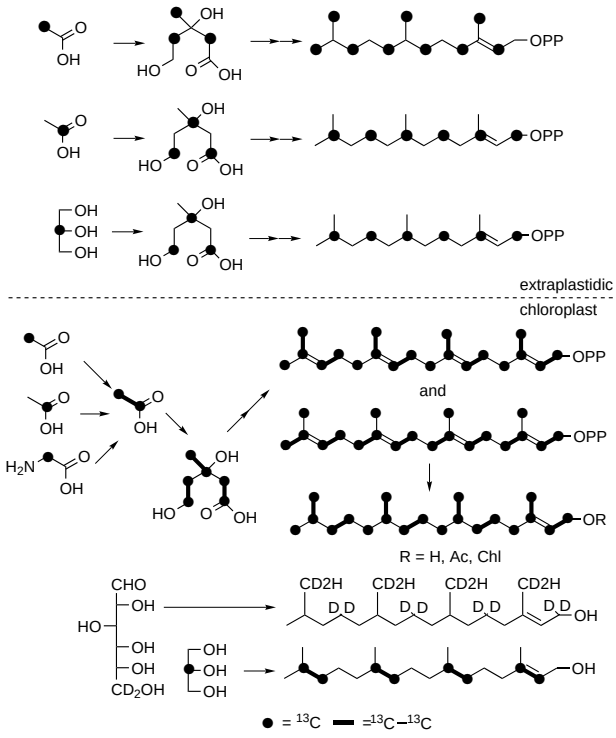

Fig. 1 Labelling patterns of the phytyl side-chain of chlorophyll *a* and sesquiterpenes incorporating ^{13}C labelled acetates, [2-^{13}C]glycerol and [6,6-$^{2}H_2$]glucose in cultured cells of *H. planus*

Table 1 ^{13}C enrichment of phytyl side-chain of chlorophyll *a* incorporating ^{13}C-labelled acetates

	Enrichment (atom% excess)		
Carbon	[1-^{13}C]acetic acid	[2-^{13}C]acetic acid	[1,2-^{13}C]acetic acid[a]
C-1	1.87	1.18	0.34
C-2	1.61	0.83	0.69
C-3	1.47	0.77	0.44
C-4	3.14	1.94	1.31
C-5	1.94	1.10	0.53
C-6	1.64	1.67	0.67
C-7	2.94	2.15	1.32
C-8	2.77	2.37	0.85
C-9	1.87	1.58	0.67
C-10	1.74	1.45	0.20
C-11	1.80	1.50	0.66
C-12	2.40	1.84	0.41
C-13	1.89	1.39	0.77
C-14	2.91	2.83	1.09
C-15	2.93	2.45	1.45
C-16	3.18	3.30	1.80
C-17	2.41	2.52	1.03
C-18	2.45	2.96	1.32
C-19	3.12	2.96	1.36
C-20	1.49	1.14	0.59
Average	2.28	1.90	0.89

[a] $J_{^{13}C-^{13}C}$/Hz: C-1–C-2, C-3–C-20, C-5–C-6, C-7–C-19, C-9–C-19, C-11–C-18, C-13–C-14, C-15–C-17 have been previously reported. C-3–C-4 = 34.2, C-7–C-8 = 34.2, C-11–C-12 = 35.4 and C-15–C-16 = 35.4.

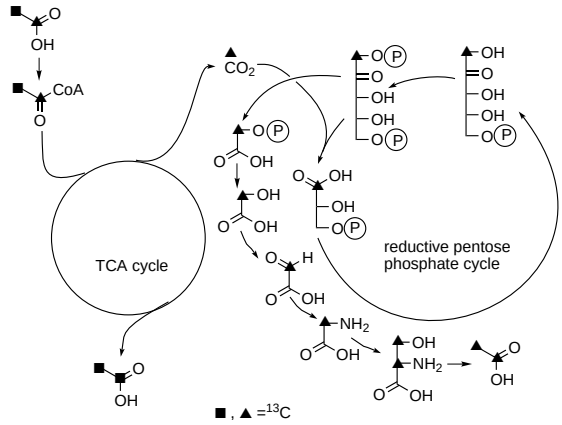

Fig. 2 Pathway for [1,2-$^{13}C_2$]acetate from [1-^{13}C]acetate *via* the TCA cycle, the reductive pentose phophate cycle and the glycollate pathway

relative intensity: 0.185) was much lower (× 4.5) than that estimated (0.82) on the basis of the natural abundance of ^{13}C (1.08%) and ^{13}C enrichment (0.89 atom% excess) indicating that reformed acetate, rather than intact acetate, was incorporated into MVA. The α-^{2}H and β-^{2}H isotopic peaks[6] were not observed in phytol incorporating [2,2,2-$^2H_3$, 2-^{13}C]- and [2,2,2-$^2H_3$, 1-^{13}C]-acetates, respectively, indicating the complete loss of ^{2}H of the acetate during formation of [1,2-$^{13}C_2$]acetate.

The pathway leading to two contiguously labelled acetate (or three contiguously labelled propionate) molecules from [2-^{13}C]acetate, which was detected in a rare actinomycetes, *Actinomadura azurea*,[7] and cell cultures of the vascular plants *Zea mays*[8] and *Morus alba*[9] was reasonably explained by the participation of the tricarboxylic acid cycle (TCA cycle). Formation of doubly labeled acetate from [1-^{13}C]acetate, however, has not yet been reported. The results of this as well as earlier studies in which we examined the incorporation of [2-^{13}C]glycine into the phytyl moiety of chlorophyll *a*[1,2] suggest that the ^{13}C-labelled carbon dioxide evolved during [1-^{13}C]acetate assimilation through the TCA cycle is reutilized *via* the reductive pentose phosphate cycle. Carbon-13 labelled CO_2 was incorporated into ribulose 1,5-bisphosphate at C-1 by the mechanism shown in Fig. 2. The ^{13}C label at C-1 in ribulose 1,5-bisphosphate was further translocated into the methylene carbon (C-2) of glycine *via* the glycolic pathway. The doubly labelled acetyl-CoA was formed from two C-2 carbons of the endogenously formed [2-^{13}C]glycine *via* the glycolic pathway. There is indirect evidence that reassimilation of CO_2 liberated during acetate photoassimilation could be reutilized in glycolate production in *Chlorella*[10] other eukaryotic algae and vascular plants.[11]

Label was detected at C-2, C-3, C-6, C-7, C-10, C-11, C-14 and C-15 of the phytyl side-chain incorporating [2-^{13}C]glycerol, all of which showed $J_{^{13}C-^{13}C}$ coupling ($J_{2,3}$ = 73.2, $J_{6,7}$ = 34.8, $J_{10,11}$ = 34.8 and $J_{14,15}$ = 34.2 Hz). When [6,6-$^2H_2$]glucose was added, the label was incorporated into C-1 (δ_D 4.15) and C-20 (δ_D 1.66) together with three methylene carbons (C-5, C-9 and C-13, δ_D 1.35–1.55, unresolved) and three methyl carbons (C-17, C-18, C-19, δ_D 0.8–0.9, unresolved). The labelling pattern indeed confirmed the operation of the non-mevalonate pathway in biosynthesis of the phytyl side-chain. We found that the phytyl side-chain was also formed from MVA.[1,2] Thus, it is suggested that biosynthesis of all compounds derived from geranylgeranyl diphosphate within liverwort chloroplasts proceeds *via* both the classical mevalonate pathway[1,2,12] and the novel non-mevalonate pathway. The simultaneous operation of the mevalonate and the non-mevalonate pathways has also been detected in microorganisms without organelles.[13]The labelling pattern of β-barbatene, a predominant sesquiterpene hydrocarbon in *H. planus*,[14] incorporating labelled glycerol and glucose revealed that biosynthesis of the cytoplasmic terpenoids proceeds *via* the mevalonate pathway but not *via* the non-mevalonate pathway.

We present here evidence that CO_2 evolved from acetate assimilation is reutilized to biosynthesize isoprenoids in chloroplasts. The enzymes involved in the TCA cycle, the reductive pentose cycle and the glycolic acid pathway are separately localized in mitochondria, chloroplasts and peroxisomes, respectively. Thus reconstruction of acetate in chloroplasts requires consideration of the flux of CO_2 and the intermediates through the TCA cycle in mitochondria.

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