Isoprenoid biosynthesis in higher plants and in *Escherichia coli*: on the branching in the methylerythritol phosphate pathway and the independent biosynthesis of isopentenyl diphosphate and dimethylallyl diphosphate

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In the bacterium *Escherichia coli*, the mevalonic-acid (MVA)-independent 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway is characterized by two branches leading separately to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The signature of this branching is the retention of deuterium in DMAPP and the deuterium loss in IPP after incorporation of 1-[4-^{2}H]deoxy-D-xylulose ([4-^{2}H]DX). Feeding tobacco BY-2 cell-suspension cultures with [4-^{2}H]DX resulted in deuterium retention in the isoprene units derived from DMAPP, as well as from IPP in the plastidial isoprenoids, phytoene and plastoquinone, synthesized via the MEP pathway. This labelling pattern represents direct evidence for the presence of the DMAPP branch of the MEP pathway in a higher plant, and shows that IPP can be synthesized from DMAPP in plant plastids, most probably via a plastidial IPP isomerase.

Key words: 1-deoxy-D-xylulose 5-phosphate, isopentenyl diphosphate (IPP) isomerase, 2-*C*-methyl-D-erythritol 4-phosphate, mevalonic acid, tobacco BY-2 cell.

INTRODUCTION

In plant cells, two pathways are involved in the biosynthesis of isoprenoids: the mevalonic acid (MVA) pathway localized in the cytoplasm, and the [2-*C*-methyl-D-erythritol (ME) 4-phosphate (MEP)] pathway (Scheme 1) that occurs in the plastids [1–4]. Evidence for the presence of the latter, long-overlooked biosynthetic route has been obtained by extensive feeding experiments using mainly ^{13}C-labelled acetate or glucose isotopomers and performed first with bacteria [5–7], and later with algae [8] and higher-plant systems [9–12]. The first five steps leading from pyruvate (**1**) and glyceraldehyde 3-phosphate (**2**) to ME cyclodiphosphate (**7**) are now fairly well documented ([13,14], and references cited therein). They constitute a linear sequence involving [1-deoxy-D-xylulose (DX) 5-phosphate (DXP)] (**3**), MEP (**4**), 4-diphosphocytidyl-ME (**5**), as well as the corresponding 2-phosphate (**6**). Evidence for a branching of the MEP pathway has been found in the bacterium *Escherichia coli*: isopentenyl diphosphate (IPP) (**9**) and dimethylallyl diphosphate (DMAPP) (**10**) are synthesized separately from an unidentified common ME cyclodiphosphate derivative (Scheme 1) [15]. First evidence for such a possible branching was obtained by incorporation of [4-^{2}H]DX [16] or free [3,5,5,5-$^2H_4$]ME [17] into the prenyl chains of ubiquinone and menaquinone from *E. coli*. The isoprene units derived from DMAPP quantitatively retained the deuterium, whereas those derived from IPP were characterized by a complete loss of deuterium. Definitive evidence for the presence of such a branching was obtained using genetic methods [15]. For this purpose, an *E. coli* strain with a disrupted gene of the DX isomero-reductase was engineered for utilizing exogenous MVA supplied to the culture medium by introducing the genes of eukaryotic MVA kinase, phospho-MVA kinase and diphospho-MVA decarboxylase. Such a strain was able to grow either on exogenous ME, which was metabolized via the MEP pathway, or on MVA, which was converted into IPP (**9**) via the three enzymes derived from the introduced genes from the MVA pathway. Growth on MVA required the presence of a functional endogenous IPP isomerase (IDI) in order to convert IPP (**9**) into DMAPP (**10**). In addition, when the *idi* gene coding for IDI was disrupted no growth was possible on MVA, indicating that there was no other means to interconnect IPP (**9**) and DMAPP (**10**) than via the reaction catalysed by IDI. Growth, however, occurred normally on ME, indicating that two distinct branches, starting from a common unknown intermediate, allowed independent biosynthesis of IPP (**9**) and DMAPP (**10**) [15]. In contrast, no evidence for such a branching was obtained in plant cells. Feeding a *Catharanthus roseus* cell-suspension culture with [2-^{13}C,4-^{2}H]DX resulted in equivalent ^{13}C labelling and ^{2}H loss in all isoprene units, whether they were derived from IPP or DMAPP [18].

The last steps of the MEP pathway involve the *gcpE* and *lytB* gene products. Both gene products were shown to be essential and were located on the 'trunk line' of the pathway [19–21]. In *E. coli* strains engineered for utilizing exogenous MVA, growth was only possible after deletion of the *gcpE* or the *lytB* genes in the presence of MVA. After deletion of *lytB*, growth was also restored after complementation with an episomal copy of the gene. In an *E. coli* strain capable of utilizing exogenous MVA, ME cyclodiphosphate (**7**) was accumulated from ME after disruption of the *gcpE* gene, suggesting it was the substrate of the GcpE protein [22]. A crude cell-free system from an *E. coli* strain overexpressing the *gcpE*/*yfgA*/*yfgB* gene clusters converted ME cyclodiphosphate (**7**) into 4-hydroxy-3-methylbut-2-enyl diphosphate (**8**) ([23] and M. Rohmer, unpublished work). The latter compound was also accumulated in a *lytB*-deficient *E. coli* strain capable of utilizing exogenous MVA [24]. It was also

Abbreviations used: DMAPP, dimethylallyl diphosphate; DX, 1-deoxy-D-xylulose; DXP, DX 5-phosphate; FAB, fast atom bombardment; IPP, isopentenyl diphosphate; IDI, IPP isomerase; ME, 2-*C*-methyl-D-erythritol; MEP, ME 4-phosphate; MVA, mevalonic acid; TBY-2, Tobacco cv. Bright Yellow-2; THF, tetrahydrofuran.

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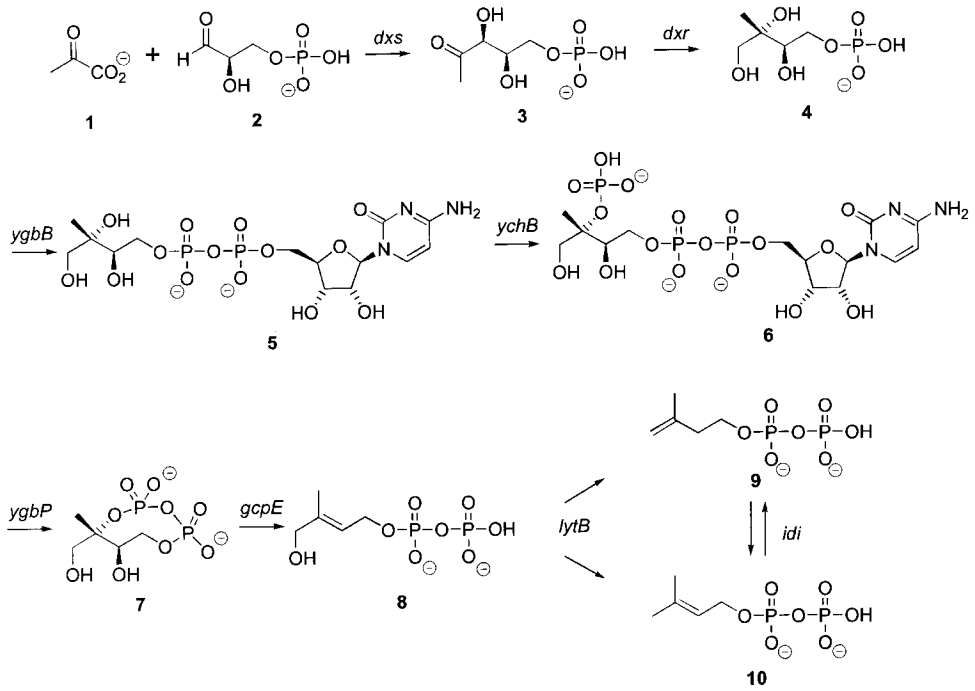


Scheme 1 MEP pathway for isoprenoid biosynthesis

Abbreviations: dxs, DXP synthase; dxr, MEP synthase; ygbB, 4-diphosphocytidyl-ME synthase; ychB, 4-diphosphocytidyl-ME kinase; ygbP, ME-2,4-cyclodiphosphate synthase; gcpE, enzyme catalysing the formation of 4-hydroxy-3-methylbut-2-enyl diphosphate synthase; lytB, enzyme catalysing the formation of IPP and DMAPP.

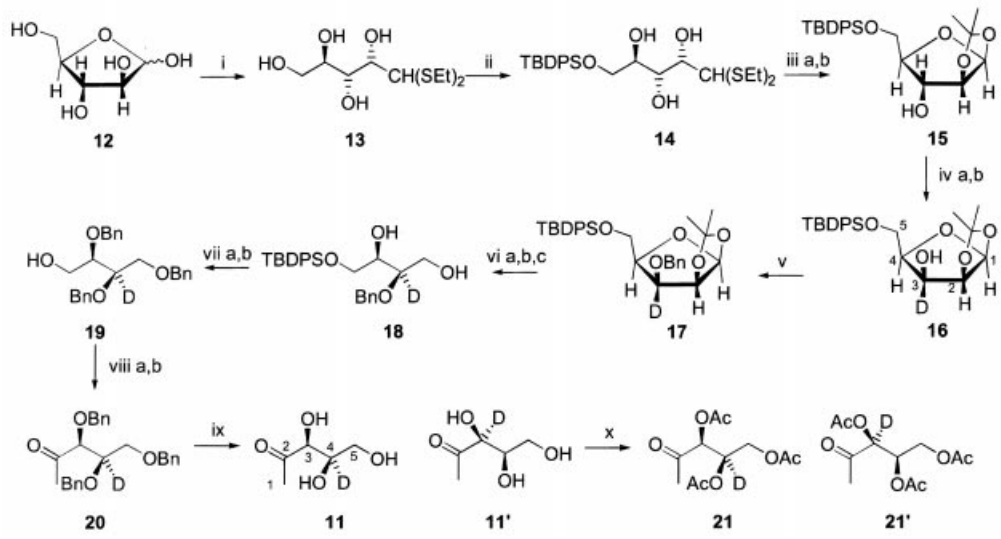
formed *in vitro* from [U-$^{13}C_5$]DX in an *E. coli* strain overexpressing the xylulose kinase gene *xylB*, which encodes an enzyme capable of phosphorylating free DX, as well as *gcpE* and all genes of the MEP pathway downstream of *gcpE* [25]. All these data suggested that ME cyclodiphosphate (**7**) and the diol diphosphate (**8**) are the substrate and the product of the GcpE enzyme respectively. Finally, the last step is apparently catalysed by the protein encoded by *lytB*. *E. coli* cells overexpressing *xylB*, as well as *lytB* and all genes of the MEP pathway downstream of *lytB*, converted [U-$^{13}C_5$]DX into [U-$^{13}C_5$]IPP as well as [U-$^{13}C_5$]DMAPP, suggesting that *lytB* represents the branch point of the MEP pathway [26]. The nature of the cofactors required for the conversion of ME cyclodiphosphate (**7**) into IPP (**9**) and DMAPP (**10**) is still a subject for investigation.

Tobacco (*Nicotiana tabacum* L.) cv. Bright Yellow-2 (TBY-2) cell cultures have proved to be a powerful biological system for investigation of isoprenoid biosynthesis in plants [27]. For instance, feeding experiments with ^{13}C-labelled glucose showed that the prenyl chain of ubiquinone was synthesized from the same IPP and DMAPP pool derived from MVA pathway as the sterols, and confirmed the contribution of the MEP pathway to the biosynthesis and of the prenyl chain of plastoquinone in the plastids (see Figure 1). It was therefore tempting to develop this rapidly growing cell suspension for further investigations on the MEP pathway in higher plants. TBY-2 cells were fed with [4-^{2}H]DX (**11**, Scheme 2) in order to check whether deuterium retention, as a signature of the branching in the MEP pathway, or loss occurred in the isoprene units. While these feeding experiments were in the stage of completion, similar incorporation experiments using [4-^{2}H]DX were being performed with eucalyptus (*Eucalyptus globulus*) twigs [28]. That study indicated a very low, but significant, deuterium incorporation in the monoterpene cineol on the carbon atom derived from C-2 of DMAPP, suggesting the presence of such a branching in the MEP pathway in a plant.

EXPERIMENTAL

General methods and materials

All non-aqueous reactions were run in dry solvents under an argon atmosphere. 'Dried and concentrated' refers to removal of residual amounts of water with anhydrous Na_2SO_4 followed by evaporation of solvent on a rotary evaporator. Flash chromatography [29] (Merck silica gel, 40–63 μm) and TLC were performed using the solvent systems indicated. TLC plates were developed by heating after spraying with an ethanolic solution of *p*-anisaldehyde (2.5 %), sulphuric acid (3.5 %) and acetic acid (1.6 %), or with an ethanolic solution of phosphomolybdic acid reagent (10 %, w/v). NMR spectra were recorded: (i) on a Bruker AC200 spectrometer at 200 MHz for ^{1}H-NMR and at 50.32 MHz for ^{13}C-NMR; (ii) on a Bruker WP400 spectrometer at 400 MHz for ^{1}H-NMR and at 100 MHz for ^{13}C-NMR; and (ii) on an ARX500 spectrometer at 500 MHz for ^{1}H-NMR and at 125 MHz for ^{13}C-NMR. NMR experiments were performed in C^2HCl_3, ^2H_2O or $C^2H_3O^2H$ using, as an internal standard, $CHCl_3$ ($\delta = 7.26$ p.p.m.), ^2HHO ($\delta = 4.56$ p.p.m.) and CH_3O^2H ($\delta = 3.30$ p.p.m.) for ^{1}H-NMR, and C^2HCl_3 ($\delta = 77.03$ p.p.m.) and $C^2H_3O^2H$ ($\delta = 49.06$ p.p.m.) for ^{13}C-NMR. ^{2}H-NMR spectra were recorded in chloroform solution ($\delta = 7.26$ p.p.m.) on a Bruker WP400 spectrometer at 61 MHz. Fast atom bombardment (FAB) MS was performed on a ZAB-HF spectrometer with an acceleration potential of 8 keV, using poly(ethylene glycol) and a solution of sodium iodide in glycerol as matrix. Electron-impact MS was performed by direct inlet on



Scheme 2 Synthesis of [4-^{2}H]DX (11)

Reagents for the chemical steps shown were as follows: (**i**) CH_3CHSH/H_2Cl (70%); (**ii**) terbutyldiphenylsilyl chloride (TBDPSCl), imidazole, dimethylformamide, 0 °C (94%); (**iiia**) HgO, $HgCl_2$, acetone; (**iiib**) $CuSO_4$, acetone, H^+ (84%); (**iva**) $(COCl)_2$, DMSO, triethanolamine (TEA), CH_2Cl_2, −78 °C to −35 °C; (**ivb**) NaB^2H_4, ethanol (71%); (**v**) NaH, tetra-*N*-butylammonium iodide (Bu_4NI), 18-crown-6, BnBr, THF (80%); (**via**) 80% OH, H_2O (83%); (**vib**) $NaIO_4$, H_2O, methanol; (**vic**) $NaBH_4$, ethanol (97%); (**viia**) NaH, Bu_4NI, 18-crown-6, BnBr, THF; (**viib**) tetra-*N*-butylammonium fluoride (Bu_4NF), THF (74%); (**viiia**) $(COCl)_2$, DMSO, TEA, THF, −78 °C to −35 °C and CH_3MgCl, −10 °C (98%); (**viiib**) $(COCl)_2$, DMSO, TEA, CH_2Cl_2, −78 °C to −35 °C (91%); (**ix**) H_2, Pd/C, Methanol (98%); (**x**) Ac_2O, pyridine, room temperature, 95%. See the text for identification of structures **11**–**21**′.

a Finnigan-MAT TSQ 700 spectrometer with a 70 eV ionization energy. Products were re-crystallized from an ether/hexane mixture. Melting points were measured on a Reichert Thermovar microscope and are uncorrected.

Synthesis of [4-^{2}H]DX (11, Scheme 2)

D-Arabinose diethyldithioacetal (**13**)

To a solution of D-arabinose (**12**) (50 g, 0.33 mol, 1 equiv.) in 6 M hydrochloric acid (500 ml) was added in a dropwise manner ethyl mercaptan (41.4 g, 067 mol, 2.1 equiv.) over 20 min. After stirring for 2.5 h, the mixture was cooled to 0 °C and filtered. The collected solid was washed with cold water (500 ml) and dried, giving rise to colourless plates (62 g, 70%). The melting-point temperature was measured as 126–128 °C. ^{1}H-NMR (200 MHz, C^2HCl_3): δ (in p.p.m.) = 1.25 (3H, t, J = 7.4 Hz, CH_3), 1.26 (3H, t, J = 7.4 Hz, CH_3), 2.59–2.76 (4H, m); 3.58 (1H, dd, $J_{1a,1b}$ = 10.6 Hz, $J_{1a,2}$ = 5.7 Hz, 1-H_a); 3.65–3.78 (2H, m, 1-H_b, 2-H); 3.82 (1H, dd, $J_{4,5}$ = 9.1 Hz, $J_{3,4}$ = 2.0 Hz, 4-H); 3.95 (1H, dd, $J_{2,3}$ = 8.1 Hz, $J_{3,4}$ = 2.0 Hz, 3-H); 4.03 (1H, d, $J_{4,5}$ = 9.1 Hz, 5-H). For ^{13}C-NMR (50 MHz, C^2HCl_3): δ (in p.p.m.) = 14.90 ($2\times CH_3$); 25.46 ($3\times CH_2$); 56.27, 65.19, 72.07, 72.86 and 73.22.

5-*O*-(*t*-Butyldiphenylsilyl)-D-arabinose diethyldithioacetal (**14**)

t-Butylchlorodiphenyldiphenylsilane (22.3 ml, 85.9 mmol, 1.1 equiv.) was added to a solution of D-arabinose diethyldithioacetal (**13**) (20 g, 78.1 mmol, 1 equiv.) and imidazole (10.6 g, 0.156 mol, 2 equiv.) in dry dimethylformamide (100 ml) at 0 °C, and left for 6 h at the same temperature. The solvent was evaporated, and the residue was dissolved in chloroform and washed with water (3×100 ml). The organic layer was collected and dried. After evaporation to dryness, the residue was purified by flash chromatography to afford a colourless solid [36.5 g, 94%, R_F = 0.37, ethyl acetate/hexane, 60:40 (v/v)]. The melting-point temperature was measured as 51–54 °C. ^{1}H-NMR (200 MHz, C^2HCl_3): δ (in p.p.m) = 1.09 (9H, s, Me_3C); 1.28 (6H, t, CH_2CH_3); 2.42 (1H, d, J = 9.3 Hz, OH); 2.82 (5H, m, SCH_2 and OH); 3.35 (1H, m, OH); 3.79 (1H, m); 3.87 (3H, m); 4.04 (1H, d, J = 9.3 Hz); 4.10 (1H, d, J = 8.4 Hz, 1-H); 7.41 (6H, m, aromatic H); and 7.66 (4H, m, aromatic H). ^{13}C-NMR (50 MHz, C^2HCl_3): δ (in p.p.m.) = 14.49 and 14.62 ($\mathbf{CH_3}CH_2$); 19.27 ($Me_3\mathbf{C}$); 23.57 and 25.66 ($CH_3\mathbf{CH_2}$); 26.88 ($C\mathbf{CH_3}$); 55.82 (C-1); 65.13 (C-5); 70.02, 70.51 and 72.44 (C-2, C-3 and C-4 respectively); 127.81, 129.87, 133.05 and 135.58 (aromatic C).

5-*O*-(*t*-Butyldiphenylsilyl)-1,2-isopropylidene-β-D-arabinofuranose (**15**)

5-*O*-(*t*-Butyldiphenylsilyl)-D-arabinose diethyldithioacetal (**14**) (30 g, 61 mmol, 1 equiv.) was dissolved in aq. acetone (250 ml, 10% water). Under efficient stirring, red mercury(II) oxide (26.31 g, 121 mmol, 2 equiv.) and mercury(II) chloride (41.2 g, 152 mmol, 2.5 equiv.) were added successively. After 6 h, the mixture was filtered through celite. The filtrate was treated with a 33% aq. solution of $NaHCO_3$ (100 ml). After evaporation of the solvents, the residue was taken up in chloroform ($CHCl_3$). The solution was washed successively with water (100 ml), aq. KI [10% (w/v); 200 ml] and saturated NaCl (100 ml). The organic layer was collected, dried, filtered and concentrated. The resulting oil was dissolved in dry acetone (250 ml) and treated with a catalytic amount of DL-camphor sulphonic acid (1.4 g, 6.1 mmol, 0.1 equiv.) and anhydrous copper(II) sulphate (30 g, 188 mmol, 3.1 equiv.). After 24 h, the mixture was neutralized with sodium carbonate, filtered through celite, and the solvent was evaporated. The residue was purified by flash chromatography to afford a colourless oil [24.4 g, 84%, R_F = 0.42, ethyl acetate/hexane, 50:50, (v/v)]. ^{1}H-NMR (200 MHz, C^2HCl_3): δ (in p.p.m.) = 1.06 (9H, s, Me_3C); 1.29 (3H, s, CMe_2); 1.32 (3H, CMe_2); 3.82 (2H, m, 5-H); 4.05 (1H, ddd, $J_{3,4}$ =

2.5 Hz, $J_{4,5a} = J_{4,5b} = 6.6$ Hz, 4-H); 4.42 (1H, d, $J_{3,4} = 2.5$ Hz, 3-H); 4.54 (1H, d, $J_{1,2} = 4.2$ Hz, 2-H); 5.88 (1H, d, $J_{1,2} = 4.2$ Hz, 1-H); 7.40 (6H, m, aromatic H); and 7.67 (4H, m, aromatic H). ^{13}C-NMR (50 MHz, C^2HCl_3): δ (in p.p.m.) = 19.24 (Me_3**C**); 26.16 and 26.88 (**Me**$_3$C); 63.75 (C-5); 76.41 (C-3); 87.09 and 87.49 (C-2 and C-4); 105.58 (C-1); 112.53 (**C**Me$_2$); 127.77, 129.81, 133.22 and 135.61 (aromatic C).

[3-^{2}H]-5-*O*-(*t*-Butyldiphenylsilyl)-1,2-isopropylidene-β-D-xylofuranose (**16**)

To a stirred solution of oxalyl chloride (5.9 ml, 67.5 mmol, 2.5 equiv.) in CH_2Cl_2 (200 ml) at -78 °C was added DMSO (8.6 ml, 121.5 mmol, 4.5 equiv.). The reagent was allowed to warm up to -35 °C for 5 min and cooled again to -78 °C. After addition of a solution of the alcohol (**15**) (11.6 g, 27 mmol, 1 equiv.) in CH_2Cl_2 (75 ml), the reaction mixture was allowed to warm to -35 °C, and after 15 min was treated with triethylamine (22.7 ml, 162 mmol, 6 equiv.) for 1 h. After addition of a saturated aqueous solution of ammonium chloride (75 ml), the reaction mixture was extracted with $CHCl_3$ (3×100 ml). The combined extracts were dried, filtered, concentrated and purified by filtration over a bed of silica gel (approx. 7 cm thickness, 3 cm diameter) on a sintered-glass funnel to give a colourless oil (ethyl acetate/hexane, 50:50).

Sodium borodeuteride (718 mg, 18.9 mmol, 0.7 equiv.) was added to an ice-cooled solution of the resulting crude ketone (11.5 g, 27 mmol, 1 equiv.) in ethanol (100 ml). Stirring was continued at room temperature. After 2 h, the reaction mixture was diluted with water (20 ml), treated with a saturated aq. solution of NH_4Cl (50 ml), and extracted with $CHCl_3$ (3×100 ml). The combined extracts were dried, filtered and concentrated. The residue afforded, after flash chromatography, [3-^{2}H]-5-*O*-(*t*-butyldiphenylsilyl)-1,2-isopropylidene-β-D-xylofuranose (**16**) as a colourless oil [8.2 g, 71 %, $R_F = 0.32$, ethyl acetate/hexane, 70:30 (v/v)]. ^{1}H-NMR (200 MHz, C^2HCl_3): δ (in p.p.m.) = 1.07 (9H, s, CMe_3); 1.36 (3H, s, CMe_2); 1.43 (3H, s, CMe_2); 3.06 (1H, s, 3-OH); 3.88 (1H, dd, $J_{5a,5b} = 13.3$ Hz, $J_{4,5a} = 8.9$ Hz, 5-H_a); 4.14 (1H, t, $J_{4,5a} = J_{4,5b} = 8.9$ Hz, 4-H); 4.15 (1H, dd, $J_{5a,5b} = 13.3$ Hz, $J_{4,5b} = 8.9$ Hz, 5-H_b); 4.61 (1H, d, $J_{1,2} = 3.9$ Hz, 2-H); 5.72 (1H, d, $J_{1,2} = 3.9$ Hz, 1-H); 7.39 (6H, m, aromatic H); and 7.71 (4H, m, aromatic H). ^{13}C-NMR (50 MHz, C^2HCl_3): δ (in p.p.m.) = 19.17 (**C**Me$_3$); 26.55 and 26.84 (C**Me**$_3$ and C**Me**$_2$); 63.20 (C-5, γ shift: +33 p.p.b.); 79.78 (C-2: β shift: -66 p.p.b.); 81.29 (C-4, β shift: -33 p.p.b.); 104.96 (C-1, γshift: +33 p.p.b.); 114.14 (**C**Me$_2$); 127.74, 129.74, 133.08, 133.28, 135.06 and 135.67 (aromatic C).

[3-^{2}H]-3-*O*-Benzyl-5-*O*-(*t*-butyldiphenylsilyl)-1,2-isopropylidene-β-D-xylofuranose (**17**)

A solution of [3-^{2}H]-5-*O*-(*t*-butyldiphenylsilyl)-1,2-isopropylidene-β-D-xylofuranose (**16**) (7.7 g, 18.1 mmol, 1 equiv.) in dry tetrahydrofuran (THF; 20 ml) was added dropwise to a suspension of NaH (870 mg, 36.2 mmol, 2 equiv.) in THF (80 ml) at 0 °C. The THF solution was stirred for 1 h at the same temperature. Tetrabutylammonium iodide (1.34 g, 3.62 mmol, 0.2 equiv.), a catalytic amount of 18-crown-6 (45 mg, 0.12 mmol, 6.6×10^{-3} equiv.) and benzyl bromide (4.3 ml, 36.2 mmol, 2 equiv.) were successively added to the reaction mixture. The reaction was stirred overnight at room temperature before being quenched by addition of a saturated solution of ammonium chloride (50 ml). After extraction with $CHCl_3$ (3×100 ml) and evaporation of the solvents, the residue was purified by flash chromatography to afford (**17**) as a colourless oil [7.5 g, 80 %, $R_F = 0.35$, ethyl acetate/hexane, 20:80 (v/v)]. ^{1}H-NMR (200 MHz, C^2HCl_3): δ (in p.p.m.) = 1.08 (9H, s, CMe_3); 1.29 (3H, s, CMe_2); 1.37 (3H, s, CMe_2); 4.01 (1H, dd, $J_{5a,5b} = 11.1$ Hz, $J_{4,5a} = 6.8$ Hz, 5-H_a); 4.17 (1H, dd, $J_{5a,5b} = 11.2$ Hz, $J_{4,5b} = 4.4$ Hz, 5-H_b); 4.29 (1H, dd, $J_{4,5a} = 6.8$ Hz, $J_{4,5b} = 4.4$ Hz, 4-H); 4.55 (1H, d, $J_{1,2} = 4.0$ Hz, 2-H); 4.60 [2H, s, CH_2Ph (where 'Ph' represents phenyl)]; 5.70 (1H, d, $J_{1,2} = 4.0$ Hz, 1-H); 7.30 (5H, m, aromatic H); 7.37 (6H, m, aromatic H); 7.70 (4H, m, aromatic H). ^{13}C-NMR (50 MHz, C^2HCl_3): δ (in p.p.m) = 19.24 (**C**Me$_3$); 26.09, 26.45 and 26.88 (C**Me**$_3$ and C**Me**$_2$); 63.82 (C-5); 72.44 (**C**H_2Ph); 78.57 (C-2, β shift: -98 p.p.b.); 82.01 (C-4, β shift: -66 p.p.b.); 104.57 (C-1); 113.48 (**C**Me$_2$); 127.64, 127.81, 128.43, 129.51, 133.61, 133.87, 135.67, 135.74 and 137.58 (aromatic C).

[2-^{2}H]-2-*O*-Benzyl-4-*O*-(*t*-butyldiphenylsilyl)-D-threitol (**18**)

A solution of [3-^{2}H]-3-*O*-benzyl-5-*O*-(*t*-butyldiphenylsilyl)-1,2-isopropylidene-β-D-xylofuranose (**17**) (6.95 g, 13.4 mmol) in 80 % aq. acetic acid (150 ml) was heated for 30 min at 75 °C. After addition of ice, the solvent was removed under reduced pressure to yield the crude mixture of the two anomers of [3-^{2}H]-3-*O*-benzyl-5-*O*-(*t*-butyldiphenylsilyl)-D-xylofuranose, which were purified, but not separated one from the other, by flash chromatography [5.32 g, 83 %, $R_F = 0.42$, ethyl acetate/hexane, 40:60 (v/v)].

To a solution of [3-^{2}H]-3-*O*-benzyl-5-*O*-(*t*-butyldiphenylsilyl)-D-xylofuranose (5.15 g, 10.8 mmol, 1 equiv.) in methanol (50 ml) was added a solution of sodium metaperiodate (3.45 g, 16.1 mmol, 1.5 equiv.) in water (50 ml). The resulting suspension was stirred for 15 h at room temperature, neutralized with sodium hydrogen carbonate and extracted with $CHCl_3$ (3×100 ml). The combined extracts were dried, filtered and concentrated to give a colourless oil, which was not purified further [$R_F = 0.72$, ethyl acetate/hexane, 50:50 (v/v)]. To an ice-cooled solution of the resulting aldehyde in ethanol (50 ml) was added sodium borohydride (410 mg, 10.8 mmol, 1 equiv.). Stirring was continued at room temperature for 2 h. After addition of water (20 ml) and of a saturated aq. solution of NH_4Cl (20 ml) the reaction mixture was extracted with $CHCl_3$ (3×100 ml). After evaporation of the solvent, the residue was purified by flash chromatography to afford [2-^{2}H]-2-*O*-benzyl-4-*O*-(*t*-butyldiphenylsilyl)-D-threitol (**18**) as a colourless oil [4.7 g, 97 %, $R_F = 0.48$, ethyl acetate/hexane, 50:50 (v/v)]. ^{1}H-NMR (200 MHz, C^2HCl_3): δ (in p.p.m.) = 1.06 (9H, s, CMe_3); 3.67–3.89 (5H, m); 4.58 (1H, d, $J = 11.6$ Hz, CH_2Ph); 4.68 (1H, d, $J = 11.6$ Hz, CH_2Ph); 7.28 (5H, m, aromatic H); 7.38 (6H, m, aromatic H); 7.67 (4H, m, aromatic H). ^{13}C-NMR (50 MHz, C^2HCl_3): δ (in p.p.m.) = 19.21 (**C**Me$_3$); 26.88 (C**Me**$_3$); 62.05 (C-1, β shift: -33 p.p.b.); 64.44 (C-4); 72.57 (C-3, β shift: -66 p.p.b.); 72.83 (**C**H_2Ph); 127.81, 128.53, 129.87, 133.05, 133.31 and 135.58 (aromatic C).

[3-^{2}H]-2,3,4-*O*-Tribenzyl-D-threitol (**19**)

A solution of [2-^{2}H]-2-*O*-benzyl-4-*O*-(*t*-butyldiphenylsilyl)-D-threitol (**18**) (4.5 g, 10.0 mmol, 1 equiv.) in dry THF (20 ml) was added dropwise to a suspension of NaH (600 mg, 25 mmol, 2.5 equiv.) in THF (50 ml) at 0 °C, and the THF solution was stirred for 1 h at the same temperature. Tetrabutylammonium iodide (740 mg, 2 mmol, 0.2 equiv.), a catalytic amount of 18-crown-6 (24 mg, 0.066 mmol, 6.6×10^{-3} equiv.) and benzyl bromide (3 ml, 25 mmol, 2.5 equiv.) were successively added to the reaction mixture, which was stirred overnight at room temperature. After quenching by addition of a saturated solution of

ammonium chloride (50 ml), the reaction was extracted with $CHCl_3$ (3×100 ml). After evaporation, the crude product was used without purification [$R_F = 0.34$, ethyl acetate/hexane, 10:90 (v/v)].

A solution of [2-^{2}H]-1,2,3-*O*-tribenzyl-4-*O*-(*t*-butyldiphenylsilyl)-D-threitol (5.1 g, 8.1 mmol, 1 equiv.) in THF was treated with Bu_4NF (3.06 g, 9.7 mmol, 1.2 equiv.). After 3 h, the solvent was removed, and the residue purified by flash chromatography to afford (**19**) as a colourless oil [2.8 g, 74 %, $R_F = 0.50$, ethyl acetate/hexane, 50:50 (v/v)]. ^{1}H-NMR (200 MHz, C^2HCl_3): δ (in p.p.m.) = 2.30 (1H, m, OH); 3.69 (4H, m); 3.80 (1H, m); 4.54 (2H, s, CH_2Ph); 4.60 (1H, d, $J = 11.8$ Hz, CH_2Ph); 4.62 (1H, d, $J = 11.6$ Hz, CH_2Ph); 4.67 (1H, d, $J = 11.6$ Hz, CH_2Ph); 4.75 (1H, d, $J = 11.8$ Hz, CH_2Ph); 7.35 (15H, m, aromatic H). ^{13}C-NMR (50 MHz, C^2HCl_3): δ (in p.p.m.) = 61.53 (C-1, γ shift: -33 p.p.b.); 69.46 (C-4, β shift: -66 p.p.b.); 72.80, 72.87 and 73.49 (**C**H_2Ph); 79.13 (C-2, β shift: -98 p.p.b.); 126.92; 127.58, 127.68, 127.77, 127.91, 127.97, 128.40, 137.94 and 138.26 (aromatic C).

[4-^{2}H]-3,4,5-*O*-Tribenzyl-DX (**20**)

To a stirred solution of oxalyl chloride (0.78 ml, 8.89 mmol, 1.3 equiv.) in THF (30 ml) at -78 °C was added DMSO (0.78 ml, 10.9 mmol, 1.6 equiv.). The solution was allowed to warm up to -35 °C for 5 min, and then cooled down again to -78 °C. After addition of a solution of the alcohol (**19**) (2.68 g, 6.84 mmol, 1 equiv.) in THF (40 ml), the reaction mixture was allowed to warm up to -35 °C, and treated after 15 min with triethylamine (4.8 ml, 34.2 mmol, 5 equiv.). The reaction mixture was stirred for 15 min at room temperature and treated at -10 °C with a solution of 3 M methyl magnesium chloride in THF (11.4 ml, 34.2 mmol, 5 equiv.). After 1 h, a saturated aq. solution of NH_4Cl (50 ml) was added, and the mixture was extracted with $CHCl_3$ (3×50 ml). After evaporation of the solvent, the residue was purified by flash chromatography to give a colourless oil [2.72 g, 98 %, $R_F = 0.52$, ethyl acetate/hexane, 40:60 (v/v)].

To a stirred solution of oxalyl chloride (0.70 ml, 8.02 mmol, 1.3 equiv.) in CH_2Cl_2 (30 ml) at -78 °C was added DMSO (0.70 ml, 9.87 mmol, 1.6 equiv.). The solution was allowed to warm up to -35 °C for 5 min and then cooled again to -78 °C. A solution of the two resulting alcohols (2.50 g, 6.17 mmol, 1 equiv.) in CH_2Cl_2 (40 ml) was added to the reaction mixture. The resulting solution was allowed to warm up to -35 °C and treated after 15 min with triethylamine (4.32 ml, 30.9 mmol, 5 equiv.). After 1 h, a saturated aq. solution of NH_4Cl (50 ml) was added and the mixture was extracted with $CHCl_3$ (3×50 ml). The combined extracts were dried, filtered, concentrated and purified by flash chromatography to give (**20**) as colourless oil [2.28 g, 91 %, $R_F = 0.42$, diethylether/hexane, 40:60 (v/v)]. ^{1}H-NMR (C^2HCl_3): δ (in p.p.m.) = 2.16 (3H, s, 1-H); 3.62 (2H, m, 5-H); 3.99 (1H, s, 3-H); 4.40 (1H, d, $J = 12.3$ Hz, CH_2Ph); 4.45 (1H, d, $J = 11.8$ Hz, CH_2Ph); 4.46 (1H, d, $J = 12.3$ Hz, CH_2Ph); 4.52 (1H, d, $J = 11.8$ Hz, CH_2Ph); 4.65 (1H, d, $J = 11.8$ Hz, CH_2Ph); 4.69 (1H, d, $J = 1$ 1.8 Hz, CH_2Ph); 7.32 (15H, m, aromatic H). ^{13}C-NMR (50 MHz, C^2HCl_3): δ (in p.p.m.) = 27.76 (C-1); 68.80 (C-5, β shift: -66 p.p.b.); 73.29, 73.36, 73.65 (**C**H_2Ph); 84.73 (C-3, β shift: -33 p.p.b.); 127.68, 127.77, 128.04, 128.10, 128.27, 128.37, 128.46, 137.25 and 137.87 (aromatic C); 210.45 (C-2).

[4-^{2}H]DX (**11**)

[4-^{2}H]-3,4,5-*O*-tribenzyl-DX (**20**) (2 g, 4.95 mmol) was hydrogenated over 10 % Pd/C (200 mg) in methanol (30 ml) for 15 h at room temperature and atmospheric pressure. The mixture was filtered, and the filtrate concentrated to give a mixture of [4-^{2}H]DX (**11**) as the major isotopomer and [3-^{2}H]DX (**11′**) as the minor isotopomer in a 3:1 ratio. The resulting mixture was a colourless oil corresponding to a 2:1:1 mixture of the open form and two furanose anomers [660 mg, 98 %, $R_F = 0.40$, chloroform/methanol, 80:20 (v/v)], which was not purified further. ^{1}H-NMR (400 MHz, ^2H_2O): δ (in p.p.m.) = 1.37 (s, CH_3, furanose); 1.42 (s, CH_3, furanose); 2.23 (s, CH_3, open form); 3.61 (d, $J_{5a,5b} = 12.0$ Hz, 5-H_a, open form); 3.62 (d, $J_{5a,5b} = 11.7$ Hz, 5-H_a, furanose); 3.64 (d, $J_{5a,5b} = 12.0$ Hz, 5-H_b, open form); 3.65 (d, $J_{5a,5b} = 11.7$ Hz, 5-H_b, furanose); 4.35 (s, 3-H). ^{13}C-NMR (100 MHz, ^2H_2O): open form: δ (in p.p.m) = 25.76 (C-1), 25.80 [C-1 of (**11′**), γ shift: -30 p.p.b.]; 62.18 [C-5 of (**11**), CH_2, β shift: -83 p.p.b.]; 62.23 [C-5 of (**11′**), γ shift: -27 p.p.b.]; 71.09 [C-4 of (**11**), α shift: -408 p.p.b., t, $J = 21.7$ Hz]; 71.45 (C-4 of (**11′**), β shift: -111 p.p.b.]; 76.82 [C-3 (**11′**), α shift: -390 p.p.b., t, $J = 22.5$ Hz]; 77.13 [C-3 of (**11**), β shift: -77 p.p.b.]; 213.01 [C-2 of (**11′**), β shift: -69 p.p.b.]; 213.06 [C-2 of (**11**), γ shift: -21 p.p.b.]; furanoses: δ (in p.p.m) = 20.78 and 23.65 [C-1 of (**11**)]; 23.68 [C-1 of (**11′**), γ shift: -32 p.p.b.]; 69.44 [C-5 of (**11**), β shift: -91 p.p.b.]; 69.54 [C-5 (**11**), β shift: -85 p.p.b.]; 74.60 [C-4 of (**11**), α shift: -396 p.p.b., t, $J = 22.5$ Hz]; 74.94 [C-4 of (**11′**), β shift: -51 p.p.b.]; 75.71 [C-4 of (**11**), α shift: -377 p.p.b., t, $J = 23.3$ Hz]; 76.03 [C-4 of (**11′**), β shift: -56 p.p.b.]; 80.71 [C-3 of (**11**), β shift: -59 p.p.b.]; 81.14 [C-3 of (**11**), β shift: -71 p.p.b.]; 102.65 [C-2 of (**11′**), β shift: -71 p.p.b.]; 102.70 [C-2 of (**11**), γ shift: -18 p.p.b.]; 106.07 [C-2 of (**11′**), β shift: -68 p.p.b.]; 106.12 [C-2 of (**11**), γ shift: -19 p.p.b.].

Triacetate of [4-^{2}H]DX (**21**)

The [4-^{2}H]DX (**11**) (20 mg, 0.15 mmol, 1 equiv.), dissolved in pyridine (70 μl, 0.9 mmol, 6 equiv.), was acetylated at room temperature overnight with acetic anhydride (70 μl, 0.9 mmol, 6 equiv.). The solvent was removed *in vacuo*, and the residue was purified by TLC to afford the two triacetate isotopomers (**21**) and (**21′**) of DX as a colourless oil [37 mg, 95 %, $R_F = 0.24$, ethyl acetate/hexane, 30:70 (v/v)]. ^{1}H-NMR ($C^2H\ Cl_3$): δ (in p.p.m.) = 2.02 (3H, s, CH_3); 2.03 (3H, s, CH_3); 2.16 (3H, s, CH_3); 2.17 (3H, s, CH_3); 4.10 (1H, dd, $J_{5a,5b} = 11.5$ Hz, $J_{4,5a} = 6.6$ Hz, 5-H_a); 4.25 (1 H, dd, $J_{5a,5b} = 11.5$ Hz, $J_{4,5b} = 5.9$ Hz, 5-H_b); 5.21 [0.75H, 25 % (**21′**), s, 3-H]; 5.54 [0.25H, 75 % (**21**), dd, $J_{4,5a} = 6.6$ Hz, $J_{4,5b} = 5.9$ Hz, 4-H]. ^{13}C-NMR (C^2HCl_3): δ (in p.p.m) = 20.35 (CH_3); 20.46 (CH_3); 20.51 (CH_3); 26.71 [C-1 of (**21**)] and 26.73 [C-1 of (**21′**), γ shift: $+17$ p.p.b.]; 61.30 [C-5 of (**21**), β shift: -102 p.p.b.]; 61.35 [C-5 of (**21′**), γ shift: -52 p.p.b.]; 68.32 [C-4 of (**21**), α shift: -337 p.p.b., t, $J = 23.1$ Hz]; 68.57 [C-4 of (**21′**), β shift: -90 p.p.b.]; 75.92 [C-3 of (**21′**), α shift: -365 p.p.b., t, $J = 21.4$ Hz]; 76.18 [C-3 of (**21**), β shift: -100 p.p.b.]; 169.63 (CO); 169.89 (CO); 170.19 (CO); 201.32 [C-2 of (**21′**), β shift: $+30$ p.p.b.]; 201.37 [C-2 of (**21**), γ shift: -19 p.p.b.]. MS FAB: $m/z = 158$ $(M+Na)^+$.

Culture of TBY-2 cells and feeding experiments

TBY-2 cells were cultured in a modified Murashige–Skoog medium [30] (obtained from Duchefa, Haarlem, The Netherlands). The concentration of KH_2PO_4 was increased to 540 mg $\cdot$ l^{-1}. Other additives were as described by Nagata et al. [31], except for the further inclusion of *myo*-inositol (100 mg $\cdot$ l^{-1}) [32]. Cell cultures (80 ml in 250 ml Erlenmeyer flasks) were kept in the dark at 26 °C and shaken at 174 rev./min. Cells were subcultured weekly (2 ml transferred to 80 ml of new medium). In

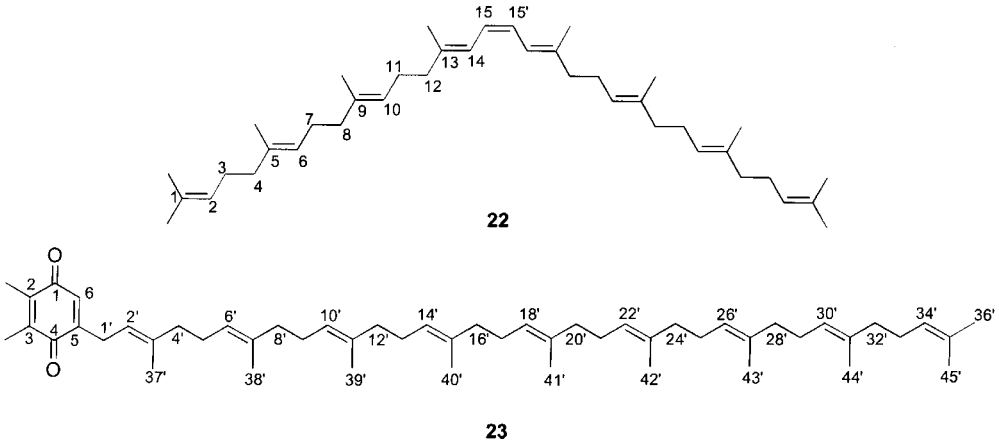


Figure 1 Major plastid isoprenoids from TBY-2 cells: *cis*-phytoene (22) and plastoquinone Q9 (23)

preceding test series, growth was determined as the increase in biomass (fresh weight), in order to optimize the composition of the culture medium and to obtain sufficient amounts of labelled isoprenoids for the analyses. Cell growth was found to be optimal with 2% (w/v) sucrose and 0.5 mM [4-^{2}H]DX (**11**) (75% isotope abundance on C-4). Twenty-six Erlenmeyer flasks (250 ml) with 80 ml cultures were used for the incorporation experiment. After 8 days, cells were harvested by vacuum filtration on a sintered glass funnel, allowing the removal of most of the culture medium. The cells [approx. 25 g (fresh weight) per culture flask] were frozen at −80 °C in a deep-freezer and freeze-dried at room temperature in a Christ Alpha 1-2 freeze-drier.

Isolation and identification of ^{2}H-labelled *cis*-phytoene (22) and plastoquinone Q9 (23) (Figure 1)

All isoprenoid samples were protected from the light during their isolation. Freeze-dried cells (18 g) were extracted four times for 1 h under reflux with chloroform/methanol [2:1 (v/v), 4 × 200 ml). The combined extracts were taken to dryness and washed thoroughly with hexane (50 ml). The hexane extract was filtered through a silica column (35 mm diameter, 40 mm height) eluted with dichloromethane. After evaporation of the solvent, the residue was separated by TLC (dichloromethane) yielding *cis*-phytoene (**22**) ($R_F = 0.76$) and plastoquinone Q9 (**23**) ($R_F = 0.67$). A second TLC purification [cyclohexane/ethyl acetate, 9:1 (v/v)] afforded *cis*-phytoene (**22**) (0.50 mg, $R_F = 0.71$) and plastoquinone Q9 (**23**) (1.4 mg, $R_F = 0.57$). The two compounds were identified by ^{1}H- and ^{13}C-NMR spectroscopies and direct-inlet MS (70 eV electron-impact ionization). All data were compared with those of reference compounds and from the literature [27,33–35].

cis-Phytoene (**22**) (^{2}H-labelled)

^{1}H-NMR (400 MHz, C^2HCl_3): δ (in p.p.m) = 1.59 (6H, s); 1.60 (6H, s); 1.62 (6H, s); 1.68 (6H, s); 1.77 (6H, s); 1.95–2.13 (24H, m, 3-, 3′-,4-, 4′-, 7-, 7′-, 8-, 8′-, 11-, 11′-, 12-, 12′-H); 5.11 (6H, m, 2-, 2′-, 6-, 6′-, 10-, 10′-H); 6.10 (2H, m, 14-, 14′-H); 6.29 (2H, dd, $J_{15,15'} = J_{14,15} = 10.9$ Hz, 15-, 15′-H). ^{2}H-NMR (61.4 MHz, $CHCl_3$): δ (in p.p.m) = 1.98 {m, 4-, 4′-, 8-, 8′-, 12-, 12′-^{2}H, derived from [3-^{2}H]DX (**11′**)}; 5.15 {m, vinylic hydrogens, 2-, 2′-, 6-, 6′-, 10-, 10′-^{2}H, derived from [4-^{2}H]DX (**11**)}. For ^{13}C-NMR data, see Table 1. UV (hexane): $\lambda_{max\ nm} = 276$, 286 and 296.

Plastoquinone Q9 (**23**) (^{2}H-labelled)

^{1}H-NMR (400 MHz, C^2HCl_3): δ (in p.p.m) = 1.60 (21H, s, 38′-, 39′-, 40′-, 41′-, 42′-, 43′-, 44′-, *cis*-45′-H); 1.62 (3H, s, 37′-H); 1.68 (3H, s, *trans*-36′-H); 1.96–2.13 [38 H, m, 4′-, 5′-, 8′-, 9′-, 12′-, 13′-, 16′-, 17′-, 20′-, 21′-, 24′-, 25′-, 28′-, 29′-, 32′-, 33′-H and 2-, 3-Me (where 'Me' represents methyl)]; 3.11 (2H, d, $J = 7.3$ Hz, 1′-H); 5.11 (9H, m, 2′-, 6′-, 10′-, 14′-, 18′-, 22′-, 26′-, 30′- and 34′-H); 6.46 (1H, t, $J = 1.8$ Hz, 6-H). ^{2}H-NMR (61.4 MHz, $CHCl_3$): δ (in p.p.m) = 1.96 {m, 4′-, 5′-, 8′-, 9′-, 12′-, 13′-, 16′-, 17′-, 20′-, 21′-, 24′-, 25′-, 28′-, 29′-, 32′-, 33′-^{2}H, derived from [3-^{2}H]DX (**11′**)}; 5.15 {m, vinylic hydrogens, 2′-, 6′-, 10′-, 14′-, 18′-, 22′-, 26′-, 30′- and 34′-H, derived from [4-^{2}H]DX (**11**)}. For ^{13}C-NMR data, see Table 2.

RESULTS

TBY-2 plastid isoprenoids

Although TBY-2 cells are not phototrophic and do not possess normal chloroplasts, they are characterized by the presence of leucoplasts [31] that still contain some typical chloroplast isoprenoids (Figure 1). Plastoquinone (**23**) was identified previously in TBY-2 cells [27]. In an attempt to isolate from the hydrocarbon fraction an orange-coloured compound, which was thought to be a carotenoid and could not be studied further because it was present in too small amounts for the NMR spectroscopy, the colourless *cis*-phytoene (**22**) was found to be accumulated in the TBY-2 cells. It was identified by both ^{1}H- and ^{13}C-NMR spectroscopy and by UV spectroscopy, and from a comparison of the data with those in the literature [33–35]. The *cis* configuration of the central double bond was confirmed by the 11 Hz coupling constant between the vinylic protons at C-15 and C-15′. Both plastoquinone (**23**) and *cis*-phytoene (**22**) are synthesized in sufficient amounts to allow ^{13}C-NMR spectroscopy. [4-^{2}H]DX is the substrate required for the detection of the branching in the MEP pathway, leading separately to IPP and DMAPP. A method of synthesis of enantiomeric pure [4-^{2}H]DX was therefore developed to perform such an incubation with the TBY-2 cells, in order to investigate the deuterium content by NMR spectroscopy.

Synthesis of [4-^{2}H]DX (11)

The synthesis of [4-^{2}H]DX (**11**) was achieved according to a previously published procedure [36] involving a highly stereo-

Table 1 ^{13}**C-NMR spectrum of *cis*-phytoene (22)**

Me, methyl.

Carbon atom	Natural abundance, δ (p.p.m.)	Deuterium labelled, δ (p.p.m.)	β or γ shifts (p.p.b.)
Me-1 (*cis*)	17.712	17.696	−16
Me-1 (*trans*)	25.731	25.667	−64
Me-5	16.068	16.084	+16
Me-9	16.024	16.040	+15
Me-13	16.548	16.561	+13
C-1	131.278	–	–
C-2	123.981	–	–
C-3	26.685	26.609	−76
C-7, C-11	26.754; 26.779	–	–
C-4, C-8	39.739	39.723	−17
C-5	134.958	–	–
C-6	124.233	–	–
C-9	135.348	135.318	−30
C-10	124.424	–	–
C-12	40.518	40.502	−16
C-13	139.565	139.471	−94
C-14	120.212	–	–
C-15	123.362	123.311	−51

selective reduction reaction with $NaBH_4$ on the α-face of the ketone at C-3, which corresponded to C-4 of the DX (**11**) (Scheme 2). The first step of the synthetic route was the conversion of arabinose (**12**) into its thioacetal [37]. Treatment of the protected arabinose (**13**) with *t*-butyldiphenylsilyl chloride in dimethylformamide in the presence of imidazole resulted in a highly selective protection of the primary hydroxy group to afford the silyl ether (**14**) in a yield of 94%. Deprotection of the thioacetal moiety in (**14**) by treatment with a mercury(II) derivative in acetone was followed by acetonation in the presence of a catalytic amount of acid to give the arabinofuranose (**15**) with only the C-3 hydroxy group remaining unprotected [38]. The stereochemistry was inverted at C-3 by Swern oxidation [39] to the intermediate ketone, followed by a highly stereoselective reduction with sodium borohydride to yield the xylofuranoside (**16**) [36]. The presence of the 1,2-*O*-isopropylidene group on the β-face of the furanoside directed the reduction of the oxo group by the deuteride from the less hindered α-face to afford the xylofuranose (**16**) with the required configuration [36]. The resulting secondary alcohol (**16**) was protected with a benzyl group to afford compound (**17**). The acetonide protection of the pentose derivative (**17**) was hydrolysed with 80% aq. acetic acid to yield a mixture of the two anomers in 83% yield [40]. Smooth oxidative cleavage occurred upon treatment of diol with sodium metaperiodate in aqueous methanol to give an aldehyde in almost quantitative yield. This aldehyde was immediately reduced with sodium borohydride to afford the diol (**18**). Benzylation of this diol (**18**) and deprotection of the silyl ether yielded the [2-^{2}H]-1,2,3-*O*-tribenzyl-D-threitol (**19**). One-pot oxidation and nucleophilic addition of methyl magnesium chloride was achieved using the Swern–Ireland procedure [41]. A Swern oxidation of the resulting mixture of diastereomeric alcohols afforded [4-^{2}H]-3,4,5-*O*-tribenzyl-DX (**20**). Deprotection of the triol derivative (**20**) by hydrogenation over 10% Pd/C in methanol at room temperature and atmospheric pressure gave [4-^{2}H]DX (**11**). Whereas the deuterium position was clearly defined in the intermediates (**16**–**20**) according to their ^{1}H- and ^{13}C-NMR spectra, an unexpected partial deuterium migration from C-4 to C-3 occurred during the hydrogenolysis of (**20**). In the FAB mass spectrum of the free deuterated DX (**11**), the presence of a unique $(M+Na)^+$ cluster ion (m/z 158) confirmed that only isotopomers bearing a single deuterium were present. NMR spectroscopy of the deuterated DX triacetate (**21**) permitted the localization of the deuterium. In the ^{13}C-NMR spectrum, the relative intensities of the deuterium-β-shifted and -γ-shifted signals of C-5 and C-2 indicated a 3:1 ratio for the DX isotopomers, with deuterium labelling at C-4 and C-3 respectively. This ratio was confirmed by ^{1}H-NMR spectroscopy by the relative intensities of the 4-H and 3-H signals. In spite of the presence of some deuterium-labelled isotopomer at C-3, this product was well suited to the incorporation experiments planned with the TBY-2 cells.

Incorporation of [4-^{2}H]DX into plastoquinone and phytoene

Feeding TBY-2 cells with [4-^{2}H]DX (**11**) resulted in a considerable deuterium labelling of their two major plastid terpenoids. First indications of the efficient incorporation of deuterium-labelled DX were obtained by MS. This technique did not, however, allow the localization of the labelling, especially since the [4-^{2}H]DX (**11**) samples also contained some [3-^{2}H]DX (**11′**) as the minor isotopomer. Only the m/z 189 fragment in the mass spectrum of plastoquinone (**23**) yielded interesting information, corresponding to a fragment retaining only four carbon atoms derived from IPP (i.e. C-1, C-2, C-3 and C-5) and linked to the benzoquinone ring [42]. As the C-4 carbon atom of IPP, which is derived from C-3 of DX, is missing, any deuterium contribution from [3-^{2}H]DX (**11′**) can be excluded. After incorporation of [4-^{2}H]DX (**11**), the intensity of the m/z 190 fragment was enhanced by 31%, suggesting the retention of one deuterium atom in an IPP-derived isoprene unit.

Deuterium atoms were precisely localized by ^{13}C-NMR spectroscopy using the deuterium-induced shifts of the ^{13}C signals. α Shifts were not observed; the intensities of the triplets corresponding to the carbon atoms bearing a deuterium were too weak to be observed. β and γ Shifts were, however, easily measured.

In the ^{13}C-NMR spectrum of plastoquinone (**23**) (Table 2), a γ shift (−16 p.p.b.) was observed for C-5 of the quinone ring, and a β shift (−100 p.p.b.) for C-1′. This indicated the presence of a deuterium atom on C-2′ in an isoprene unit derived from IPP. Two additional γ shifts (−34 p.p.b. and −18 p.p.b.) were found for two signals corresponding to the cluster of the signals

Table 2 ^{13}C-NMR spectrum of plastoquinone Q9 (23)

Me, methyl.

Carbon atom	Natural abundance, δ (p.p.m.)	Deuterium labelled, δ (p.p.m.)	β or γ shift (p.p.b.)
Me-2	12.046		–
Me-3	12.388		–
C-37′, C-38′, C-39′, C-40′, C-41′,	15.996		–
C-42′, C-43′ or C-44′	16.022		–
	16.066		–
	16.139		–
C-45′	17.681	17.665	−16
C-36′	25.699	25.632	−67
C-5′, C-9′, C-13′, C-17′, C-21′, C-25′,	26.476		–
C-29′ and C-33′	26.658		–
	26.692		–
	26.702		–
	26.753		–
C-1′	27.454	27.354	−100
C-4′, C-8′, C-12′, C-16′, C-20′, C-24′,	39.681	39.647	−34
C-28′, C-32′	39.717	39.699	−18
	39.739		–
C-2′, C-6′, C-10′, C-14′, C-18′, C-22′,	118.084		–
C-26′, C-30′, C-34′	123.790		–
	124.181		–
	124.391		–
C-6	132.009		–
C-3′, C-7′, C-11′, C-15′, C-19′, C-23′,	134.883		–
C-27′, C-31′, C-33′, C-35′	134.911		–
	134.931		–
	134.939		–
	134.976		–
	135.418		–
C-3	140.556		–
C-2	140.959		–
C-5	147.948	147.932	−16
C-1	187.687		–
C-4	187.832		–

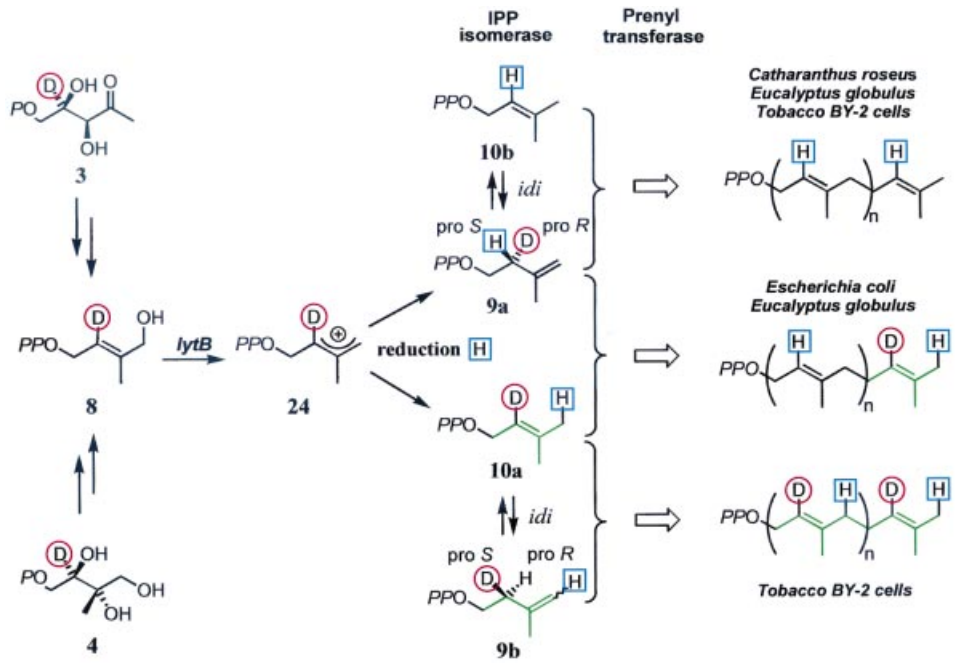
from carbon atoms C-4′, C-8′, C-12′, C-16′, C-20′, C-24′, C-28′ and C-32′ of the prenyl chain. This was in accordance with the presence of a deuterium on C-6′, C-10′, C-14′, C-18′, C-22′, C′-26, C-30′ and/or C-34′ in the prenyl chain, i.e. again in isoprene units derived from IPP. γ Shifts were measured for the *trans* (−67 p.p.b.) and *cis* (−16 p.p.b.) terminal methyl groups (C-36′ and C-45′), indicating the presence of a deuterium atom on the C-34′ carbon atom, i.e. in the DMAPP-derived isoprene unit. Additional γ shifts (approx. −16 p.p.b.) were observed for the signals of the methyl groups at C-37′, C-38′, C-39′, C-40′, C-41′, C-42′, C-43′ and/or C-44′ in accordance with the presence of a deuterium atom at C-6′, C-10′, C-14′, C-18′, C-22′, C-26′ and/or C′-30. Because of the overlapping of the signals, they could not be examined in detail.

In the ^{13}C-NMR spectrum of *cis*-phytoene (**22**) (Table 1), which is a symmetrical molecule, γ shifts were observed for the *cis* (−64 p.p.b.) and *trans* (−16 p.p.b.) terminal methyl group signals, as in the spectrum of plastoquinone (**23**), indicating the presence of a deuterium atom at C-2. Four shifted signals (−76 p.p.b., −30 p.p.b., −94 p.p.b. and −51 p.p.b.) were observed for C-3, C-9, C-13 and C-15, corresponding to β shifts induced by the presence of deuterium atoms at C-2, C-10, C-14. Finally, γ shifts for the methyl group Me-5 (+16 p.p.b.), Me-9 (+15 p.p.b.) and Me-13 (+13 p.p.b.) confirmed the presence of a deuterium at C-6, C-10 and C-14.

Presence of a deuterium in the β or γ position did not modify significantly the relaxation times of ^{13}C. According to the relative intensities of the shifted and non-shifted ^{13}C signals, a mean of 15% deuterium abundance was estimated for each measurable labelled position in *cis*-phytoene (**22**), as well as in plastoquinone (**23**). This deuterium abundance was quite similar in all isoprene units, irrespective of whether they were derived from DMAPP (**10**) or from IPP (**9**).

DISCUSSION

Important deuterium retention (approx. 15%) was observed on all carbon atoms corresponding to C-2 of IPP (**9**) and DMAPP (**10**) in all isoprene units, whether they were derived from IPP (**9**) or from DMAPP (**10**). The deuterium retention from [4-^{2}H]-DX or from [3-^{2}H]ME in the DMAPP-derived isoprene units, accompanied by a deuterium loss in those derived from IPP [16,17], was considered in *E. coli* to be the signature of the branching in the MEP pathway, with two different routes leading to IPP and DMAPP, starting from an unidentified MEP metabolite [15]. Such a weak, but significant, deuterium retention was reported for the biosynthesis of the monoterpene cineol from [4-^{2}H]DX in eucalyptus twigs, suggesting that branching may occur in higher plants [28]. The present study demonstrated that deuterium retention from [4-^{2}H]DX was much more exemplified in TBY-2 plastid isoprenoids than in the eucalyptus monoterpene. In addition, the labelling pattern was quite different from those observed in the *E. coli* and eucalyptus isoprenoids. In the TBY-



Scheme 3 Hypothetical biogenesis of IPP (9) and DMAPP (10) in the MEP pathway: the last steps

IDI and prenyl transferase remove the pro-*R* hydrogen of IPP.

2 cells, deuterium retention from [4-^{2}H]DX (**11**) in the DMAPP-derived isoprene units of plastoquinone (**23**) and phytoene (**22**) most likely corresponds to the same biosynthetic sequence leading to DMAPP as that found in *E. coli* (DMAPP branch). In the branch leading to IPP, as found in *E. coli* (IPP branch), the deuterium from [4-^{2}H]DX is lost. Deuterium retention in acyclic isoprenoids on carbon atoms corresponding to C-2 of IPP is possible, when IPP is synthesized by isomerization of C-2 deuterium-labelled DMAPP, assuming that the enantioselectivity of the IDI and the prenyl transferase in tobacco are identical with those of the enzymes examined in other living organisms. All known IDIs and *trans*-prenyl transferases, isolated from animals, fungi or bacteria, possess the same enantioselectivity ([43,44], and literature cited therein). They remove the pro-*R* hydrogen atom of IPP. Such an IPP isomerization into DMAPP was not observed in *E. coli*. Indeed, it had been shown that the *idi* gene present in *E. coli* is not essential, since its disruption was not lethal [45], whereas insertional inactivation of the single *idi* gene in *Saccharomyces cerevisiae* proved to be lethal [46]. When measured in crude extracts, IDI activity in *E. coli* is close to the limit of detection (A. Hemmerlin and T.J. Bach, unpublished work). Overexpression of the *E. coli idi* gene revealed that the corresponding 20.5 kDa recombinant enzyme is less active than yeast IDI, indicated by a 20-fold-lower V_{max}, which, however, might possibly be compensated for by a lower K_m [45]. The non-essential nature of IDI in *E. coli* was confirmed further by a recent study [15], in which a genetically engineered *E. coli* strain having the DXP synthase gene disrupted, and being transformed with a set of genes coding for enzymes converting MVA into IPP, was only able to grow on MVA when IDI was active, and did not grow on MVA after deletion of the *idi* gene. This strain was, however, capable of growing on ME in the absence of an active IDI [15].

The different labelling patterns of IPP (**9**) and DMAPP (**10**) observed in *E. coli* after incorporation of [4-^{2}H]DX or [3-^{2}H]ME, and in plant systems after feeding of [4-^{2}H]DX, are most likely to result from the last step of the MEP pathway, catalysed by the *lytB* gene product and from the reactions catalysed by the IDI and the prenyl transferase (Scheme 3). In *E. coli*, hydrogen atoms at C-1, C-4 and C-5 of ME are fully conserved and are found on carbon atoms C-4, C-1 and C-5 of IPP or DMAPP respectively [17,47,48]. Feeding *Zymomonas mobilis* with [1-^{2}H]glucose as the only source of carbon and energy was a convenient means to synthesize *in vitro* ^{2}H-labelled NAD(P)H, and, accordingly, other reducing cofactors [49]. Analysis of the deuterium content in the triterpenoids of the hopane series from this bacterium revealed the presence of two reduction sites in the formation of the isoprene units: one, which was expected, on carbon atoms corresponding to C-4 of IPP and DMAPP, which was the signature of the NADPH-dependent reduction step catalysed by the DXP isomero-reductase, with the other being an unidentified site on carbon atoms corresponding to C-2 of the isoprene units. The latter step most likely corresponds to the reduction occurring during the conversion of methylbutenediol diphosphate (**8**) into IPP [23]. Indeed, the intermediate (**24**) formed after an elimination reaction on the methylbutenediol diphosphate (**8**) would afford either IPP after reduction at C-2, or DMAPP after reduction at C-4 (Scheme 3). The deuterium from [4-^{2}H]DX or from [3-^{2}H]-ME is conserved in the diphosphate (**8**). Elimination of water or of a better leaving group from diphosphate (**8**) would afford an allylic cationic intermediate (**24**), which upon reduction at C-2 from the *si* face affords (2*R*)-[2-^{2}H]IPP (**9a**) or, upon reduction at C-4, affords [2-^{2}H]DMAPP (**10a**). The deuterium is thus conserved in DMAPP during the biosynthesis of acyclic prenyl chains by the prenyl transferases. Isomerization of DMAPP isotopomer (**10a**) affords (2*S*)-[2-^{2}H]IPP (**9b**), which also retains its deuterium during the chain elongation, with only the pro-*R* proton being eliminated. In contrast, the deuterium of (2*R*)-[2-^{2}H]IPP (**9a**) is lost either after isomerization by the IDI into non-labelled DMAPP (**10b**) or after chain elongation by the prenyl

transferase, both enzymes removing the pro-*R* deuterium and yielding finally unlabelled isoprene units.

IDI is localized unequivocally in *Arabidopsis thaliana* in the cytosol, in the mitochondria and in the plastids [50]. This enzyme is of eukaryotic origin, most likely using alternative methionine codons for initiation of translation, thereby allowing the synthesis of isoenzymes containing N-terminal extensions for targeting into compartments. In the cyanobacterium *Synechocystis* PCC 6803, no open reading frame could be identified coding for a related IDI [50]. This holds true for all the bacterial genomes sequenced so far, with the exception of *E. coli* and *Mycobacterium tuberculosis*, but in these two latter cases it cannot be excluded that *IDI* may have been secondarily acquired from a eukaryotic host [50]. However, not only the functional identification of a structurally and mechanistically different IDI (referred to as class II IDI) in *Streptomyces* sp. strain CL190, but also the detection of class II IDI homologues (29–46 % identity) in a variety of Gram-positive bacteria, cyanobacteria, and even archaebacteria [51], gives rise to a new topic of debate with regard to the 'non-essential' and non-limiting role of the IDI reaction, at least under specific environmental and physiological conditions. In any case, by application of a colour screen, using *E. coli* cells transformed with a set of genes permitting the synthesis of lycopene from GGPP, it revealed that further transformation with *idi* genes from various organisms always led to higher production of this carotenoid [50,52,53].

Previously, it was assumed that, in peppermint secretory cells, IPP is the only terminal product of the MEP pathway [54], but in a more recent study, Lange et al. [55] discuss those earlier observations much less restrictively and do not rule out that downstream from MEP there is a bifurcation that gives rise to both IPP and DMAPP, as found in *E. coli* [15], on the basis of an accumulation of both IPP and, to a lesser degree, of DMAPP in 2-(dimethylamino)ethyl diphosphate-treated cells. 2-(Dimethylamino)ethyl diphosphate had previously been identified as a transition-state analogue of the intermediate in the IDI-catalysed reaction [56]. Arigoni et al. [18] postulated that DMAPP is not a committed precursor of IPP during terpenoid biosynthesis from DXP in *C. roseus*, but that is not contradictory to our observations with TBY-2 cells. Furthermore, a slight retention of deuterium on the carbon atom derived from C-2 of IPP might have occurred in the cineol from *E. globulus*, but could not be unambiguously confirmed by ^{13}C-NMR [28].

In conclusion, the results of the [4-^{2}H]DX incorporation into the plastidial isoprenoids of TBY-2 cells are in agreement with the presence of an active DMAPP branch of the MEP pathway and of a plastidial class I IDI [50]. They do not give any indication on the presence of the IPP branch, as this one branch is expected to be 'transparent' in this type of investigation via its characteristic deuterium loss. The isotope abundance was much lower in the labelled positions of the plastid isoprenoids (approx. 15 %) than in the deuterium-labelled DX used as precursors (approx. 75 % at C-4). Such a dilution may have occurred via two different mechanisms: *de novo* synthesis of unlabelled DMAPP and IPP from unlabelled sucrose, which represented the main carbon source in the culture medium, and/or biosynthesis of unlabelled IPP from [4-^{2}H]DX via the IPP branch of the MEP pathway, and isomerization into unlabelled DMAPP. In any case, TBY-2 cells represent an excellent material for the investigation of this part of the MEP pathway, which was not detected in *C. roseus* and was hardly detectable in *E. globulus* [28].

We thank Dr R. Graf and Mr J. D. Sauer for the NMR measurements, and Mr R. Huber and Mrs E. Mastio for the MS analyses. This work was supported by a grant to M. R. from the 'Institut Universitaire de France'.

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Received 27 February 2002/26 April 2002; accepted 15 May 2002
Published as BJ Immediate Publication 15 May 2002, DOI 10.1042/BJ20020337