Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway

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Abstract Isopentenyl diphosphate (IPP) is the biological C_5 precursor of isoprenoids. By labeling experiments using [1-^{13}C]glucose, higher plants were shown to possess two distinct biosynthetic routes for IPP biosynthesis: while the cytoplasmic sterols were formed via the acetate/mevalonate pathway, the chloroplast-bound isoprenoids (β-carotene, lutein, prenyl chains of chlorophylls and plastoquinone-9) were synthesized via a novel IPP biosynthesis pathway (glyceraldehyde phosphate/pyruvate pathway) which was first found in eubacteria and a green alga. The dichotomy in isoprenoid biosynthesis in higher plants allows a reasonable interpretation of previous odd and inconclusive results concerning the biosynthesis of chloroplast isoprenoids, which so far had mainly been interpreted in the frame of models using compartmentation of the mevalonate pathway.

Key words: Carotenoid; ^{13}C-NMR-spectroscopy; Glyceraldehyde phosphate; Pyruvate; Isopentenyl diphosphate; Phytol; Plastoquinone-9; Sterol

1. Introduction

Isoprenoids belong to a highly studied natural product family [[1] and references cited therein]. Their universal biological precursor is isopentenyl diphosphate (IPP) [[2,3] and references cited therein]. Its formation, first investigated in yeast and in mammalian liver tissue, had been described as the acetate/mevalonate pathway which was later accepted as ubiquitous in all living organisms [2,3]. Recently, a totally different route, in which mevalonate is not a precursor and where IPP is formed from glyceraldehyde phosphate and pyruvate, was found in bacteria and green algae [4–6].

In plant cells, isoprenoids like carotenoids and phytol, the side chain of chlorophylls, are synthesized and accumulated inside the plastid compartment, whereas sterols are formed in the cytoplasm/endoplasmatic reticulum [7–11]. Mevinolin, a highly specific inhibitor of mevalonate formation (i.e. of formation of IPP), strongly inhibits sterol biosynthesis in higher plants (vascular plants) [12–14]. However, the biosynthesis of chlorophylls (containing a phytol side chain), carotenoids and plastoquinone-9 (with a C_{45} prenyl side chain) was unaffected by mevinolin [12–14]. Therefore the question arises if in higher plants mevalonate is involved in the synthesis of plastidic isoprenoids or if plastids possess a separate IPP biosynthetic pathway. Recent findings on a novel IPP biosynthesis in bacteria [4] and a green alga [5] suggested that plastids, being of prokaryotic endosymbiotic origin, might possess their own mevalonate-independent IPP biosynthesis. This topic is matter of the investigation described here.

2. Experimental

2.1. General procedure

Three species of higher plants were investigated for the early steps of biosynthesis of plastidic isoprenoids: two monocotyledonous plants (barley, *Hordeum vulgare* L., and duckweed, *Lemna gibba* L.) and one dicotyledonous plant (carrot, *Daucus carota* L.). All of them were grown heterotrophically in sterile cultures on D-[1-^{13}C]glucose (Omicron Biochemical Inc., South Bend, IN) as sole carbon and energy source. After isolation and purification of the sterols, phytol, β-carotene, lutein and plastoquinone-9, the ^{13}C-labeling pattern of the different compounds were determined by ^{13}C-NMR-spectroscopy. In the labeling experiments with *H. vulgare* and *D. carota* only phytol, sitosterol and stigmasterol were analyzed while in the *L. gibba* experiment, which was performed at a larger scale, also β-carotene, lutein, and plastoquinone-9 could be analyzed in addition to phytol, sitosterol and stigmasterol.

2.2. Growth conditions

Axenic cultures of duckweed (*L. gibba*) were cultivated for 12 days heterotrophically on a mineral medium [15] containing labelled [1-^{13}C]glucose (0.3% w/v, 10% isotopic abundance). Low light (Osram L fluorescent lamps, 40 μmol photons m^{-2} s^{-1}) stimulated growth and greening of plants. One hundred and fifty grams of fresh weight was harvested from a 3.6 l culture.

Green tissue cultures of carrot (*D. carota*) were grown for 24 days under stimulatory light (40 μmol photons m^{-2} s^{-1}) on a standard medium [16] containing 2 ppm indole acetic acid, 1 ppm kinetin and 1 ppm 2,4-dichloro-phenoxy acetic acid. Sucrose (3% w/v) was supplemented by [1-^{13}C]glucose (0.35% w/v, isotopic abundance 99%) in order to yield 10% isotopic abundance in C-1 of the metabolized hexose. One hundred and fifty grams of fresh weight was harvested.

Barley grains (*H. vulgare*, 110 g) were germinated aseptically after pretreatment with 50% H_2SO_4 for 1 h. After 24 h, 700 embryos were dissected from the endosperm and placed in petri dishes in nutrient solution [17] (500 ml total volume) containing [1-^{13}C]glucose (1% w/v, 15% isotopic abundance) instead of sucrose [17]. The embryos were kept and grown under continuous red light (λ_{max} 660 nm, 5 μmol photons m^{-2} s^{-1}) for 5 days. Four hundred pale green primary leaves (17 g, fresh weight) were harvested. In order to reduce photosynthesis, all plants were grown under very low irradiances, which permitted greening of the plants but did not allow a sufficient phototrophic growth.

2.3. Extraction, purification and analysis of isoprenoid lipids

In all experiments, the extraction of sterols, phytol, β-carotene, lutein and plastoquinone-9 was performed as previously described [5]. The acetylated sterol fractions were separated by silver ion TLC and reverse-phase HPLC into acetates of stigmasterol, sitosterol and campesterol as described for the separation of the acetates of *Scenedesmus* sterols [5]. Due to the presence of large amounts of long-chain alcohols, final purification of phytol acetate had to be performed by TLC on silver nitrate-impregnated silica gel (cyclohexane/toluene 85:15, 3 migrations). The assignment of ^{13}C-NMR chemical shifts for sitosterol acetate and stigmasterol acetate was done by comparison with literature data [18]. NMR-measurements were performed on a Bruker W 400 Spectrometer, and the evaluation of isotopic enrichments was done as previously described [4,5] by comparison with

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Abbreviations: IPP, isopentenyl diphosphate; NMR, nuclear magnetic resonance; TLC, thin layer chromatography

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spectra of unlabeled reference compounds. The natural abundance of ^{13}C amounts to 1.1%. Higher abundances represent a labeling of the C-atoms with ^{13}C.

3. Results and discussion

From the applied [1-^{13}C]glucose all isoprenic units, found in each analyzed isoprenoid (e.g. 4 in phytol, 8 in β-carotene) were identically and significantly labeled with ^{13}C (Fig. 1). For the sake of clarity only average isotopic abundances were indicated for each of the five carbon atoms of the isoprenic skeleton represented by IPP (Table 1). Assuming a main breakdown of [1-^{13}C]glucose by glycolysis, metabolites such as [2-^{13}C]acetyl-CoA, which is the direct precursor in the mevalonate IPP pathway as well as [3-^{13}C]glyceraldehyde 3-phosphate and [3-^{13}C]pyruvate, which are the precursors in the alternative IPP pathway, will show up (Fig. 2a).

3.1. ^{13}C-labeling of sterols

According to the acetate/mevalonate pathway, IPP is distinctly labeled from [1-^{13}C]glucose at carbon positions C-2, C-4 and C-5 (Fig. 2b). Indeed, the main ^{13}C-labeling pattern found in all three higher plants (*L. gibba*, *H. vulgare* and *D. carota*) for the isoprenic units of the cytoplasmic sterols (Fig. 2a and Table 1) was in accordance, as expected, with the formation of the C_5 isoprenic skeleton by condensation of [2-^{13}C]acetyl-CoA obtained by glucose breakdown via glycolysis. The differences in the ^{13}C-abundance of C-2, C-4 and C-5 of the isoprenic units in sterols between the three higher plants (Table 1) were due to differences in the isotopic abundance of the applied [1-^{13}C]glucose. A slight ^{13}C-labeling was also detected in all carbon atoms derived from C-1 of IPP (Table 1 and Fig. 2a, small black dots). This might be explained by incorporation of $^{13}CO_2$ (derived e.g. from the oxidative pentose phosphate pathway) via leucine catabolism or in the mevalonate shunt by carboxylation of methyl crotonyl coenzyme A [19]. The label from $^{13}CO_2$, being formed from [1-^{13}C]glucose, shows up in C-1 of the degradation product acetoacetate which is readily incorporated into cytoplasmic plant isoprenoids [20,21]. An alternative reason for the partial labeling of C-1 of isoprenic units would be a significant incorporation of isopentenyl diphosphate, which was synthesized in chloroplasts via the new non-mevalonate pathway (see below) into sterols. This interpretation would, however, be in contradiction with the isoprenoid biosynthesis observed in *Ginkgo biloba*, in which case no transfer of plastidic isoprenoid precursors into sterol biosynthesis in the cytoplasm was observed [22].

Table 1
Average ^{13}C-isotopic abundances measured in isoprenic units (represented by IPP) of higher plant isoprenoids after feeding of [1-^{13}C]glucose with differential percentage of isotopic abundance

Isoprenoid	^{13}C-isotopic abundances in C-atoms of IPP				
	C-1	C-2	C-3	C-4	C-5
Lemna gibba: carbon source [1-^{13}C]glucose (10% isotopic abundance)					
Sitosterol	2.0	**2.5**	1.5	**2.7**	**3.0**
Stigmasterol	2.0	**2.7**	1.4	**2.8**	**2.8**
Phytol	**3.1**	1.4	1.5	1.6	**3.1**
β-Carotene	**1.9**	1.0	0.9	1.2	**1.9**
Lutein	**2.6**	1.4	1.3	1.4	**2.8**
Plastoquinone-9	**1.9**	1.1	1.1	1.1	**2.0**
Hordeum vulgare: carbon source [1-^{13}C]glucose (15% isotopic abundance)					
Sitosterol	1.7	**3.8**	1.3	**3.9**	**4.2**
Stigmasterol	1.4	**3.3**	1.3	**3.2**	**3.1**
Phytol	**4.7**	1.0	1.1	1.4	**4.5**
D. carota: carbon source sucrose (3% w/v)+[1-^{13}C]glucose (0.353% w/v, 99% isotopic abundance)					
Sitosterol	2.5	**5.1**	1.7	**4.9**	**5.5**
Stigmasterol	1.7	**3.8**	1.1	**4.2**	**4.0**
Phytol	**3.2**	1.3	1.4	1.7	**3.2**

Numbering of the carbon atoms 1–5 of IPP refers to Fig. 2b,c. The numbers in bold face represent a highly significant ^{13}C-abundance.

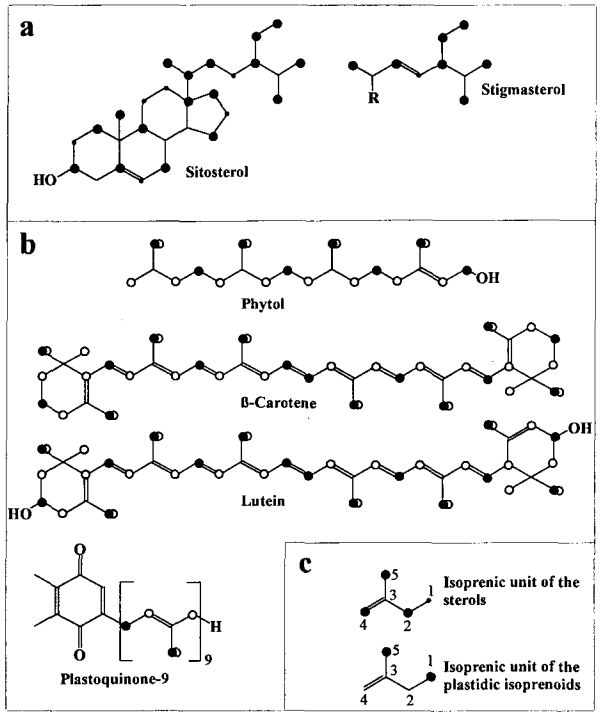

Fig. 1. Labeling pattern (●) of isoprenoids found after heterotrophic growth of *L. gibba* L., *D. carota* L. and *H. vulgare* L. on [1-^{13}C]glucose. a: The ^{13}C-labeling of sitosterol and stigmasterol, which was obtained from all three plants, is indicated. b: ●, ^{13}C-labeling of phytol (obtained from all three plants) as well as from β-carotene, lutein and plastoquinone-9 (from *L. gibba*). ○, The expected ^{13}C-labeling pattern if the compounds would have been formed via the acetate/mevalonate pathway. c: The ^{13}C-labeling pattern of isoprenic units is indicated as it can be deduced from (a) and (b).

3.2. ^{13}C-labeling of plastidic isoprenoid lipids

The ^{13}C-labeling patterns of phytol (*L. gibba*, *D. carota*, *H. vulgare*), β-carotene, lutein (both *L. gibba*) and the isoprenoid side chain of plastoquinone-9 (*L. gibba*) are indicated in Table 1 and Fig. 1b and 2c.

In contrast to the isoprenic units of the cytoplasmic sterols, those of phytol (from *L. gibba*, *D. carota* and *H. vulgare*) as well as those of β-carotene, lutein and the C_{45} prenyl side chain of plastoquinone-9 (all from *L. gibba*) were labeled in a quite different way. They showed the same unusual ^{13}C-labeling as those detected for isoprenoids from bacteria [4] and for the green algal species *Scenedesmus obliquus* [5], *Chlorella vulgaris* and *Chlamydomonas reinhardtii* [Schwender et al. unpublished results] when grown on [1-^{13}C]glucose. This

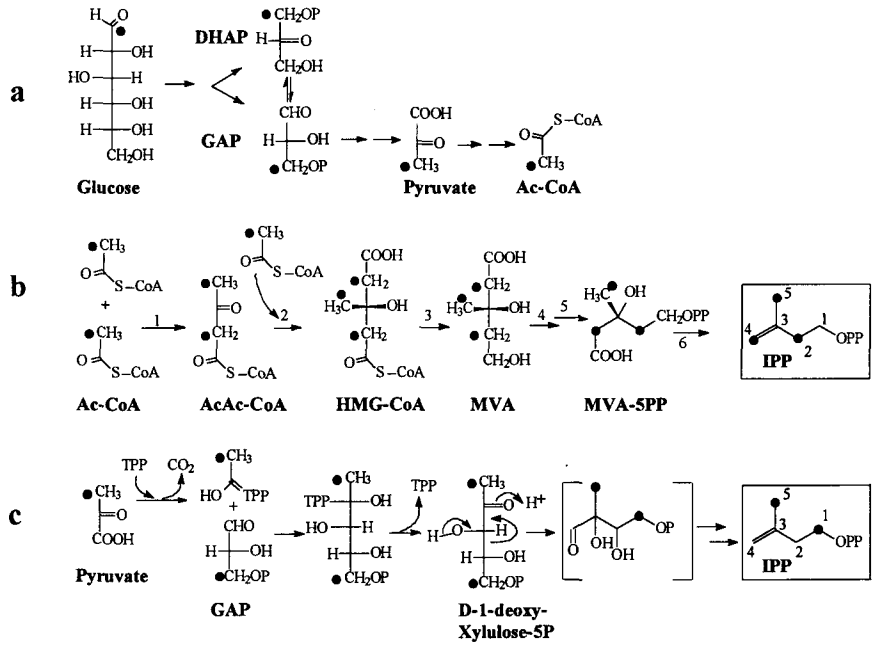

Fig. 2. Glycolysis of [1-^{13}C]glucose and formation of isopentenyl diphosphate (IPP) via two different pathways. ●, The ^{13}C-label, which arises from feeding of [1-^{13}C]glucose to plant seedlings and cell cultures. a: [3-^{13}C]glyceraldehyde 3-phosphate (GAP) and [3-^{13}C]pyruvate derive from [1-^{13}C]glucose via glycolysis. [2-^{13}C]acetyl-CoA derives from [3-^{13}C]pyruvate by the pyruvate dehydrogenase complex. b: According to the classical acetate/mevalonate pathway, observed in sterol biosynthesis, IPP is built up from acetyl-CoA ([2-^{13}C]acetyl-CoA) by the following main reactions: (1) Two molecules acetyl-Coenzyme A (Ac-CoA) form acetoacetyl-CoA (AcAc-CoA). (2) Addition of a third Ac-CoA yields hydroxymethylglutaryl-CoA (HMG-CoA). (3) HMG-CoA is reduced to mevalonic acid (MVA). (4 and 5) MVA is phosphorylated twice at C-5. (6) MVA-5-diphosphate yields IPP via a decarboxylation/elimination step. c: The novel IPP-biosynthesis pathway is based on ^{13}C-incorporation studies performed with bacteria [4,6,23] and the green alga *S. obliquus* [5]. According to this pathway, also found for the formation of chloroplast isoprenoids, the addition of a C_2 precursor (derived from pyruvate, most likely by formation of hydroxyethyl thiamine, TPP = thiamine diphosphate) to a C_3 precursor (glyceraldehyde 3-phosphate, GAP) yields a first C_5 intermediate, most likely D-1-deoxyxylulose-5-phosphate [23]. The carbon skeleton of this intermediate or another related C_5-derivative undergoes a rearrangement reaction which provides the branched carbon skeleton of IPP.

indicates that all the plastidic isoprenoids of duckweed, carrot and barley were formed via the new non-mevalonate IPP pathway from pyruvate and glyceraldehyde 3-phosphate as shown in Fig. 2c [4–6].

3.3. Degree of ^{13}C-abundance in labeled positions of isoprenoids

Pyruvate and glyceraldehyde 3-phosphate are mainly obtained by glycolysis. In the Embden-Meyerhof-Parnas pathway, glucose is converted into fructose 1,6-bisphosphate which is cleaved into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Fig. 2a). As these two triose phosphates are interconvertible via the triosephosphate isomerase, half of the isotopic abundance of glucose would be expected in all triose phosphate derivatives if only glycolysis occurs (compare Table 1). Since the labeling degree of isoprenoids was somewhat lower, loss of ^{13}C-labeling occurred most probably by liberation of $^{13}CO_2$ via the oxidative pentose phosphate pathway as already observed for some bacteria and the green alga *Scenedesmus* [4,5].

3.4. Compartmentation of IPP biosynthesis in higher plants

According to our ^{13}C-labeling experiments the cytoplasmic sterols are formed in all three higher plants via the acetate/mevalonate pathway, whereas the plastidic isoprenoids are synthesized via a new non-mevalonate IPP pathway. In this new pathway, IPP is formed from pyruvate and glyceraldehyde 3-phosphate [6] yielding, after condensation, 1-deoxyxylulose-5-phosphate, which is most likely the first C_5 intermediate in the alternative IPP biosynthesis pathway [23] (Fig. 2c). A transposition [4] yields finally the branched isoprenic skeleton from the straight-chain deoxypentulose framework (Fig. 2c). Our ^{13}C-labeling experiments described here strongly suggest that this mevalonate-independent IPP route is not restricted to bacteria and green algae, but also possesses a wide distribution in plastids of higher plants. Further evidence for the presence of the new IPP biosynthesis pattern in higher plants was also found in *Ginkgo biloba* for the biosynthesis of the unusual diterpenes, the ginkgolides [22], and for the formation of taxane diterpenes in *Taxus* [24]. The subcellular localization of the biosynthesis of these diterpenoids is, however, not clear and has hitherto not yet been studied. In our ^{13}C-labeling experiments with higher plants the novel IPP-biosynthesis pathway is clearly associated with the typical plastidic isoprenoids, which are formed and accumulated in all green plastids (chlorophyll phytol in *D. carota* and *H. vulgare* as well as β-carotene, lutein, chlorophyll phytol and prenyl chain of plastoquinone-9 in *L. gibba*).

The segregation of two different IPP-biosynthetic pathways in higher plants into the cytoplasmic compartment on the one hand and the plastidic compartment on the other hand explains some of our former observations of the non-inhibition of accumulation of plastidic isoprenoids by mevinolin [12–14] as well as some odd and inconclusive former results in literature concerning the ^{14}C-labeling of plant isoprenoids. For instance it had been shown for seedlings of maize, oat, barley,

pea, lettuce and pine [25–29] that plastidic isoprenoid compounds, such as β-carotene, the phytol side chain of chlorophylls and the prenyl side chain of plastoquinone-9, were preferentially labeled from $^{14}CO_2$. In contrast, [^{14}C]mevalonate labeled only the cytosolic sterols at good rates [25–29]. In addition, it had been demonstrated that in maize ^{14}C-labeled pyruvate was incorporated into β-carotene at good rates, whereas acetate was not [30]. These older results can now be fully explained by the occurrence of the new alternative glyceraldehyde phosphate/pyruvate pathway of IPP formation in higher plants as described here. This pathway had completely been overlooked in the past. From the various evidences reported here it appears that in higher plants the chloroplasts, the presumed prokaryotic endosymbionts, have maintained and preserved their 'bacterial' IPP biosynthesis pathway. Further studies with isolated chloroplasts and etioplasts from mosses, ferns and higher plants will be performed in order to demonstrate that the new biosynthetic pathway of IPP formation is common to all plants and linked to the plastid compartment.

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