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Review

Isoprenoid biosynthesis *via* 1-deoxy-D-xylulose 5-phosphate/2-*C*-methyl-D-erythritol 4-phosphate (DOXP/MEP) pathway✪

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Higher plants, several algae, bacteria, some strains of *Streptomyces* and possibly malaria parasite *Plasmodium falciparum* contain the novel, plastidic DOXP/MEP pathway for isoprenoid biosynthesis. This pathway, alternative with respect to the classical mevalonate pathway, starts with condensation of pyruvate and glyceraldehyde-3-phosphate which yields 1-deoxy-D-xylulose 5-phosphate (DOXP); the latter product can be converted to isopentenyl diphosphate (IPP) and eventually to isoprenoids or thiamine and pyridoxal. Subsequent reactions of this pathway involve transformation of DOXP to 2-*C*-methyl-D-erythritol 4-phosphate (MEP) which after condensation with CTP forms 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME). Then CDP-ME is phosphorylated to 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate (CDP-ME2P) and to 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate (ME-2,4cPP) which is the last known intermediate of the DOXP/MEP pathway. For-

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Abbreviations: DOXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; MVA, mevalonate; for other abbreviations see Legend to Fig. 2 and Fig. 3.

The names of the pathway – "non-mevalonate" or "alternative" found in the literature should not be used any more. According to the recommendation of the Terpnet (Barcelona, 1999) the following nomenclature is acceptable: DOXP/MEP, MEP or Rohmer's-pathway.

mation of IPP and dimethylallyl diphosphate (DMAPP) from ME-2,4cPP still requires clarification.

This novel pathway appears to be involved in biosynthesis of carotenoids, phytol (side chain of chlorophylls), isoprene, mono-, di-, tetraterpenes and plastoquinone whereas the mevalonate pathway is responsible for formation of sterols, sesquiterpenes and triterpenes. Several isoprenoids were found to be of mixed origin suggesting that some exchange and/or cooperation exists between these two pathways of different biosynthetic origin. Contradictory results described below could indicate that these two pathways are operating under different physiological conditions of the cell and are dependent on the developmental state of plastids.

INTRODUCTORY REMARQUES

Isoprenoids are a broad group of natural compounds with carbon skeleton built of branched C_5 isoprenoid units. Isoprenoids are widespread among living organisms, both eukaryotes and prokaryotes, functionally important for many aspects of cell metabolism, and they influence also membrane structure and function. Many important biological roles of numerous isoprenoids have been described in: photosynthesis (carotenoids, chlorophylls, plastoquinone), respiration (ubiquinone), hormonal regulation of metabolism (sterols), regulation of growth and development (giberellic acid, abscisic acid, brassinosteroids, cytokinins, prenylated proteins), defense against pathogen attack, intracellular signal transduction (Ras proteins), vesicular transport within the cell (Rab proteins) and as coenzymes (dolichols). Several isoprenoids are also known to influence membrane structure (sterols, dolichols, carotenoids etc.) (Sacchettini & Poulter, 1997; Bach *et al.*, 1999).

The early steps of isoprenoid biosynthesis have been studied first *in vitro* using cell-free homogenates obtained from rat liver and yeast cells by Bloch and Lynen (Chaykin *et al.*, 1958, Lynen *et al.*, 1958). The specific precursor of all isoprenoids, mevalonate (MVA) was found to be synthesized by the condensation of three acetyl-CoA molecules *via* acetoacetyl-CoA and 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA), yielding after phosphorylation and decarboxylation isopentenyl diphosphate (IPP). All the enzymes involved in the MVA pathway were isolated and studied in many animal and plant systems, however some results obtained with the labeled precursor – [^{3}H]mevalonate were difficult to explain. Current biosynthetic evidence cames from the application of ^{13}C-labeled precursors and subsequent exact NMR analysis of the position of the ^{13}C-atoms incorporated within the isoprenoid carbon skeleton. These results suggest the existence of an MVA-independent pathway for IPP formation in bacteria (Rohmer *et al.*, 1993), green algae (Lichtenthaler *et al.*, 1995; Schwender *et al.*, 1996) and higher plants (Lichtenthaler *et al.*, 1997; Schwender *et al.*, 1997) and also in the malaria parasite *Plasmodium falciparum* (Jomaa *et al.*, 1999).

On the basis of labeling patterns (Fig. 1) of isoprenoid carbon skeletons derived from ^{13}C-labeled metabolites of glycolysis as well as [^{13}C]acetate, it has been shown that IPP in chloroplasts is synthesized from pyruvate and glyceraldehyde-3-phosphate and not from MVA.

BIOSYNTHESIS OF THE ISOPRENOID PRECURSOR IPP *VIA* THE NOVEL, DOXP/MEP PATHWAY

As suggested by ^{13}C-labeling experiments, the pathway starts with the addition of a pyruvate-derived C_2-unit to glyceraldehyde-3-phosphate (GA-3-P) (Rohmer *et al.*, 1993; Schwender *et al.*, 1996) (Fig. 2). This was supposed to take place in a transketolase-type enzymatic reaction. After TPP-catalyzed decarboxylation of pyruvate, the TPP-bound acetaldehyde is added to the carbonyl group of

GA-3P yielding 1-deoxy-D-xylulose 5-phosphate (DOXP) as the first intermediate (Schwender *et al.*, 1997; Arigoni *et al.*, 1997). The starting enzyme of this pathway is 1-deoxy-D-xylulose 5-phosphate synthase (DXS). The *dxs* gene has been cloned from several higher plants (Lange *et al.*, 1998; Bouvier *et al.*, 1998), *Escherichia coli* (Lois *et al.*, 1998; Sprenger *et al.*, 1997), green alga – *Chlamydomonas* (Lichtenthaler, 1999) and from a strain of *Streptomyces* (Kuzuyama *et al.*, 2000a). The enzyme requires thiamine diphosphate and divalent cations such as Mg^{2+} or Mn^{2+} for its activity (Sprenger *et al.*, 1997; Bouvier *et al.*, 1998). Based on sequence data from nucleic acid data bases it can be concluded that the DXS-like sequences are highly conserved in evolution. DXS-like gene (CLA1) found in *Arabidopsis thaliana* (Mandel *et al.*, 1996) is supposed to be a single copy gene regulated by light. The mutation of the CLA1 impairs the proper development of chloroplasts, arresting these organelles at an early stage of development. In addition, 1-deoxy-D-xylulose is also an intermediate in the biosynthesis of coenzymes thiamine and pyridoxal phosphate (Julliard & Douce, 1991) (Fig. 2). The second enzymatic step – a C–C-skeleton rearrangement and reduction of 1-deoxy-D-xylulose-5-phosphate to 2-*C*-methyl-D-erythritol 4-phosphate (MEP) is catalyzed by *dxr* gene product, namely DOXP-reductoisomerase (DRI or DXR) in the presence of NADPH and Mn^{2+} (Fig. 2). The enzyme transforming DOXP to MEP has been characterized in *E. coli* (Kuzuyama *et al.*, 2000b). A cloning strategy was also developed for isolation of the gene encoding a plant homolog of this enzyme from peppermint (Lange & Croteau, 1999), *A. thaliana* (Schwender *et al.*, 1999), blue-green alga *Synechocystis* (Proteau, 1998) and the parasite *Plasmodium falciparum* (Jomaa *et al.*, 1999). Unlike the microbial reductoisomerase, the plant ortholog encodes a preprotein bearing an N-terminal plastidial transit peptide that directs the enzyme to the plastids. It was found that DXR activity was strongly and specifically inhibited by fosmidomycin, an antibiotic possessing formyl and phosphonate groups in the molecule (Kuzuyama *et al.*, 1998). It was also shown that fosmidomycin inhibits the biosynthesis of carotenoids and chlorophylls in greening leaves, as well as the isoprene emission (Zeidler *et al.*, 1998). Recently it has been reported by Jomaa *et al.* (1999) that fosmidomycin and its derivative inhibit DXR from *P. falciparum* and that this inhibitor cured mice infected with the rodent malaria parasite *P. vickei*. Thus, this inhibitor can be possibly effective in chemotherapy of malaria.

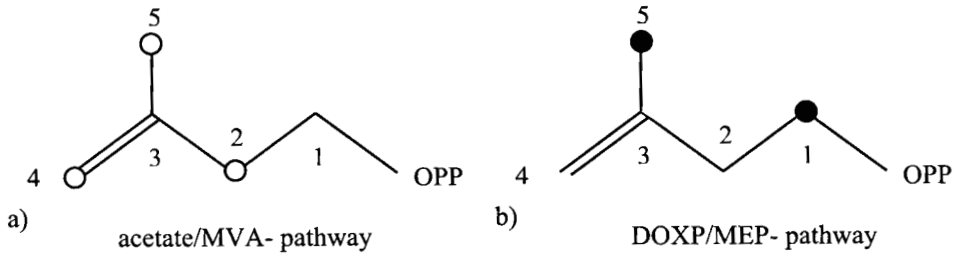

Figure 1. **^{13}C-Labeling patterns of isopentenyl diphosphate (IPP) from [1-^{13}C]glucose formed *via* a, the acetate/MVA pathway (white circles); b, the DOXP/MEP pathway (black circles).**

The further biosynthetic step consists in the conversion of MEP to 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME) in a CTP-dependent reaction by the *ispD* gene product 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol synthase (CMS) (Fig. 2). This enzyme is active in the presence of Mg^{2+} or Mn^{2+} and has been cloned in *E. coli* (Rohdich *et al.*, 1999) and *A. thaliana* (Rohdich *et al.*,

2000a). The following two steps of the pathway were initially identified using bioinformatics methods. Hypothetical candidate, *ispE* gene product, catalyzing the fourth step 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase (CMK) which phosphorylates catalyzes last characterized step in the pathway – the conversion of CDP-ME-2P into 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate (ME-2,4cPP). This product is accumulated under stress conditions in several bacteria (Ostrovsky *et al.*, 1998). The latter enzyme

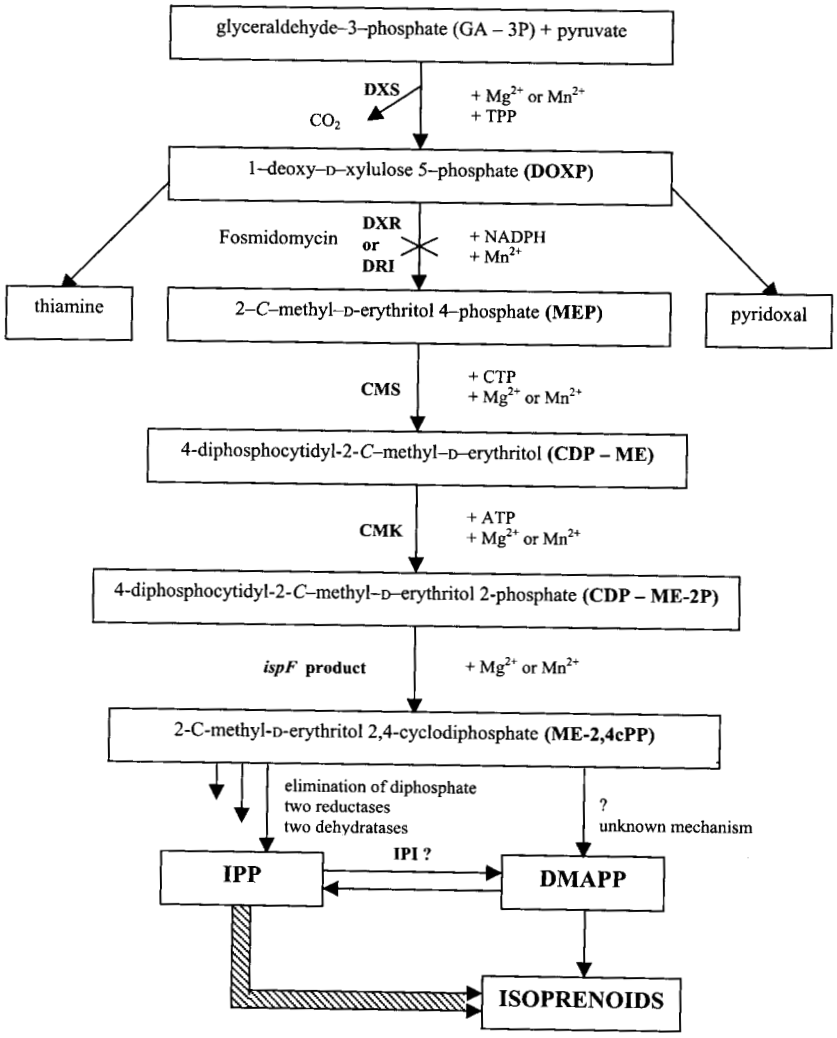

Figure 2. Formation of isopentenyl diphosphate (IPP) *via* the DOXP/MEP pathway (Lichtenthaler, 1998).

Abbreviations: TPP, thiamine diphosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR or DRI, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; CMS, 4-(cytidine-5-diphospho)-2-*C*-methyl-D-erythritol synthase; CMK, 4-(cytidine-5-diphospho)-2-*C*-methyl-D-erythritol kinase; IPI, isopentenyl diphosphate isomerase.

CDP-methyl-D-erythritol (CDP-ME) at the 2-hydroxy group in an ATP-dependent reaction yielding 2-*C*-methyl-D-erythritol-2-phosphate (CDP-ME-2P) has been described recently in *E. coli* (Lüttgen *et al.*, 2000). Plant orthologue (tomato) has also been proposed (Rohdich *et al.* 2000b). The *ispF* gene product (*ispF* gene product) is active only in the presence of Mg^{2+} or Mn^{2+} (Herz *et al.*, 2000). The final steps of the pathway, IPP and DMAPP formation from ME-2,4cPP is still unexplored area. The enzymatic reactions, possibly initiated by intramolecular elimination of diphosphate, require two NADPH-dependent reduc-

tases and two dehydratases yielding IPP which is then isomerized to DMAPP – the starter molecule of isoprenoid biosynthesis (Lichtenthaler *et al.*, 2000). Growing body of evidence indicate that IPP and DMAPP are biosythesized *via* independent mechanisms in the late steps of the presented pathway (Rodriguez-Concepcion *et al.* 2000).

OCCURRENCE OF THE MEP PATHWAY IN DIFFERENT ORGANISMS

The mevalonate-independent pathway for IPP synthesis was first discovered by Flesch & Rohmer (1988) in their studies on biosynthesis of hopanoids (i.e. pentacyclic triterpenic sterol surrogates of different bacterial species) using [^{13}C]acetate. This biosynthetic scheme for IPP formation was later found in *E. coli, Alicyclobacillus acidocaldarius, Methylobacterium organophilum, Zymomonas mobilis* (Flesch & Rohmer, 1988; Rohmer *et al.*, 1993). Several observations showed that in a few Archaebacteria (*Pyrococcus horikoshii, Methanococcus jannaschii, Methanobacterium thermoautotrophicum*) only the mevalonate pathway is operating (Lichtenthaler *et al.*, 2000). In most bacterial strains only one of the two IPP biosynthetic pathways seems to appear. *Helicobacter pylori* may be an exception since its genome contains also a HMG-CoA reductase ortholog (Lichtenthaler *et al.*, 2000).

The ^{13}C-labeling studies showed that in red algae (*Cyanidum*), chrysophyte (*Ochromonas*) and oxygenic photosynthetic blue-green bacteria (*Synechocistis*) cytoplasmic sterols are formed *via* the mevalonate pathway. In contrast, in all cases the plastid-bound isoprenoids, such as phytol, β-carotene, lutein and the side chain of plastoquinone-9 exhibited the MEP-pathway labeling pattern (Schwender *et al.*, 1997). Further studies on the green algae *Scenedesmus obliquus, Chlorella fusca* and *Chlamydomonas reinhardtii* showed that not only the plastidic isoprenoids but also their cytosolic sterols and mitochondrial ubiquinones (*Scenedesmus*) (Schwender *et al.*, 1996; Disch *et al.*, 1998b) were labeled *via* the DOXP/MEP pathway. In addition, the labeled MVA was not incorporated into the sterols of these green algae (Schwender *et al.*, 1997) indicating that these organisms have lost during evolution, or they never possessed, the classical mevalonate pathway. However, it is also possible that, a low rate of biosynthesis *via* the mevalonate pathway might have been undetected by the ^{13}C-labeling technique (Lichtenhaler *et al.*, 1997). In the case of *Euglena gracilis* the plastidic phytol as well as the cytosolic ergosterol were labeled from [1-^{13}C]glucose and from ^{13}C-labeled mevalonate according to the pattern specific for the mevalonate pathway (Disch *et al.*, 1998b).

Investigations with specific intermediates of both pathways such as [1-^{2}H]DOX and [2-^{13}C]MVA showed in *Cyanidum, Ochromonas* and *Euglena* that small amounts of not only [^{13}C]sterols but also [^{13}C]phytol could be formed from [2-^{13}C]MVA while [1-^{2}H]DOX was found to serve as the precursor of both phytol and ergosterol; this points to the possibility of exchange between the two pools of IPP of different biosynthetic origin (Lichtenthaler, 1998) (Fig. 3).

In higher plants the labeling experiments with ^{13}C-labeled mevalonate and ^{2}H-labeled 1-deoxy-D-xylulose, suggested that biosynthesis of IPP occurs at two sites. The first one is located in the cytoplasm operates *via* the mevalonate pathway, and the produced IPP is subsequently distributed to the endoplasmic reticulum and mitochondria for the formation of triterpenoids (including sterols), sesquiterpenoids and the prenyl chain of ubiquinone. The second site of IPP biosynthesis, located to the chloroplasts, produces *via* MEP pathway IPP for the formation of all isoprenoids involved in photosynthesis: phytol, plastoquinone, carotenoids (Schwender *et al.*, 1997; Arigoni *et al.*, 1997; Lichtenthaler *et al.*,

1997). It is worth noting that these experiments were performed under very low light conditions. Additionally, in the study on tobacco, the experiments were carried out using a heterotrophically grown cell culture (BY-2) system devoid of functional chloroplasts (Disch *et al.*, 1998a).

[2-^{14}C]acetate incorporation into chloroplastic isoprenoids and fatty acids was about five times higher than that of [2-^{14}C]pyruvate. These results indicate that the synthesis of IPP occurs *via* chloroplast mevalonate rather than the DOXP/MEP pathway. A further study confirmed the existence of chloroplastic

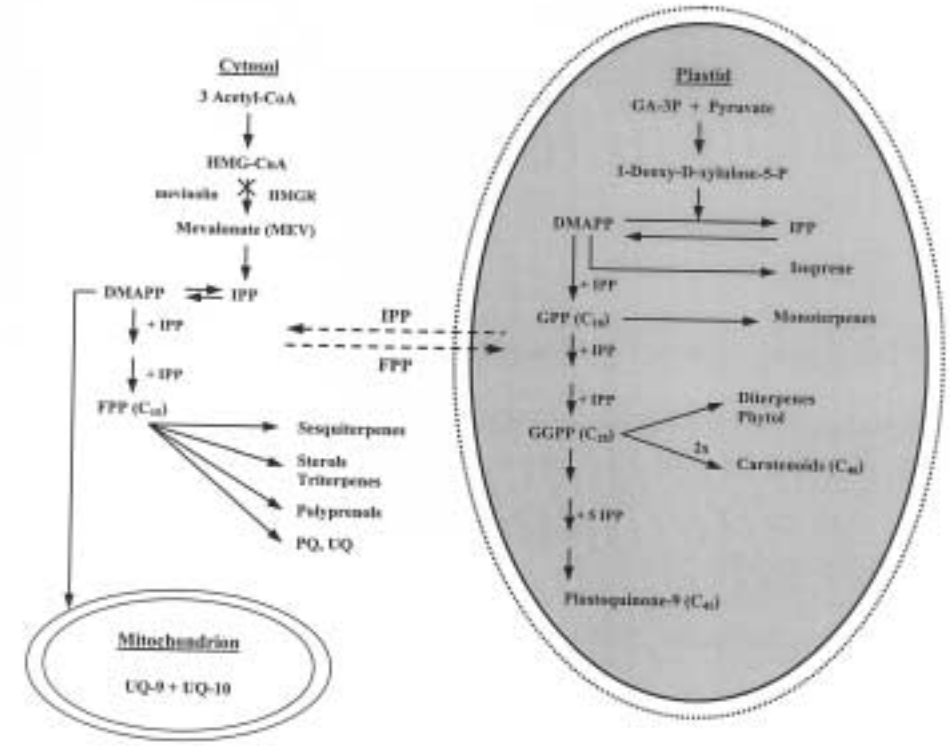

Figure 3. Compartmentation of the IPP and isoprenoid biosynthesis within plant cells between cytosol (mevalonate pathway) and plastid (DOXP/MEP pathway) (Lichtenthaler, 1999, modified).

The specific inhibitor of HMG-CoA reductase (HMGR)-mevinolin is indicated. Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; PQ, plastoquinone; UQ, ubiquinone.

Simultaneously, labeling of PQ and UQ with [^{3}H]mevalonate was observed in etiolated and non-etiolated spinach seedlings pointing to MVA-dependent origin of IPP in these experimental conditions (Wanke *et al.*, 2000). Recently it has been also found that polyisoprenoid alcohols occurring in the roots of *Coluria geoides* grown *in vitro* are formed from mevalonate (Skorupińska-Tudek, K., unpublished).

The study of chloroplasts metabolism during early developmental stages performed by Heintze *et al.* (1994) showed that in immature chloroplasts from young spinach plants mevalonate kinase, detected only after breaking of the chloroplasts (Preiss & Schultz, 1994). Evidence for nuclear genes encoding early steps of the mevalonate pathway enzymes localized in the endoplasmic reticulum (ER) but not in the plastids was obtained but it was difficult to detect their expression possibly because of its very low level at these developmental stages. Therefore Heintze *et al.* (1994) suggested that the genes encoding the chloroplastic mevalonate pathway enzymes are expressed only at an early stage of chloroplast development.

The labeling pattern of the phytyl side-chain of chlorophyll determined by incorporation of ^{2}H or ^{13}C-labeled acetates, glycerol or glucose in *Heteroscyphus planus* cells suggests simultaneous operation of the novel, DOXP/MEP and the mevalonate pathway (Nabeta *et al.*, 1997). Additionally, reports were published on the existence of a plastidic, along with an ER associated, HMG-CoA reductase (Wong *et al.*, 1982; Kim *et al.*, 1996). These, sometimes contradictory results suggest the possibility that indeed two pathways of IPP formation are operating and their activation is dependent on physiological conditions or developmental stage of the cell.

COMPARTMENTATION OF IPP BIOSYNTHESIS IN HIGHER PLANTS AND ENDOSYMBIOTIC THEORY

In higher plants the MEP pathway derived IPP is used not only for the biosynthesis of isoprenoids involved in photosynthesis but also for the formation of the volatile hemiterpene isoprene (Schwender *et al.*, 1997), taxol (Eisenreich *et al.*, 1996) and marrubiin (Knöss *et al.*, 1997).

Fast incorporation of $^{14}CO_2$ into isoprene suggested that isoprene synthesis was related to the photosynthetic CO_2 fixation. In addition, isoprene synthase has been found in plastids. Further evidence came from labeling experiments using deuterium labeled DOX or its methyl glycoside. High incorporation rates of this intermediate into isoprene were found in poplar (*Populus nigra*), celandine (*Chelidonium maius*) and willow (*Salix viminalis*) (Zeidler *et al.*, 1997).

According to the endosymbiotic theory, the plastid compartment is a heritage from photosynthetic prokaryotic ancestors. The fact that the phytol of chlorophyll a in the photosynthetic bacteria is also formed by the MEP pathway supports this theory. Thus, it appears that the plastids have maintained the original bacterial DOXP/MEP pathway of IPP biosynthesis during coevolution with the eukaryotic plant cells (Lichtenthaler *et al.* 2000).

COOPERATION BETWEEN TWO PATHWAYS OF IPP BIOSYNTHESIS IN HIGHER PLANTS

Several observations led to the suggestion that some exchange and cooperation between the two pathways does exist, but still it is not clear to what extent two different pools of IPP or other prenyl diphosphates such as geranyl diphosphate, farnesyl diphosphate or geranylgeranyl diphosphate are exchangeable (Fig. 3). Nabeta *et al.* (1997) studing the ^{13}C-labeling of chlorophyll and carotenoids found that cytosolic farnesyl diphosphate was transferred into plastid, where it was condensed with a DOXP derived IPP. The export of IPP or geranyl diphosphate from the plastids into the cytosol may also exist, as it was shown by inhibitor studies (Schwender *et al.*, 1997). Several observations suggest the occurrence of at least some exchange. Three isoprene units were found to be labeled *via* the MVA pathway, and the fourth isoprene unit *via* the DOXP/MEP pathway upon labeling of the diterpene ginkgolide from [^{13}C]glucose (Lichtenthaler, 1999). In the liverwort *Heteroscyphus,* the first three isoprenic units of phytol showed some label from the applied [^{13}C]MVA, whereas the fourth unit was not labeled (Nabeta *et al.*, 1997). Recent studies on chamomile also suggest that two cellular IPP pools might cooperate and exchange IPP or GPP (Adam & Zapp, 1998). As shown in these experiments, the first two C_5-units of sesquiterpene molecules were derived from [^{13}C]glucose *via* the DOXP/MEP pathway, and the third C_5-unit was labeled either by the DOXP or the MVA pathway.

Recent discoveries have contested the well established model of isoprenoid biosynthesis

in bacteria and plants. The results discussed above indicate that both the classical, MVA and the novel, DOXP/MEP pathway are present within at least some, if not all, plant cells. It is the task for the future to establish the mechanisms of spatial and temporal modulation of the activity of the enzymes involved in these two parallel pathways leading to the formation of IPP.

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