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The Deoxyxylulose Phosphate Pathway for the Biosynthesis of Plastidic Isoprenoids: Early Days in Our Understanding of the Early Stages of Gibberellin Biosynthesis

Valerie M. Sponsel

Department of Biology, University of Texas at San Antonio, 6900 North Loop 1604 West, San Antonio, Texas 78249, USA

Abstract

The identification of a novel pathway for isopentenyl diphosphate synthesis by Rohmer, Arigoni and colleagues in the early 1990's has led to a reappraisal of terpenoid biosynthesis in many organisms. It is now apparent that in plants there are two biosynthetic routes to isopentenyl diphosphate—the classical mevalonate pathway in the cytosol and the deoxyxylulose phosphate pathway in plastids. Sesquiterpenoids and sterols are predominantly synthesized in the cytosol by the mevalonate pathway whereas monoterpenoids, diterpenoids, the phytol side-chain of chlorophyll, carotenoids, and the nonaprenyl side-chain of plastoquinone-9 are synthesized within plastids by the deoxyxylulose phosphate pathway. Our assumptions that the early stages of gibberellin biosynthesis are plastid-localized has led to several attempts to demonstrate that the deoxyxylulose phosphate pathway is the biosynthetic route to gibberellins. Although definitive evidence is still not available there is a growing body of evidence, mostly from transgenic plants and from the use of the inhibitor, fosmidomycin, that gibberellins are synthesized from deoxyxylulose phosphate-derived isopentenyl diphosphate. However, there is evidence that a small amount of cross-talk between the two pathways may occur, implying that the pathways are not totally autonomous. Implications for the regulation of the early stages of gibberellin biosynthesis are discussed.

Key words: 1-Deoxy-D-xylulose 5-phosphate; Isopentenyl diphosphate; Isoprenoid metabolism; Gibberellin biosynthesis; Plastids

Introduction

Gibberellins (GAs), being diterpenes, are synthesized from isopentenyl diphosphate (IPP), the universal 5-carbon building block for all terpenoid compounds. IPP and its isomer dimethylallyl diphosphate (DMAPP) are elaborated into monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}), and polyterpenoids (>C_{40}). IPP is found in several cellular compartments, and the intracellular localiza-

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Corresponding author; e-mail: vsponsel@lonestar.utsa.edu

tion of IPP-derived compounds often has functional significance.

For many years the sole biosynthetic origin of IPP was thought to be the mevalonate (MVA) pathway. Two alternative hypotheses were put forward to account for the presence of IPP in different intracellular compartments: either (1) that there are multiple intracellular sites for the classical MVA pathway (Goodwin and Mercer 1963; Goodwin 1965) or (2) that all IPP is synthesized in the cytosol and is then transported into organelles (Kreuz and Kleinig 1981). Experimental evidence (reviewed by Gray 1987 and Kleinig 1989) did not fully support either hypothesis, leaving the intriguing possibility that there might be a novel explanation for what really happens in the plant. The origin of plastidic IPP was of particular interest to GA researchers, since the biosynthesis of *ent*-kaurenoids, which are GA precursors, was thought to occur in plastids (West 1981).

It has now been established that there are indeed two pathways for the biosynthesis of IPP in higher plants. Whereas IPP is synthesized in the cytosol by the MVA pathway, IPP in the plastids is synthesized from different precursors, and by a novel route (Rohmer and others 1993; Lichtenthaler and others 1997a and b; Rohmer 1999; Lichtenthaler 1999). The new pathway is known by several names including the non-MVA, the deoxyxylulose phosphate (DOXP), the methylerythritol phosphate, or the Rohmer pathway, but will be referred to as the DOXP pathway in this paper.

The fate of IPP synthesized by the two pathways is different (Lichtenthaler 1999). IPP synthesized in the cytosol from MVA is further assembled into sesquiterpenoids, and triterpenoids, of which brassinosteroids are an example. It is also the source of prenyl side-chains for ubiquinone synthesis in mitochondria. IPP synthesized in plastids from DOXP gives rise to monoterpenoids, diterpenoids, and tetraterpenoids, including carotenoids. Thus, plastid-derived DOXP is expected to be the biosynthetic source of both the GAs and abscisic acid. The biosynthetic origin(s) of the terpenoid side-chain of cytokinins is still under investigation (Åstot and others 2000).

This paper briefly describes the biochemical work that established the DOXP pathway first in eubacteria, then in algae and in plant plastids, and summarizes what is known to date on the DOXP pathway as the biosynthetic source of GA precursors. It also examines evidence of potential cross-talk between the MVA and DOXP pathways.

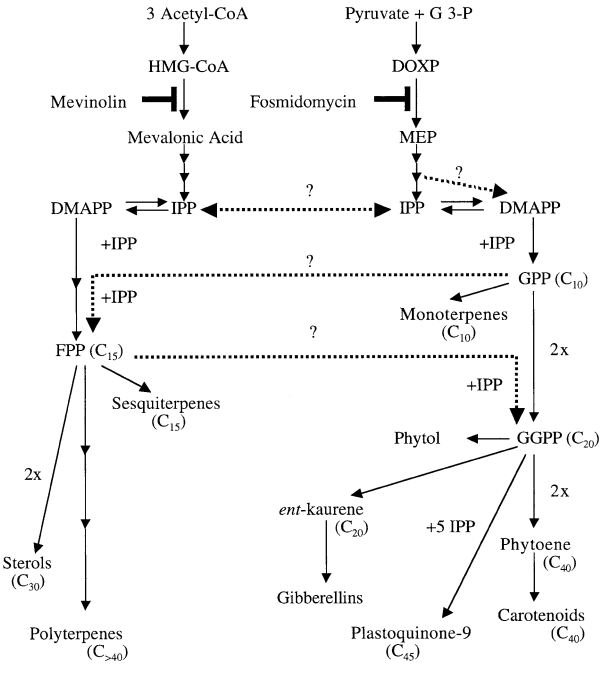

Figure 1. Parallel pathways for the biosynthesis of isopentenyl diphosphate (IPP). The mevalonate pathway (left) is localized in the cytosol of higher plants. The deoxyxylulose phosphate (DOXP) pathway (right) is localized in plastids. IPP synthesized by the two pathways is converted into different terpenoid products, as outlined in the Figure. The amount of cross-talk between the two pathways is at present unknown, interchange of intermediates having been demonstrated directly in only a few instances. DMAPP (dimethylallyl diphosphate), FPP (farnesyl diphosphate), GPP (geranyl diphosphate), GGPP (geranylgeranyl diphosphate), HMG-CoA (hydroxymethylglutaryl-coenzyme A), MEP (methylerythritol phosphate).

The Classical MVA Pathway to IPP

Evidence Inconsistent with the MVA Pathway Being the Biosynthetic Route to Plastid-localized IPP

The MVA pathway to IPP was elucidated in the 1950s and has been reviewed extensively (see, for example Goodwin 1965). Three units of acetyl-CoA condense to form 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) (Figure 1). HMG-CoA is reduced by 2 NADPH yielding mevalonic acid (MVA), which, in three ATP-requiring steps, is converted to IPP with the loss of CO_2. The enzyme HMG-CoA reductase is competitively inhibited by mevastatin (compactin) and mevinolin (6α-methylcompactin, lovastatin) (Bach and Lichtenthaler 1982). Thus, both of these compounds prevent the formation of MVA, and lead to a depletion of compounds derived from MVA (Figure 1).

There is incontrovertible evidence that the MVA pathway occurs in the cytosol of higher plants, and is the route to sesquiterpenoids and steroids (Goodwin 1965), but the origin of plastidic isoprenoids has long been questioned. [^{14}C]-MVA is readily converted into cytosolic steroids but not into plastid-localized terpenoids (Treharne and others 1966). Conversely, [^{14}C]-CO_2 is efficiently incorporated into plastidic terpenoids, but minimally into cytosolic steroids (Treharne and others 1966; Bach and Lichtenthaler 1982). Moreover, inhibitors of HMG-CoA reductase reduced sterol accumulation in intact radish (*Raphanus sativus*) plants, yet barely affected carotenoid accumulation (Bach and Lichtenthaler 1983). More recently, Chappell and others (1995) showed that unregulated expression of hamster HMG-CoA reductase in tobacco (*Nicotiana tabacum*) plants caused a large increase in cycloartenol (a steroid precursor), but the levels of carotenoids and the phytol side-chain of chlorophyll were relatively unchanged.

The common interpretation of these results was that plastid-localized enzymes for the conversion of acetate to MVA and IPP are inaccessible to substrates and inhibitors, because of an uptake barrier imposed by the plastid envelope, and/or metabolite channeling within the plastid. This interpretation was questioned when attempts to demonstrate enzymes of the MVA pathway to IPP in plastid preparations were unsuccessful (Lütke-Brinkhaus and Kleinig 1987). In addition, it was shown that in *Arabidopsis* the two genes for HMG-CoA reductase encode products that are targeted to the endosplasmic reticulum, with no evidence for either plastid- or mitochondrial-targeted forms of this enzyme (Enjuto and others 1994). Evidence for IPP uptake into intact plastids was also absent, throwing doubt on the alternative proposal, namely, that IPP synthesized in the cytosol from MVA moves into plastids for further elaboration into mono-, di-, and tetraterpenes.

In the absence of any satisfactory explanation for the biosynthetic origin of plastid-localized IPP that could reconcile the results of *in vivo* and *in vitro* substrate and inhibitor studies, Lütke-Brinkhaus and Kleinig (1987) and Kleinig (1989) acknowledged that IPP in plastids could be synthesized by a new pathway.

A Novel Pathway to IPP in Eubacteria, Green Algae, and Plastids of Higher Plants

Flesch and Rohmer (1988) provided the first clear evidence for an alternative pathway to IPP. They studied a class of triterpenoids in eubacteria known as hopanoids, which have a role in bacterial membranes similar to that of sterols in membranes from eukaryotes. Incorporation of label (directly or indirectly) from ^{13}C-labeled acetate, glucose, pyruvate, and erythrose into hopanoids and/or ubiquinones in several species of bacteria was analyzed by ^{13}C-NMR and was shown to be different from the known patterns of incorporation from MVA. Thus, instead of the conventional pathway, Rohmer and others (1993) proposed that a C_2-unit derived during decarboxylation of pyruvate is added to a triose phosphate derivative, followed by transposition to give the branched C_5-skeleton of IPP (Figures 1, 2). The fate of carbon atoms from each of the substrates and their assignment to positions in IPP and its products can be found in the original paper and in the detailed review by Rohmer (1999).

Schwender and others (1996) studied the incorporation of labeled glucose into isoprenoids in cells of the green alga, *Scenedesmus obliquus*. This organism was used because its growth and pigment formation are resistant to inhibitors of HMG-CoA reductase. *Scenedesmus* produced sterols, phytol, tetraterpenes, and plastoquinone-9 all with labeling patterns consistent with the new pathway, thus demonstrating its presence in eukaryotic as well as prokaryotic cells.

Shortly thereafter, the novel DOXP pathway to IPP was shown to exist in plants too. Lichtenthaler and others (1997b) examined the incorporation of [1-^{13}C]-glucose in photoheterotrophic cultures of *Daucus carota* (carrot), *Hordeum vulgare* (barley) embryos, and *Lemna gibba* plantlets. The labeling of chloroplastidic isoprenoids was consistent with the DOXP pathway, though the sterols were formed from MVA (Lichtenthaler and others 1997b). Hence, unlike *Scenedesmus* and other green algae in which all isoprenoids are formed by the DOXP pathway (Schwender and others 2001), in Angiosperms it is evident that both routes operate—the DOXP pathway in the plastids producing plastid isoprenoids, and the MVA pathway in the cytosol producing sterols.

The DOXP Pathway

Metabolic reactions in this pathway were demonstrated first in eubacteria, but are now known to occur in plants too. In the first step (Figure 2) a two-carbon activated acetaldehyde moiety, which is derived from pyruvate by decarboxylation, is added to glyceraldehyde 3-phosphate (G 3-P). The product is 1-deoxy-D-xylulose 5-phosphate, referred to as

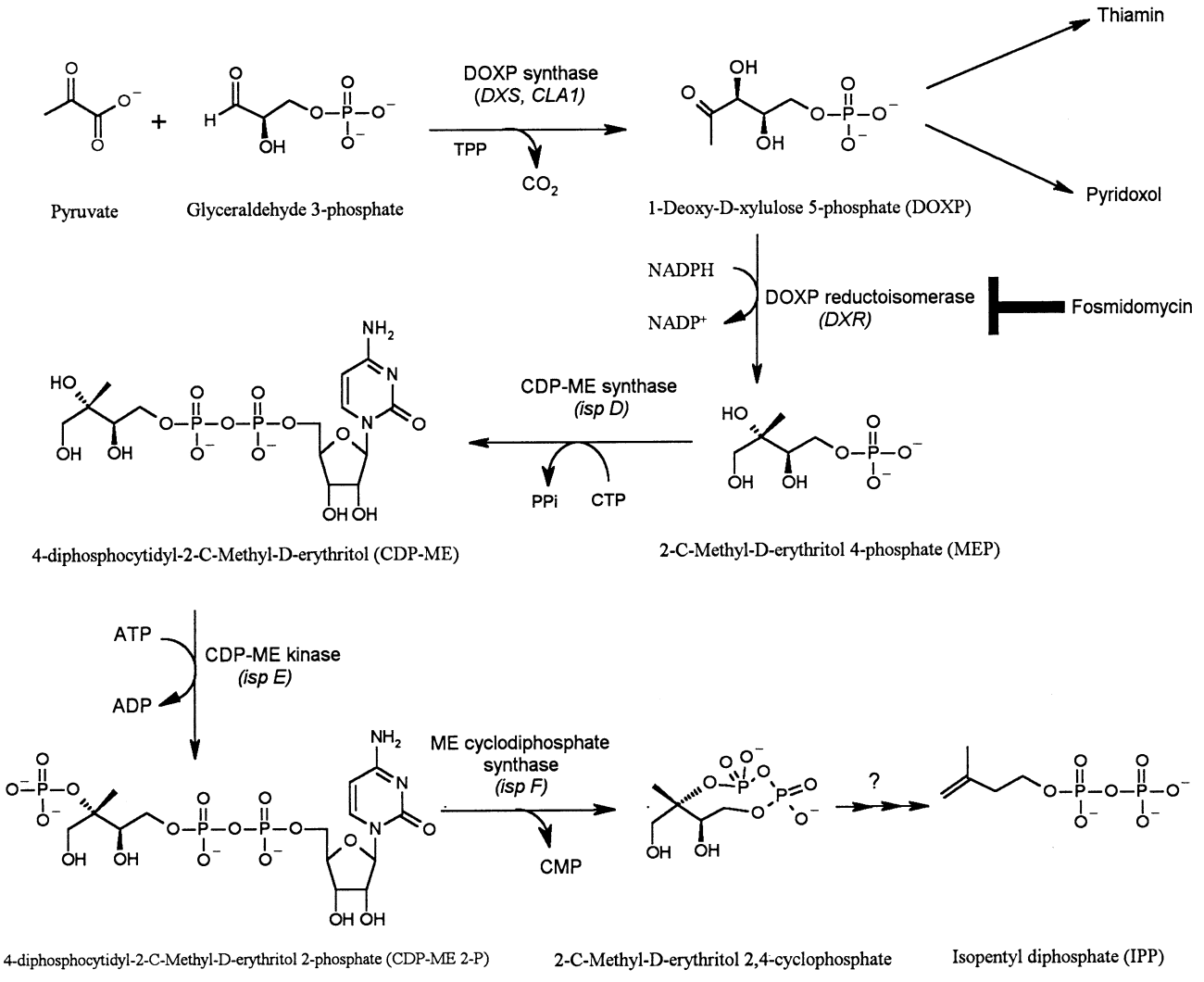

Figure 2. Metabolic conversions in the DOXP pathway. Steps between methylerythritol 2,4,cyclodiphosphate and IPP are at present unknown.

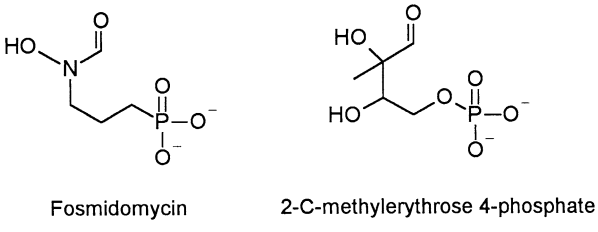

Figure 3. Fosmidomycin is a potent inhibitor of DOXP reductoisomerase, probably due to its structural resemblance to 2-C-methylerythrose 4-phosphate, which is a putative intermediate between DOXP and MEP.

deoxyxylulose phosphate (DOXP) (Rohmer and others 1993; Schwender and others 1996). The reaction requires thiamin diphosphate (TPP), indicative of a transketolase-type reaction. In *E. coli*, DOXP is an intermediate in the biosynthesis of thiamin (and hence vitamin B_1) and pyridoxol (and hence vitamin B_6), in addition to its now recognized role in isoprenoid biosynthesis (Sprenger and others 1997).

Evidence that pyruvate and G 3-P are precursors of DOXP in plants, and that DOXP is an intermediate in plastidic isoprenoid biosynthesis followed. In plants, the source of G 3-P is the Calvin cycle. Pyruvate may be synthesized in the plastids from 3-phosphoglyceric acid, or as a minor bi-product of rubisco activity (Lichtenthaler 1999; Andrews and Kane 1991). Alternatively, it may be taken up from the cytosol. Labeled-DOXP was shown to be incorporated into β-carotene, lutein, and phytol in cell cultures of *Catharanthus roseus* (Madagascar periwinkle) (Arigoni and others 1997) and into isoprene in *Populus niger* (poplar) leaves (Schwender and others 1997). In each instance the incorporation of

stable isotope (either ^{13}C or ^{2}H) from the substrate was confirmed in the product by NMR spectroscopy, and the products were all compounds known to be localized in, or in the case of isoprene, emitted from plant plastids.

From structural considerations, 2-C-methyl-D-erythritol (ME), or its 4-phosphate (MEP), was implicated as the first intermediate between DOXP and IPP (Figure 2). Labeled glucose fed to *Corynebacterium ammoniagenes* is incorporated into ME, and into the prenyl side-chain of hydroxymenaquinone, consistent with ME (or MEP) being an intermediate in the DOXP pathway (Duvold and others 1997). The conversion of the straight-chained DOXP to the branched MEP involves reduction and an intramolecular rearrangement, the mechanism of which has been described by Arigoni and others (1997).

The conversion of DOXP to ME in two plants, *Liriodendron tulipifera* (tulip tree) and *Ipomoea parasitica,* was shown by Sagner and others (1998). In plants it is most likely that MEP is the intermediate formed from DOXP by transposition and reduction, and that ME, applied exogenously, is phosphorylated first by plant kinases.

Conversion of MEP to 4-diphosphocytidyl-2-C-methyl-erythritol (CDP-ME) (Figure 2) is catalysed by a CDP-ME synthase, isolated initially from cell extracts of *E. coli* (Rohdich and others 1999; Kuzayama and others 2000a). Labeled CDP-ME, prepared using recombinant enzyme, is converted into carotenoids by isolated chromoplasts of *Capsicum annuum* (chili pepper) (Rohdich and others 1999) indicating its importance in plastidic isoprenoid synthesis. Although it cannot be discounted that the CDP-ME is hydrolyzed to MEP prior to metabolism, this is considered unlikely (Rohdich and others 1999). CDP-ME synthase has also been cloned from *Arabidopsis thaliana* (Rohdich and others 2000a).

Phosphorylation of CDP-ME (Figure 2) has been observed in *E. coli* (Kuzuyama and others 2000b; Lüttgen and others 2000), yielding 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate (CDP-ME 2-P). This compound, fed in ^{14}C-labeled form to isolated chromoplasts of *C. annum,* yielded ^{14}C-labeled phytoene and ^{14}C-labeled β-carotene as products, clearly indicating its role in plastidic isoprenoid biosynthesis (Lüttgen and others 2000).

A further step in the pathway is the conversion of CDP-ME 2-P to 2-C-methyl-D-erythritol 2,4-cyclodiphosphate after the elimination of CMP (Herz and others 2000; Takagi and others 2000) (Figure 2). This metabolic step has been demonstrated using bacterial gene products. The conversion in chromoplasts of *C. annum* has been alluded to but not reported in detail (Herz and others 2000). However, radioactivity from ME 2,4-cyclodiphosphate is incorporated into carotenoids in isolated chromoplasts of *C. annum,* lending credibility to its role in the DOXP pathway in plastids (Herz and others 2000).

Intermediates between ME 2,4-cyclodiphosphate and IPP have yet to be identified (Figure 2). However, it is postulated that several additional steps involving ring opening, dehydration, and reduction are required in the conversion of ME-cyclodiphosphate to IPP (Eisenreich and others 2001). Bioinformatics approaches to determine the nature of genes and gene products involved in steps between ME 2,4- cyclodiphosphate and IPP are discussed later. There has also been some discussion of whether DMAPP might be formed from a branch-point in the DOXP pathway, rather than from IPP in the conventional reaction catalyzed by isopentenyl isomerase (Arigoni and others 1999; Rodríguez-Concepción and others 2000) (Figure 1). The gene encoding isopentenyl isomerase (*ipi*) is absent from the genomes of many bacteria that lack the MVA pathway, though it is present in *E. coli* (Cunningham and others 2000). Therefore in those bacteria that lack *ipi,* DMAPP, which is a required compound for the subsequent synthesis of higher order terpenoids, may be formed independently of IPP. Whether the DOXP pathway in plants branches to produce DMAPP, or whether IPP in plastids is isomerized to DMAPP (as it is in the cytosol) is not currently known.

Enzymes in the DOXP Pathway, and the Genes Encoding Them

DOXP Synthase, dxs, and *CLA1*

The gene *dxs,* which encodes deoxyxylulose phosphate synthase (DOXP synthase), catalyzes the condensation of pyruvate and G 3-P, giving DOXP (Figure 2). The gene was cloned from *E. coli* after being identified in the bacterial genome because of sequence similarities to transketolases (Sprenger and others 1997). The *dxs* gene is part of an operon that also contains the *ispA* gene, encoding another enzyme involved in isoprenoid metabolism, namely, farnesyl diphosphate synthase (Lois and others 1998). The proximity of *dxs* to *ispA* is consistent with them having a functional relationship. The protein encoded by the *dxs* gene is a member of a small family that is similar, but not identical, to classical bacterial transketolases. Members of this family of DOXP synthases possess a TPP-binding motif like those in other transketolases and, indeed,

TPP is required for catalytic activity of *dxs* (Sprenger and others 1997).

The DOXP synthase gene family is present not only in eubacteria, but in algae (Schwender and others 1996; Schwender and others 2001), the Apicomplexa group of Protozoa, which includes *Plasmodium falciparum* (which is the causative agent of malaria) (Jomaa and others 1999), and plants (see, for example, Bouvier and others 1998; Lange and others 1998; Estevez and others 2000; Lois and others 2000). It does not appear to be present in animals and fungi. The DOXP synthase in *Arabidopsis* is encoded by CLA1 (*chloroplastos alterados* or altered chloroplasts). A T-DNA insertional mutant of *CLA1* was the first to be isolated, since it defined an albino phenotype with exceedingly low levels of carotenoids and chlorophyll and with arrested chloroplast development (Mandel and others 1996; Estevez and others 2000). The *CLA1* protein, which was already known to share a certain degree of similarity to transketolases (Mandel and others 1996), was later shown to encode DOXP synthase (Estevez and others 2000). It is a nuclear gene encoding a protein with a putative N-terminal chloroplast targeting sequence. Based on northern blotting and reporter gene studies, *CLA1* is expressed throughout the plant, including some organs (roots) and tissues (vascular tissues and epidermis) which are not normally sites of chlorophyll or carotenoid accumulation. However, these tissues are sites for the synthesis of other terpenoids. For example, a DOXP synthase involved in monoterpene biosynthesis in peppermint (*Mentha x piperita*) was cloned from a cDNA library prepared from epidermal oil gland mRNA. These oil gland cells, also known as glandular trichomes, were specifically chosen because they are highly specialized for isoprenoid biosynthesis (Lange and others 1998). In *Arabidopsis*, there is also evidence for post-transcriptional regulation of *CLA1* (Estevez and others 2000). Western blots indicated that the protein is localized mostly in developing tissues such as young rosette leaves and immature siliques. The regulation of DOXP synthase is discussed again later.

DOXP Reductoisomerase, *dxr* (formerly *yaeM*)

The conversion of DOXP to MEP, which involves an intramolecular rearrangement and reduction, is catalyzed by a DOXP reductoisomerase (Figure 2). The gene, formerly notated as *yaeM* but now designated *dxr,* was first cloned in *E. coli* because of its ability to complement a metabolic block in mutants that required MEP for growth (Kuzuyama and others 1998b; Takahashi and others 1998). The conversion of DOXP to MEP requires NADPH and a divalent cation (Mg^{2+}, Mn^{2+}, or Co^{2+}). It is the first committed step in IPP biosynthesis, since DOXP is a precursor not only of IPP but of thiamin and pyridoxol (Lois and others 1998). The recombinant enzyme has been studied in some detail and is characteristic of class B dehydrogenases (Radykewicz and others 2000).

Based on sequence homology to the bacterial gene, Schwender and others (1999) were able to clone a cDNA from *Arabidopsis*, which when expressed in *E. coli* was able to catalyze the NADP-dependent conversion of DOXP to MEP. DOXP reductoisomerase is a single copy gene in *Arabidopsis*. The herbicide, fosmidomycin, specifically inhibits DOXP reductoisomerase by mimicking a putative intermediate, 2-C-methyl-D-erythrose 4-phosphate (Figure 3) (Zeidler and others 1998; Kuzuyama and others 1998a; Jomaa and others 1999). Fosmidomycin therefore has utility in studies of the DOXP pathway which are comparable to those employing mevastatin and mevinolin in the MVA pathway. The use of fosmidomycin to inhibit the DOXP pathway, specifically with intent to alter GA biosynthesis, is discussed later.

CDP-ME Synthase (MEP-Cytidyltransferase), *emph* (formerly *ygbP*)

A CDP-ME synthase (also referred to as an MEP-cytidine transferase) which catalyzes the conversion of MEP to CDP-ME (Figure 2) was isolated first from *E. coli* cell extracts (Rohdich and others 1999; Kuzayama and others 2000a). Incubations of MEP in the presence of CTP yielded a product that was labeled if ^{32}P was in the α-position but not in the γ-position (Rohdich and others 1999). Postulation that the product was CDP-ME inspired a database search of bacterial genomes for enzymes transferring cytidylphosphate residues and led ultimately to the *ygbP* gene (Rohdich and others 1999). The construction of a *ygbP* hyperexpression strain of *E. coli,* which catalyzed the conversion of MEP to CDP-ME at high rate, allowed for the isolation of recombinant CDP-ME synthase and sufficient CDP-ME for structural confirmation by NMR spectroscopy (Rohdich and others 1999). Using a different approach, Kuzayama and others (2000a) transformed *E. coli* so that it could use MVA as a source of IPP, then searched for mutants that were MVA-dependent, surmising that these mutants would be defective in the DOXP pathway. They then went on

to clone genes that could complement the defects leading, *inter alia,* to the isolation of the gene encoding the CDP-ME synthase. The *ygbP* gene, which is now formally referred to as *ispD,* appears to be present only in bacteria that also posses the *dxs* and *dxr* genes, reinforcing the assertion that their gene products catalyze an integrated series of reactions.

The *Arabidopsis* ortholog of CDP-ME synthase was cloned by Rohdich and others (2000a). The plant protein has a putative plastid targeting sequence. The catalytic domain is approximately 30% identical with the *E. coli* enzyme. The *Arabidopsis* enzyme has an absolute requirement for a divalent cation, preferably Mg^{2+}, for activity. It can use several NTPs as substrate, though the maximum catalytic activity was observed with CTP. However, it is unlikely that products with other NDP moieties can act as substrates for the next enzyme in the pathway (Lüttgen and others 2000).

CDP-ME Kinase, *ispE* (formerly *emph*)

Seeking additional *E. coli* genes that complement defects in the DOXP pathway, Kuzuyama and others (2000a and b) identified the *ychB* gene, which encoded a protein able to catalyze the ATP-dependent conversion of CDP-ME to CDP-ME 2-P (Figure 2). Thus, the *ychB* gene encodes a CDP-ME kinase. Using an alternative bioinformatics approach based on the logic that genes encoding enzymes in the entire DOXP pathway will co-occur, Lüttgen and others (2000) sought genes that occurred with *dxs, dxr, ispD* (*ygbP),* and *ispF, (ygbB*) in bacterial genomes. A database search retrieved the *ychB* gene which, when hyperexpressed in *E. coli,* yielded a 31kDa protein that could catalyze the ATP-dependent phosphorylation of CDP-ME (Lüttgen and others 2000). The sequence of *ygbB* and putative orthologs shows an apparent ATP-binding site, and other homology with kinases, such as mevalonate kinase (Lüttgen and others 2000).

The *ychB* gene, which is now referred to as *ispE,* encodes a protein with 30% identity with a cDNA from *Lycopersicon esculentum* (tomato) (Rohdich and others 2000b). The recombinant tomato protein catalyzes the phosphorylation of CDP-ME at a comparable rate to that of the *E. coli* protein. The tomato protein has a putative plastid targeting sequence (Rohdich and others 2000b), and, in fact, was first identified by Lawrence and others (1997) in a chloroplast import assay as a protein of unknown function whose synthesis is up-regulated during the transition from chloroplast to chromoplast. The newly identified function of this protein as CDP-ME kinase is consistent with the massive build-up of carotenoids, including lycopene and β-carotene, that accompanies the chloroplast to chromoplast transition in tomato fruits.

ME-2,4-Cyclodiphosphate Synthase, *ispF* (formerly *ygbß*)

The *ispD* (formerly *ygbP*) and the *ispF* (formerly *ygbB*) genes are co-expressed in many bacteria, predicting their functional relationship. The *ygbB* gene for *E. coli* was expressed in an *E. coli* hyperexpression system and was shown to encode a 12kDa protein that catalyzed the conversion of CDP-ME 2-P to ME 2,4-cyclodiphosphate and CMP (Figure 2) (Herz and others 2000; Takagi and others 2000). The enzyme requires only Mg^{2+} or Mn^{2+} as a cofactor. Putative *ispD* and *ispF* orthologs are closely linked in more than 20 bacteria that possess the DOXP pathway, and the products may exist as bifunctional proteins with N-terminal *ispD* and C-terminal *ispF* domains in some bacteria (Herz and others 2000). Identification of the *ispF* ortholog in plants is to be anticipated.

Two other genes, *lytB* and *gpcE,* have been implicated in the DOXP pathway (Cunningham and others 2000). They were identified because of their co-occurrence in bacterial and *Arabidopsis* genomes, with the five genes already known to encode enzymes in the pathway. They are absent from the genomes of organisms known not to possess the DOXP pathway (Cunningham and others 2000). Based on this correlative evidence it appears very likely that these genes will be shown to encode enzymes catalyzing steps between ME-2,4-cyclodiphosphate and IPP.

DOXP Pathway in Plant Plastids-Implications for GA Biosynthesis

In eukaryotes the DOXP pathway is known to occur only in organisms that harbor plastids within their cells, namely algae, the apicoplastic protozoa and plants (Lange and other 2000). Although the genes encoding DOXP pathway enzymes are in the nuclear genome (indicative of lateral gene transfer from a former endosymbiont), the genes encode proteins with plastid targeting sequences. In addition, CLA1 transit peptide when fused to green fluorescent protein is localized to plastids of both cultured tobacco cells and tomato leaf cells (Araki and others 2000; Lois and others 2000). Moreover,

intermediates in the DOXP pathway, when fed to plants, are converted into known plastidic isoprenoids. This information leaves little doubt that the DOXP pathway operates in plant plastids, and indeed, in plants there is no evidence for the DOXP pathway being located in the cytosol.

The long-held premise that the early stages of GA biosynthesis are plastid localized is supported by evidence that shows that enzymes encoding intermediate steps in GA biosynthesis (between IPP and *ent*-kaurene) are clearly plastid- or proplastid-localized. These enzymes include IPP isomerase (Dogbo and Camara 1987), GPP synthase (Soler and others 1992), GGPP synthase (Dogbo and Camera 1987; Laferrière and Beyer 1991; Kuntz and others 1992; Okada and others 2000), *ent*-copalyl diphosphate synthase (formerly *ent*-kaurene synthase A) (Sun and Kamiya 1974), and *ent*-kaurene synthase (formerly *ent*-kaurene synthase B) (Aach and others 1995, 1997; Yamaguchi and others 1998). Some of these enzymes are found only in plastids, and others may also have extra-plastidic isoforms (Okada and others 2000). However, the preceding citations show that the entire sequence of reactions for the conversion of IPP to the GA precursor, *ent*-kaurene, does occur in plastids. Since there is no evidence for large-scale uptake of IPP from the cytosol into plastids, one must surmise that the precursor pool of IPP for *ent*-kaurene biosynthesis is formed within the plastids. The logical conclusion must therefore be that GAs are synthesized by the DOXP pathway.

Conversion of DOXP pathway intermediates into *ent*-kaurenoids and GAs has not yet been demonstrated. Nevertheless there are several sources of circumstantial evidence that strongly support the assertion that in the early stages, GA biosynthesis proceeds in large part, if not entirely, via the DOXP pathway.

One line of evidence comes from studies involving the manipulation of genes encoding enzymes in the DOXP pathway. Estevez and others (2001) have over- and under-expressed DOXP-synthase encoded by *CLA1* in *Arabidopsis*. DOXP synthase is a limiting enzyme in the pathway in bacteria, and is highly regulated in tomato fruit development (Lois and others 2000). Thus, an alteration in the level of DOXP synthase would be expected to have a significant effect on the levels of metabolites downstream (Figure 1). Estevez and others (2001) quantified chlorophyll and α-tocopherol (both incorporating phytol moieties), carotenoids and ABA (derived from phytoene), and GAs (derived from *ent*-kaurene) in *Arabidopsis* plants in which *CLA1*, or the antisense version thereof were constitutively expressed from a 35S promoter. Some of the lines that showed strong antisense expression were lethal, presumably because of severe depletion of chlorophyll and carotenoids and could not be used. Of relevance to this discussion are the levels of GAs which were estimated indirectly by measuring the expression of *GA4* (*AtGA3oxl*), a gene that is subject to feedback regulation (Chiang and others 1995). Indeed, the level of *GA4* transcript is dose-dependent (in an inverse manner) allowing transcript levels to be used as a means of approximating GA levels (Cowling and others 1998). Plants in which the level of *GA4* expression is high are expected to contain lower levels of GAs than those in which the level of *GA4* expression is low. Estevez and others (2001) reported that over-expressing DXS led to decreases in *GA4* expression, and thus (by inference) higher levels of GAs. Alternatively, suppression of DOXP synthase expression by antisense led to increases in *GA4* expression, inferring that GA levels are reduced. Other isoprenoid levels, measured more directly by spectrophotometry, were enhanced by DOXP synthase over-expression, and reduced in the antisense lines, too.

The conclusion from this study is that DOXP synthase is a limiting enzyme in the DOXP pathway in *Arabidopsis* as well as in bacteria and tomato, and that over-expression elevates the available pool of IPP, leading to elevated levels of metabolites downstream from IPP. In addition, lowering the expression of DOXP synthase further limits the availability of enzyme and reduces the levels of downstream metabolites. Although determination of GA levels directly by GC-MS would be more compelling, the work nevertheless supports the assertion that GAs are synthesized by the DOXP pathway.

Okada and others (2002) have modified the expression of another enzyme in the DOXP pathway in *Arabidopsis*. The expression of CDP-ME synthase was altered using antisense technology. Levels of chlorophyll a, chlorophyll b, carotenoids, and *ent*-kaurene were measured directly in these plants. Decreased levels of all pigments were evident. Moreover, the level of *ent*-kaurene, measured by GC-MS, was reduced in one transgenic line by 80%.

There is additional evidence for the biosynthesis of GAs by the DOXP pathway from studies utilizing tomato. Again transgenic plants were used, but in these instances the expression of phytoene synthase (*PSY*), which encodes an enzyme downstream from IPP, was manipulated (Figure 1). GGPP is at a branch point in the terpenoid pathway, with one branch leading to *ent*-kaurenoids and GAs, and another to carotenoids. PSY catalyzes condensation of

two molecules of GGPP to form carotenoids. Tomato plants in which PSY is down-regulated by the presence of antisense RNA contain elevated levels of GAs (Fraser and others 1995). Conversely, plants in which *PSY* is constitutively expressed are dwarfed and contain lower levels of GAs (Fray and others 1995). Thus, down-regulation of carotenoid synthesis spares GGPP, whereas up-regulation of carotenoid synthesis would diminish the availability of GGPP for GA biosynthesis. This inverse relationship between GAs and carotenoids suggests that they share a common precursor pool of GGPP. Since it has been clearly demonstrated that the DOXP pathway leads to carotenoids, the observations here that GAs and carotenoids appear to share the same precursor pool provides additional circumstantial evidence for the biosynthesis of GAs by the DOXP pathway.

Another line of evidence comes from studies with fosmidomycin Fig. 3, which is an antibiotic produced by *Streptomyces lavendulae* that inhibits DOXP reductoisomerase, and hence blocks the conversion of DOXP to MEP (Kuzuyama and other 1998a; Zeidler and others 1998). Since blocking the formation of MEP inhibits phytol and carotenoid biosynthesis, fosmidomycin is also a potent herbicide. It is therefore difficult to design experiments to test the efficacy of fosmidomycin to inhibit GA biosynthesis totally, because a dose designed to eliminate biosynthesis of GAs by the DOXP pathway would also block the biosynthesis of chlorophyll and carotenoids and thus would be lethal. Okada and others (2002) have shown that fosmidomycin treatment of *Arabidopsis* plants reduced *ent*-kaurene levels in WT plants by approximately 60%, and fosmidomycin treatment of transgenic plants expressing antisense CDP-ME synthase reduced *ent*-kaurene levels to less than 20% of the level in untreated wildtype plants.

Taken together, the work described above provides strong evidence that GAs are formed, at least in part, via the DOXP pathway. However, definitive evidence must come from studies that demonstrate, using spectroscopic methods, the unequivocal incorporation of label from DOXP pathway intermediates into GAs.

Factors that Can Affect Flux Through the DOXP Pathway in Plants

HMG-CoA reductase catalyzes a highly regulated step in the MVA pathway. In animals, the regulation of this enzyme is known to be multifaceted and has been studied in detail (Goldstein and Brown 1990). In plants it appears that several factors, including light, hormones, pathogens, and ectopic expression of an HMG-CoA reductase transgene can all affect native HMG-CoA reductase levels, and consequently have profound effects on IPP biosynthesis (McGarvey and Croteau 1995; Re and others 1995).

In the DOXP pathway it is now apparent that DOXP synthase is the first point of regulation, and it is anticipated that there may be a multiplicity of ways by which this enzyme, like HMG-CoA reductase, may be regulated. The formation of DOXP is known to be a rate-determining step in the pathway, since increasing or decreasing the DOXP synthase levels in *Arabidopsis* plants can cause major fluctuations in the levels of several isoprenoid endproducts, including GAs (Estevez and others 2001). In *Arabidopsis* there is evidence of both transcriptional and posttranscriptional regulation of *CLA1*, which encodes DOXP synthase (Estevez and others 2000). Northern blots showed *CLA1* mRNA in all tissues examined, though western blots showed a propensity of translated product in young leaves, buds, and immature siliques, and little or none in roots and imbibing seeds. In tomato, too, the expression of *DXS* is organ-specific and developmentally regulated (Lois and others 2000), showing close correlation to the timing of fruit ripening. In peppermint, the timing and level of DOXP synthase expression closely correlates with the peak of monoterpenoid biosynthesis in young leaves (Lange and other 1998).

DOXP is a precursor not only for isoprenoids, but for thiamin and pyridoxol. It is likely, therefore, that metabolic steps beyond DOXP would also be regulated, to control the flux through individual branch pathways. The reaction catalyzed by DOXP reductoisomerase is thought not to be regulated (Rodríguez-Concepción and others 2001) even though it is the first commited step in the pathway from DOXP to IPP. At present there is no information regarding potential regulation of subsequent metabolic steps between MEP and IPP.

Clearly there are many points of regulation in the branch pathways beyond IPP, resulting in vastly different levels for the individual isoprenoids formed from a single pool of plastid-localized IPP. Whereas it was previously thought that carotenoid biosynthesis in tomato fruits was regulated by phytoene synthase (*PSY1*) activity alone, it is now thought that there is coordinated regulation of *DXS* and *PSY1*, which leads to a fine-tuning of carotenoid levels in tomato fruits (Lois and others 2000). In the GA pathway *ent*-copalyl diphosphate synthase, en-

coded by *GA1* in *Arabidopsis,* is thought to fulfill a gate-keeper role, regulating the flux between GGPP and GA_{12} (Silverstone and others 1997). It is conceivable that DOXP synthase, encoded by *CLA1*, and *GA1* show coordinated regulation, like that of *DXS* and *PSY1* in tomato, though if this occurred it would likely be in tissues in which other plastidic isoprenoids are not required in excessive amounts. Coordination of expression could be achieved by feedforward and/or feedback regulation. Clearly the observations that *CLA1* transcript abundance is high in young actively growing tissues and organs of *Arabidopsis* (Estevez and others 2000) correlates well with what is already known about the temporal and spatial pattern of *GA1* expression (Silverstone and others 1997).

Potential Interaction of MVA and DOXP Pathways

The possible cross-talk between the cytosolic MVA pathway and the plastidic DOXP pathway is a topic of intense current interest. Cross-talk could occur by the transfer of a common intermediate from either pathway into the other compartment. However, there are no common intermediates in the MVA and DOXP pathways prior to IPP, so direct cross-talk would require the transfer of IPP itself, or a metabolite downstream from IPP, between compartments. This is already known not to occur on a large scale, although it may occur to some limited extent. Historically, feeding labeled intermediates in the MVA pathway does give labeling of plastidic isoprenoids, albeit at very low levels and only at certain times in plastid development (Treharne and others 1966). Similarly, Arigoni and others (1997) have demonstrated that label from [^{13}C]-deoxyxylose is incorporated into sitosterol, albeit at approximately 15 times less than incorporation into phytol and carotenoids.

More recent evidence for possible uptake of IPP from the MVA pathway into the plastids comes from work with *cla1* mutants. Although the *cla1* mutation is null, and seedlings are albino, they are not totally devoid of carotenoids or chlorophyll. These observations led Estevez and others (2000) to suggest that in this mutant MVA-derived IPP could provide a small amount of substrate for plastidic isoprenoid synthesis. However, this interpretation is open to question since Araki and others (2000) have reported that there is a second gene in *Arabidopsis* that has high homology to *CLA1*.

Specific inhibitors of the DOXP pathway can give additional information on potential cross-talk. However, the use of fosmidomycin cannot completely eliminate carotenoids, chlorophyll, or *ent*-kaurene in *Arabidopsis* (Okada and others 2002). Whether this is because a concentration that totally eliminates their synthesis would be lethal, or whether such complete elimination cannot be achieved because of cross-talk has yet to be determined. Moreover, there is no guarantee that cross-talk is a normal physiological feature, even if it might exist between the two pathways under such severely compromising circumstances as a near-lethal dose of herbicide.

Perhaps the best evidence for concerted cross-talk between the two pathways in plants comes from studies on the biosynthesis of some sequiterpenoids in chamomile (*Matricaria recutita*) flowers (Adam and Zapp 1998). These authors studied the incorporation of [^{13}C]-glucose into bisabolane-A and chamazulene (a break-down product of matricin), and were able to assign the origin of the three isoprenoid units that comprise each of these sesquiterpenoids based on the incorporation of ^{13}C at each position. Intriguingly, they found that in some of the sequiterpenoid molecules two of the isoprenoid units were formed via the DOXP pathway and one was of mixed MVA/DOXP pathway origin. The interpretation of these results is that geranyl diphosphate (GPP) is synthesized in the plastid via the DOXP pathway and is transported to another cellular compartment, perhaps the plastid intermembrane space or the cytosol. In this new location it appears that GPP is converted to farnesyl diphosphate (FPP, the parent molecule of sesquiterpenoids) by the addition of an IPP unit derived either via the DOXP pathway or via the MVA pathway. Clearly, this situation is exceptional as there is evidence that sesquiterpenoids in a large number of other plants are derived in totality from MVA-derived IPP.

Additional evidence of chimeric molecules derived from isoprenoid precursors synthesized from the two pathways comes from the liverwort *Heteroscyphus planus.* Nabeta and others (1997) have reported that [^{13}C]-mevalonate fed to this liverwort is actually incorporated into phytol, and in such a way that suggests that a FPP moiety biosynthesized by the cytosolic MVA pathway is combined with a isoprene unit derived from DOXP. Lichtenthaler (1999) also cites work in which the diterpene ginkgolide in *Gingko biloba* (maidenhair tree) was shown to be composed of an FPP moiety from the MVA pathway combined with a DOXP-derived isoprenoid unit. These initial reports present a sce-

nario of concerted, co-ordinated interplay between the two pathways in a manner that is deserving of continued investigation.

In addition to the potential for transfer of intermediates between pathways, there is also the potential for an intermediate in one pathway to be a regulatory factor in the other. There is distinct evidence for such a situation. Crowell and others (Dr. D. N. Crowell, personal communication) have demonstrated that a *cla1* mutant of *Arabidopsis* (formerly designated *lvr111*) that possesses all the phenotypic features of a plant in which the DOXP pathway is blocked (an albino phenotype and dwarfism) also has altered levels of HMG-CoA reductase, and elevated sterol content. In addition, Fray and others (1995) reported that an alteration in PSY levels in ripening tomato fruits not only gave observed changes in plastidic isoprenoids, but also an elevation of cytosolic sterols. However, this finding, unlike that of Crowell and others, does not pinpoint regulation to any particular position in the MVA pathway, nor is the identity of the regulatory factor or factors apparent in either case. Moreover, since blocking the DOXP pathway has such profound effects on growth it will be necessary to distinguish between indirect and direct effects on the MVA pathway.

Of particular interest to the present discussion would be a possible regulatory effect of one or more intermediates in the MVA pathway on plastidic isoprenoid biosynthesis. It is anticipated that this would be rather small, because of results that suggest that mevastatin has only a minimal effect on chlorophyll and carotenoid levels, and its effects on growth are not GA-reversible (Bach and Lichtenthaler 1982). However, this potential method of regulating plastidic isoprenoid biosynthesis should be re-examined in the light of our current knowledge of the DOXP pathway.

Conclusion

Progress towards elucidating the DOXP pathway to plant plastidic IPP has been rapid in the past few years. Five intermediates have been characterized, and the genes encoding five enzymes in the pathway have been cloned and expressed in heterologous systems. Although GAs have not been shown unequivocally to be derived from DOXP-derived IPP, the evidence presented here is supportive of such an assertion. However, definitive evidence must come from studies that demonstrate the unequivocal incorporation of label from DOXP pathway intermediates into *ent*-kaurenoids and GAs. Whether GAs are synthesized *only* by the DOXP pathway may be very hard to determine. Since IPP derived from the DOXP pathway is converted into such vital isoprenoids as the side-chains of chlorophyll and plastoquinone, one must assume that mutations and chemicals that knock out the DOXP pathway completely would be lethal. Hence, it may be very difficult to determine the contribution of MVA-derived intermediates to GA biosynthesis, though initial results suggest that the contribution, if any, is small.

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