Elucidation of the Methylerythritol Phosphate Pathway for Isoprenoid Biosynthesis in Bacteria and Plastids. A Metabolic Milestone Achieved through Genomics[1]

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Plants synthesize an enormous variety of metabolites that can be classified into two groups based on their function: primary metabolites, which participate in nutrition and essential metabolic processes within the plant, and secondary metabolites (also referred to as natural products), which influence ecological interactions between plants and their environment (Croteau et al., 2000). Isoprenoids (also called terpenoids) are the most functionally and structurally varied group of plant metabolites. Isoprenoids are synthesized in all organisms but are especially abundant and diverse in plants, with tens of thousands of compounds reported to date (Chappell, 1995, 2002; McGarvey and Croteau, 1995; Croteau et al., 2000). Many isoprenoids are present in all plants and act as primary metabolites with roles in respiration, photosynthesis, and regulation of growth and development. However, the highest variety of isoprenoids is secondary metabolites that function in protecting plants against herbivores and pathogens, in attracting pollinators and seed-dispersing animals, and as allelochemicals that influence competition among plant species (Croteau et al., 2000; Chappell, 2002). Many compounds with important commercial value as flavors, pigments, polymers, fibers, glues, waxes, drugs, or agrochemicals are secondary metabolites of isoprenoid origin. Each plant species synthesizes a specific array of isoprenoid secondary metabolites, and most of them (including rubber and the anticancer drug taxol) are produced only in a few wild or semiwild plant species. Although genetic engineering appears to be a powerful tool to direct the production of both primary and secondary isoprenoid products in plants, only a partial knowledge of the pathways involved in the biosynthesis of their precursors was available until very recently.

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ISOPRENOID BIOSYNTHESIS. A TALE OF TWO PATHWAYS

Despite their diversity of functions and structures, all isoprenoids derive from the common five-carbon (C_5) building units isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), also called isoprene units (Fig. 1). The simplest isoprenoids, like isoprene (a volatile product released from photosynthetically active tissues that participates in the formation of tropospheric ozone), contain a single C_5 unit and are called hemiterpenes. More complex isoprenoids are usually formed by "head-to-tail" or "head-to-head" addition of isoprene units. Monoterpenes are C_{10} isoprenoids that consist of two isoprene units and are components of the essences of flowers, herbs, and spices. The isoprenoids that derive of three isoprene units are C_{15} sesquiterpenes, which can be found in essential oils and act as antimicrobial phytoalexins and antifeedants. The diterpenes (C_{20}) include the side chain of chlorophyll, phylloquinones and tocopherol, gibberellins, phytoalexins, and taxol. The triterpenes (C_{30}), such as phytosterols, brassinosteroids, and some phytoalexins, toxins, and waxes, are generated by the joining of two C_{15} chains. The most prevalent tetraterpenes (C_{40}) are carotenoids, which are pigments in many flowers and fruits, contribute to light harvesting, and protect the photosynthetic apparatus from photooxidation. Polyterpenes contain more than eight isoprene units and include prenylated electron carriers (ubiquinone and plastoquinone) and polyprenols such as rubber and dolichol (required for protein glycosylation). The products of partial isoprenoid origin, including cytokinins or prenylated proteins, are called meroterpenes (McGarvey and Croteau, 1995; Croteau et al., 2000).

After the discovery of the mevalonic acid (MVA) pathway in yeast and animals in the 1950s, it was assumed that IPP was synthesized from acetyl-CoA via MVA and then isomerized to DMAPP in all organisms (Chappell, 1995; McGarvey and Croteau, 1995). In many cases, however, the experimental data on the biosynthesis of specific isoprenoids in plants and microorganisms could not be explained from the

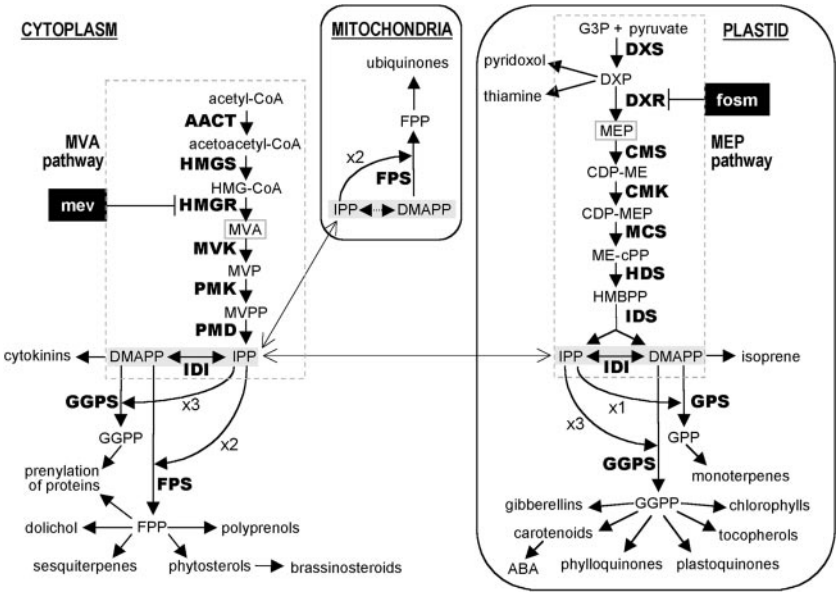

Figure 1. Isoprenoid biosynthesis pathways in the plant cell. HMG-CoA, Hydroxymethylglutaryl CoA; MVP, 5-phosphomevalonate; MVPP, 5-diphosphomevalonate; HBMPP, hydroxymethylbutenyl 4-diphosphate; FPP, farnesyl diphosphate; ABA, abscisic acid. The first intermediate specific to each pathway is boxed. Enzymes are indicated in bold: **AACT**, acetoacetyl CoA thiolase (EC 2.3.1.9); **HMGS**, HMG-CoA synthase (EC 4.1.3.5); **HMGR**, HMG-CoA reductase (EC 1.1.1.88); **MVK**, MVA kinase (EC 2.7.1.36); **PMK**, MVP kinase (EC 2.7.4.2); **PMD**, MVPP decarboxylase (EC 4.1.1.33); **IDI**, IPP isomerase (EC 5.3.3.2); **GPS**, GPP synthase (EC 2.5.1.1); **FPS**, FPP synthase (EC 2.5.1.10); **GGPS**, GGPP synthase (EC 2.5.1.29); **DXS** (EC 4.1.3.37); **DXR**, DXP reductoisomerase (EC 1.1.1.267); **CMS** (EC 2.7.7.60); **CMK** (EC 2.7.1.148); **MCS** (EC 4.6.1.12); **HDS**; **IDS**, IPP/DMAPP synthase. The steps specifically inhibited by mevinolin (mev) and fosmidomycin (fosm) are indicated.

exclusive operation of the MVA pathway (for review, see Lichtenthaler et al., 1997, 1999; Eisenreich et al., 1998, 2001; Rohmer, 1999). A few years ago, an alternative MVA-independent pathway for the biosynthesis of the isoprene building units was identified by labeling experiments in bacteria (Flesch and Rohmer, 1988; Rohmer et al., 1993; Broers, 1994) and plants (Schwarz, 1994). This pathway was originally named non-mevalonate pathway or Rohmer pathway. After the identification of the first steps of the pathway, its name was changed to indicate the substrates (pyruvate/glyceraldehyde 3-phosphate [G3P] pathway) or the first intermediate, deoxyxylulose (DX) 5-phosphate (DXP pathway). However, it is becoming more accepted to name the pathway after what is currently considered its first committed precursor, methylerythritol 4-phosphate (MEP), following the same rule used to name the MVA pathway.

Isoprenoids are synthesized in all living organisms, but experimental evidence accumulated since the discovery of the MEP pathway has shown that most organisms only use one of the two pathways for the biosynthesis of their precursors. Thus, the MEP pathway is the only one present in most eubacteria and the malaria parasite *Plasmodium falciparum*, but it is absent from archaebacteria, fungi and animals, which synthesize their isoprenoids exclusively through the operation of the MVA pathway. By contrast, plants use both the MEP pathway and the MVA pathway for isoprenoid biosynthesis, although they are localized in different compartments (Fig. 1; Lichtenthaler et al., 1997; Eisenreich et al., 1998, 2001; Lichtenthaler, 1999; Rohmer, 1999). The MEP pathway synthesizes IPP and DMAPP in plastids, whereas the MVA pathway produces cytosolic IPP (Fig. 1). Mitochondrial isoprenoids are synthesized from MVA-derived IPP that is imported from the cytosol (Lichtenthaler, 1999). Some exchange of IPP or a common downstream intermediate does also appear to take place between the plastids and the cytoplasm (for review, see Eisenreich et al., 1998, 2001; Lichtenthaler et al., 1997; Lichtenthaler, 1999; Rohmer, 1999). This limited exchange may explain in part why the MEP pathway was completely overlooked until very recently, because labeled precursors of the MVA pathway could be incorporated (although with very low efficiency) into most plastid isoprenoids. The now uncovered MEP pathway for the biosynthesis of isoprenoids may represent one of the last evolutionarily conserved metabolic pathways which remained to be unraveled.

SIMPLER IS BETTER. ELUCIDATION OF THE MEP PATHWAY IN *ESCHERICHIA COLI*

E. coli, the metabolically best studied bacterium, has served as a powerful model system for the elucidation of the MEP pathway (Fig. 2), which has been achieved thanks to multidisciplinary approaches that included organic chemistry, microbial genetics, biochemistry, molecular biology, and bioinformatics. However, the impressively fast identification of the genes involved in the pathway in bacteria and plants would not have been possible without recently developed genomic tools such as the availability of full genome sequences and expressed sequence tag (EST) collections. The elucidation of the MEP pathway is also a beautiful example of how genomics can be readily integrated with traditional approaches to identify whole metabolic pathways in distant organisms.

1996 to 1998. From the Precursors to the Identification of the First Genes of the Pathway

Although evidences of a MVA-independent pathway for IPP biosynthesis were found independently

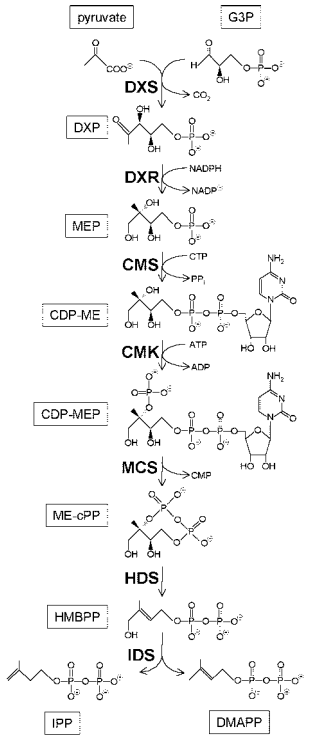

Figure 2. The MEP pathway. See Figure 1 for abbreviations.

by different research groups (Flesch and Rohmer, 1988; Rohmer et al., 1993; Broers, 1994; Schwarz, 1994), Rohmer and collaborators were the first in publishing their work in refereed journals. These authors used labeled precursors to study the biosynthesis of the bacterial isoprenoids hopanoids, and they observed labeling patterns that suggested the addition of a C_2 unit derived from pyruvate by decarboxylation to a C_3 triose phosphate (or a derivative) in a transketolase type reaction (Flesch and Rohmer, 1988; Rohmer et al., 1993). G3P and pyruvate were afterward identified as the direct precursors of IPP by labeling experiments with *E. coli* mutants defective in enzymes of the triose phosphate metabolism (Rohmer et al., 1996). These experiments suggested that the first reaction of the novel pathway involved the head-to-head condensation of (hydroxyethyl) thiamin derived from pyruvate with the C1 aldehyde group of G3P to yield DXP (Fig. 2), a compound that also serves as a precursor in the biosynthesis of the vitamins B_1 (thiamine) and B_6 (pyridoxol) in bacteria and plastids (Fig. 1). Other studies in different research groups confirmed the incorporation of labeled DX into bacterial and plant plastidial isoprenoids (for review, see Lichtenthaler et al., 1997; Eisenreich et al., 1998; Lichtenthaler, 1999; Rohmer, 1999).

Once this information was available, three independent approaches led to the identification of the first gene of the MEP pathway, encoding DXP synthase (DXS; Sprenger et al., 1997; Lange et al., 1998; Lois et al., 1998). Synthesis of DXP according to the mechanism described above required an acyloin condensation reaction whereby pyruvate is decarboxylated. This type of reaction was well documented as a secondary activity of thiamine diphosphate-dependent transketolases or the E1 component of pyruvate dehydrogenase or pyruvate decarboxylase. Taking advantage of the recent advent of full genomic sequence information for *E. coli*, Sprenger et al. (1997) and Lois et al. (1998) independently found a bacterial gene encoding a product with homology to transketolase and E1. Expression of the corresponding protein in *E. coli* and determination of its ability to form only DXP from pyruvate and G3P confirmed that it encoded a DXS enzyme (Sprenger et al., 1997; Lois et al., 1998). DXS-like sequences were found widespread in bacteria and plants but were absent from animal and yeast genomes. The Arabidopsis homolog had been previously described as CLA1, a plastid-targeted protein of unknown function encoded by a nuclear gene whose disruption caused an albino phenotype (Mandel et al., 1996). Following a homology-based approach, Lange et al. (1998) identified another plant transketolase-like sequence in a cDNA library from peppermint (*Mentha piperita*) oil gland secretory cells, which are highly specialized for monoterpene production and are therefore an enriched source of transcripts from genes involved in isoprenoid biosynthesis. The identified gene encoded a protein with DXS activity that was most similar to Arabidopsis CLA1, suggesting a role in the biosynthesis of plastid isoprenoids essential for photosynthesis and chloroplast function.

Rohmer et al. (1996) had proposed that an intramolecular rearrangement of DXP followed by an unspecified reduction process could produce MEP in the next reaction of the pathway (Fig. 2). Subsequent experiments showed that chemically synthesized ME was directly incorporated into *E. coli* isoprenoids (Duvold et al., 1997). A genetic strategy based on this information succeeded in identifying the bacterial gene encoding DXP reductoisomerase (DXR), the enzyme that converts DXP into MEP (Kuzuyama et al., 1998; Takahashi et al., 1998). Because MEP is only known to be a precursor for isoprenoids, these authors hypothesized that *E. coli* auxotrophic mutants requiring ME should be specifically affected in MEP and isoprenoid biosynthesis. After isolating mutants that grew on minimal medium with ME but not in the absence of this compound, they identified *yaeM* (now designated *dxr* or *ispC*) as the gene complementing ME auxotrophy in all the mutants and demonstrated that its product was a DXR enzyme involved in isoprenoid biosynthesis (Kuzuyama et al., 1998; Takahashi et al., 1998).

1999 to 2000. Bioinformatics and Comparative Genomics Identify New Candidate Genes

For the identification of the next gene of the MEP pathway, Rohdich et al. (1999) incubated radiola-

beled MEP with *E. coli* cell extracts and purified enzyme fractions and observed that a radioactive product was produced when the reaction mixture contained a nucleotide 5′-triphosphate (CTP was the preferred substrate). On the basis of NMR spectroscopy data, the structure of the new metabolite was assigned as 4-diphosphocytidyl ME (CDP-ME; Fig. 2). A database search with CDP and pyrophosphorylase as keywords retrieved a gene encoding a bacterial enzyme that catalyzes the formation of CDP-ribitol from ribitol 5-phosphate and CTP. Subsequent database searches with this sequence uncovered a number of similar genes from organisms with the MEP pathway, including Arabidopsis (in which the corresponding protein encompassed a putative plastid leader sequence). Activity assays with the recombinant product of the *E. coli* gene (*ygbP*, also designated *ispD*) demonstrated that it encoded a CDP-ME synthase (CMS) that specifically produced CDP-ME from MEP and CTP (Fig. 2). Furthermore, incubation of radiolabeled CDP-ME with pepper (*Capsicum annuum*) chromoplasts resulted in the incorporation of radioactivity into carotenoids, suggesting that this metabolite was an intermediate of the MEP pathway (Rohdich et al., 1999).

The identification of the *E. coli dxs*, *dxr*, and *ygbP* genes provided sequence information that established the basis for a comparative genomics procedure that eventually led to the elucidation of the entire MEP pathway: the bioinformatic search for genes that were conserved in eubacteria and plants (the latter showing a N-terminal extension that could serve as a plastid targeting signal) but absent in archaebacteria, yeast, and animals (which synthesize their isoprenoids exclusively from MVA). Thus, whole genome comparisons to identify genes after the distribution of the identified MEP pathway genes retrieved the next two genes of the MEP pathway, *ychB* and *ygbB*; Herz et al., 2000; Lüttgen et al., 2000). A procedure similar to that developed for *ygbP* was used to study the activity of the encoded proteins and their involvement in the MEP pathway. The purified recombinant enzyme encoded by the *E. coli ychB* gene was shown to be a CDP-ME kinase (CMK) that catalyzes the ATP-dependent phosphorylation of CDP-ME to CDP ME 2-phosphate (CDP-MEP). This compound was then converted into ME 2,4-cyclodiphosphate (ME-cPP) by the enzyme ME-cPP synthase (MCS), encoded by the *E. coli ygbB* gene (Fig. 2). As expected for MEP pathway enzymes, the plant homologs showed putative plastid signal peptides. In addition, incorporation experiments with pepper chromoplasts suggested that both CDP-MEP and ME-cPP were intermediates of the MEP pathway (Herz et al., 2000; Lüttgen et al., 2000). A plant gene homologous to *ychB* had previously been retrieved in a bioinformatic approach designed to identify ESTs encoding metabolite kinases in a cDNA library from peppermint oil gland secretory cells (Lange and Croteau, 1999a). Although these authors proposed that the encoded protein could phosphorylate isopentenyl monophosphate to IPP in the putative last step of the MEP pathway, further experiments with the recombinant enzymes from *E. coli* and tomato (*Lycopersicon esculentum*) showed that they catalyzed the phosphorylation of CDP-ME to CDP-MEP at a much higher rate, indicating that this is the true metabolic role of the enzyme (Rohdich et al., 2000a).

2000 to 2001. Strains Engineered to Synthesize IPP from MVA Demonstrate the Branching of the Pathway and Confirm the Role of the Previously Identified Genes

Although the results described above strongly suggested that *ygbP* (*ispD*), *ychB* (*ispE*),and *ygbB* (*ispF*) encoded enzymes directly involved in the MEP pathway, a clear-cut demonstration was provided by the development of a neat experimental system originally designed for the cloning of unknown MEP pathway genes in *E. coli* (Kuzuyama et al., 2000a, 2000b; Takagi et al., 2000; Campos et al., 2001a). To rescue lethal mutants in the MEP pathway genes, *E. coli* cells were genetically engineered with a recombinant MVA operon containing heterologous genes for the last three enzymes of the MVA pathway: MVA kinase, MVP kinase, and MVPP decarboxylase (see Fig. 1). These cells do not synthesize MVA, but they can take it from the growth medium and use it as an alternative source of IPP, which could be then converted to DMAPP by the *E. coli* IPP isomerase encoded by the *idi* gene (Hahn et al., 1999). By using this system Rodríguez-Concepción et al. (2000) demonstrated that *idi* is the only gene encoding an enzyme with IPP isomerase activity in *E. coli* and showed that this enzyme plays a role in isoprenoid biosynthesis in vivo. However, *idi* is not an essential gene in *E. coli* (Hahn et al., 1999; Rodríguez-Concepción et al., 2000). The work with strains harboring the MVA operon supported previous evidence from labeling experiments (Giner et al., 1998; Charon et al., 2000) demonstrating that the MEP pathway branched at some point after MEP leading to the separate synthesis of IPP and DMAPP (Rodríguez-Concepción et al., 2000). The MVA operon system was also used by two independent groups to provide genetic evidence that the enzymes encoded by *ygbP*, *ychB*, and *ygbB* catalyze reactions of the MEP pathway before the proposed branching, because the disruption of these genes was lethal, indicating that they were not acting in the proposed branches to IPP or DMAPP) and could be rescued with MVA (Kuzuyama et al., 2000a, 2000b; Takagi et al., 2000; Campos et al., 2001a).

2001 to 2002. Identification of the Last Two Genes of the Pathway

Although the described system with the MVA operon was a good genetic tool for the discovery of

the rest of the MEP pathway genes, they were first described by bioinformatic approaches of comparative genomics (Cunningham et al., 2000; Campos et al., 2001b). The *E. coli* genes annotated as *gcpE* (*ispG*) and *lytB* (*ispH*) were putatively ascribed to the MEP pathway because they were conserved in plants and eubacteria with this pathway but were absent from archaebacteria, yeast, and animal genomes. In addition, the corresponding plant gene products contained an N-terminal domain that could act as a plastid targeting signal. Directed deletion of *gcpE* (Altincicek et al., 2001b; Campos et al., 2001b) or *lytB* (Altincicek et al., 2001a) in *E. coli* strains engineered with the MVA operon resulted in cells that were able to grow only when the medium was supplemented with MVA, demonstrating that both genes were required specifically for IPP biosynthesis in *E. coli*. Subsequent studies (Hecht et al., 2001; Seemann et al., 2002a, 2002b; Wolff et al., 2002) contributed to reveal that the *gcpE* gene product encoded an enzyme (hydroxymethylbutenyl 4-diphosphate [HMBPP] synthase [HDS]) that catalyzes the formation of HMBPP from ME-cPP (Fig. 2). The role of *lytB* is less clear, but it appears to encode an enzyme (IDS) that directly converts HMBPP into a 5:1 mixture of IPP and DMAPP (Fig. 2; Rohdich et al., 2002). Therefore, the activity of this enzyme could be identified as responsible for the branching, which had been previously predicted by biochemical and genetic approaches (Giner et al., 1998; Charon et al., 2000; Rodríguez-Concepción et al., 2000). The branching is an important difference with the MVA pathway, in which IPP and DMAPP are generated sequentially, the latter arising from the former in a reaction catalyzed by IPP isomerase (Fig. 1).

THE MEP PATHWAY IN PLANTS

The recent development of genomic tools is revolutionizing the study of plant metabolism. As described above, the MEP pathway is a good example of how bioinformatics and comparative genomics have made relatively fast and simple to identify the genes potentially involved in a metabolic pathway in different organisms based only on sequence information. Searches on The Arabidopsis Information Resources database (http://www.Arabidopsis.org) indicate that genes encoding proteins with homology to all the *E. coli* MEP pathway enzymes are present in Arabidopsis (Table I). The ChloroP algorithm (http://www.cbs.dtu.dk/services/ChloroP) predicts that all of these proteins contain a putative plastid targeting peptide of variable length (Table I), consistent with their predicted role in plastid isoprenoid biosynthesis. Functional genomics approaches consisting of the generation and screening of collections of T-DNA and transposon insertion mutants have led to the identification of Arabidopsis mutants defective in the genes encoding DXS, DXR, and CMS (Budziszewski et al., 2001). All of these mutants have a seedling-lethal albino phenotype, confirming that the MEP pathway is essential for plant life. With the increasing availability on public on-line databases of plant functional genomics tools (including collections of ESTs and DNA microarrays), it will soon become possible to even deduce accurate gene expression data that may provide some clues as to their biological role. However, only functional analysis of each proposed plant protein ortholog with biochemical and genetic approaches will ascertain its contribution to the biosynthesis of plastid isoprenoids.

Plant Genes and Enzymes

Genes and ESTs corresponding to all the MEP pathway enzymes (Table I) can be found in the availableArabidopsis databases. The most abundant ESTs are those from the genes encoding DXS and HDS (about 0.2‰ of all the ESTs in the collections), followed by IDS (0.1‰). These ESTs are widely distributed in the available Arabidopsis collections (which are made from a variety of tissues and developmental stages) suggesting that the corresponding genes are expressed throughout the plant. From all of the tentative orthologs of the *E. coli* MEP pathway enzymes that can be found in the Arabidopsis genome,

Table I. *Arabidopsis MEP pathway proteins*

The enzymes that have been demonstrated to be involved in plastid isoprenoid biosynthesis are shown in bold. The length of the proteins and their predicted plastid targeting peptides (PTP) is indicated with the number of amino acid residues. The total number of ESTs in GenBank Arabidopsis collections (113,330 ESTs) is also shown.

Protein	Other Names	Accession No.	Length (PTP)	ESTs
DXS	CLA1	At4g15560	717 (58)	23
DXS2		At3g21500	628 (35)	3
DXS3		At5g11380	700 (47)	1
DXR	ISPC,YAEM	At5g62790	477 (49)	4
CMS	ISPD,YGBP	At2g02500	302 (61)	1
CMK	ISPE,YCHB	At2g26930	383 (41)	6
MCS	ISPF,YGBB	At1g63970	223 (52)	4
HDS	ISPG,GCPE	At5g60600	740 (38)	24
IDS	ISPH,LYTB	At4g34350	452 (38)	13

only DXS might be encoded by more than one gene (Table I). Arabidopsis *cla1* mutants defective in DXS (At4g15560) show an albino phenotype and a very early arrest of chloroplast development that can be rescued with DX (Mandel et al., 1996; Araki et al., 2000; Estévez et al., 2000). However, mutant plants can still accumulate low levels of plastid isoprenoids such as chlorophylls and carotenoids, suggesting either an import of cytosolic MVA-derived isoprenoid precursors to the plastids or the presence of extra DXS enzymes (Araki et al., 2000; Estévez et al., 2000). Two other Arabidopsis proteins, predicted from genomic and EST sequences and tentatively named DXS2 (At3g21500) and DXS3 (At5g11380), show homology to DXS (Fig. 3). Only a few ESTs from these genes have been found in green siliques (three ESTs from *DXS2*) and roots (one EST from *DXS3*), suggesting that their expression is low and may be restricted to certain tissues or developmental stages. By contrast, the gene encoding DXS is widely expressed in the Arabidopsis plant, as deduced from the number and distribution of ESTs (Table I) and the analysis of mRNA and protein accumulation (Estévez et al., 2000). The differential expression pattern could explain why DXS-deficient seedlings show a block in plastid isoprenoid synthesis (which causes the albino phenotype) that is not rescued by the other two putative DXS isoforms. Although both DXS2 and DXS3 contain N-terminal sequences predicted by the ChloroP program to target them to plastids (Table I; Fig. 3), it is not known whether they are functional DXS enzymes with a role in the MEP pathway. The deduced mature proteins lack stretches of amino acids that are present in all the bacterial and plant DXS enzymes (Lois et al., 1998, 2000), and a conserved His residue essential for DXS activity (Querol et al., 2001) is not present in the DXS3 protein (Fig. 3). A functional analysis is therefore needed to confirm the predictions generated by the sequence-based analysis and to demonstrate their biological function.

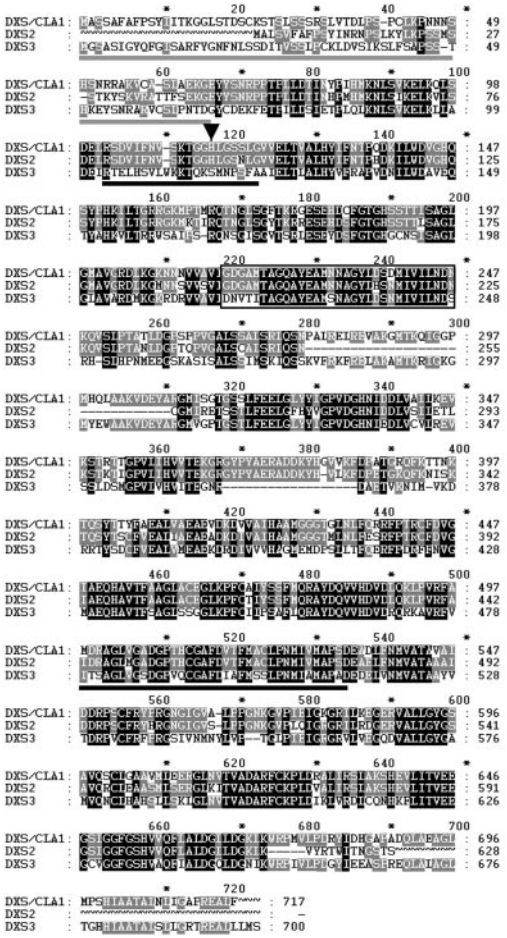

Figure 3. Multiple alignment of Arabidopsis DXS-like proteins. Sequences from Arabidopsis DXS (At4g15560), DXS2 (At3g21500), and DXS3 (At5g11380) were aligned using the ClustalW program (http://www2.ebi.ac.uk/clustalw). Identical residues are highlighted in black boxes (when present in all three sequences) or gray boxes (those only present in two sequences). The N-terminal region absent from bacterial DXS proteins is underlined in gray. Sequence signatures of transketolase and DXS enzymes (Querol et al., 2001) include the thiamine diphosphate-binding domain (boxed) and two other conserved motifs (black bar), one of which contains a His residue required for activity (arrowhead).

The rest of the Arabidopsis MEP pathway enzymes (Table I) appear to be encoded by a single gene, and functional data supporting their role in plastid isoprenoid biosynthesis are available for DXR (Lange and Croteau, 1999b; Schwender et al., 1999; Carretero-Paulet et al., 2002), CMS (Rohdich et al., 2000b; Okada et al., 2002), and HDS (Querol et al., 2002). A CMK ortholog from tomato has also been described (Rohdich et al., 2000a). The most obvious difference between plant and *E. coli* MEP pathway enzymes is the presence of N-terminal extensions of variable sequence and length (Table I), which have been shown to function as plastidial signal peptides for plant DXS (Araki et al., 2000; Lois et al., 2000), DXR (Rodríguez-Concepción et al., 2001; Carretero-Paulet et al., 2002), and HDS (Querol et al., 2002). The mature proteins produced after cleavage of these peptides are similar to the bacterial enzymes except in the case of HDS (GCPE), which contains a large plant-specific domain (Querol et al., 2002). The mature Arabidopsis HDS protein is able to complement the lethal deletion of the *gcpE* gene in *E. coli*, but it is possible that because of this extra domain, the plant protein may have distinct regulatory or catalytic functions. Most of the work on the characterization of the MEP pathway enzymes has been done with the *E. coli* proteins, including the resolution of the crystal structure of the enzymes DXR (Reuter et al., 2002; Yajima et al., 2002), CMS (Kemp et al., 2001; Richard et al., 2001), and MCS (Kemp et al., 2002; Richard et al., 2002; Steinbacher et al., 2002). By contrast, only limited knowledge about the catalytic properties of the plant enzymes is available (for review, see Eisenreich et al., 2001).

Regulation of the Metabolic Flow through the MEP Pathway

Despite the impressive progress in the elucidation of the MEP pathway in bacteria and plants, much work is still ahead to analyze the contribution of the different enzymes to the control of the flux of intermediates through the pathway that will eventually determine the supply of IPP and DMAPP for the synthesis of plastid isoprenoid end products. The first studies have been carried out with DXS and DXR (Mandel et al., 1996; Bouvier et al., 1998; Lange et al., 1998; Lange and Croteau, 1999b; Schwender et al., 1999; Araki et al., 2000; Chahed et al., 2000; Estévez et al., 2000, 2001; Lois et al., 2000; Veau et al., 2000; Walter et al., 2000; Mahmoud and Croteau, 2001; Rodríguez-Concepción et al., 2001; Carretero-Paulet et al., 2002). To date, DXS is the only enzyme of the MEP pathway that has been shown to have a limiting role for isoprenoid biosynthesis in all the systems analyzed, including Arabidopsis (Estévez et al., 2001), tomato (Lois et al., 2000), and bacteria (Harker and Bramley, 1999; Miller et al., 1999, 2000; Kuzuyama et al., 2000c; Matthews and Wurtzel, 2000). The role of DXR is less clear. Overexpression studies suggest that DXR activity is not limiting for isoprenoid biosynthesis in bacteria (Miller et al., 2000). The dramatic accumulation of carotenoids that takes place during tomato fruit ripening does not require increased levels of *DXR* transcripts and encoded protein either (Rodríguez-Concepción et al., 2001). By contrast, overexpression of DXR in peppermint led to increased isoprenoid synthesis (Mahmoud and Croteau, 2001), and a positive correlation was found between enhanced isoprenoid biosynthesis and accumulation of transcripts encoding both DXS and DXR in monocot roots (Walter et al., 2000) and periwinkle (*Catharanthus roseus*) cell cultures (Veau et al., 2000). The distribution of *DXR* and *DXS* transcripts in the Arabidopsis plant is similar, with highest levels in light-grown seedlings and inflorescences (Carretero-Paulet et al., 2002). However, *DXS* expression precedes that of *DXR* in some organs, such as developing inflorescences, suggesting that DXR instead of DXS might be limiting for the onset of plastid isoprenoid biosynthesis in this case (Carretero-Paulet et al., 2002). Together, the results support a general regulatory role for DXS in controlling the metabolic flux through the MEP pathway, whereas DXR activity may be limiting or not depending on the species, organ, and/or developmental stage. It is likely that other enzymes of the MEP pathway may also contribute to regulate the supply of intermediates for plastid isoprenoid biosynthesis, but this remains to be established.

Coordination with Related Metabolic Pathways

The MEP pathway produces plastidial IPP and DMAPP precursors that are then used as building blocks for the production of isoprenoid end products by many different pathways (Fig. 1). A central question is how the downstream pathways are coordinated with the MEP pathway (and among them) to make sure that the required precursors will be supplied when needed. Expression of some of the MEP pathway genes has been shown to either precede or parallel the activation of specific pathways for the production of monoterpenes in peppermint oil gland secretory cells (Lange et al., 1998), monoterpenoid indole alkaloids in periwinkle cell cultures (Veau et al., 2000), apocarotenoids in monocot roots (Walter et al., 2000), and carotenoids in pepper and tomato fruit (Bouvier et al., 1998; Lois et al., 2000). In the last case, it has been shown that the expression of tomato *DXS* can be regulated by changes in the carotenoid composition of the fruit (Lois et al., 2000). Furthermore, changes in the levels of MEP pathway intermediates in tomato fruit fed with DX or treated with fosmidomycin (a specific inhibitor of DXR activity; Fig. 1) induced the expression of *DXS* but also of *PSY1*, the gene encoding the committed enzyme that catalyzes the first step of the carotenoid pathway in fruit (Lois et al., 2000; Rodríguez-Concepción et al., 2001). These results suggest a significant coordination between both the MEP pathway and the carotenoid pathway through the control of the expression of key genes, which may contribute to a fine regulation of carotenoid accumulation. Interference with this balanced regulation by overexpression of *PSY1* under the *35S* promoter in transgenic tomato led to the production of dwarf plants because the geranylgeranyl diphosphate available for gibberellin synthesis was redirected into the carotenoid pathway (see Fig. 1; Fray et al., 1995). This exemplifies how our limited knowledge on the mechanisms by which the MEP pathway and the downstream pathways are coordinated represents an important obstacle to modify precisely the production of specific isoprenoid end products.

The unique compartmentalization of isoprenoid biosynthesis in plants involves the existence of additional plant-specific regulatory mechanisms. Although the MEP pathway and the MVA pathway are independent pathways that are physically separated, they usually coexist within the plant cell (Fig. 1). In fact, a limited exchange of isoprene building units (IPP and DMAPP) or a common downstream intermediate takes place between compartments, and some isoprenoid end products are built from precursors supplied by both the MEP pathway and the MVA pathway (for review, see Eisenreich et al., 1998, 2001; Lichtenthaler et al., 1997; Lichtenthaler, 1999; Rohmer, 1999). Although the extent of this crossflow depends on the plant species, it has been estimated to be below 1% in intact plants under physiological conditions (Eisenreich et al., 2001). In experiments carried out with seedlings, the rate of exchange of intermediates appears not to be high enough to fully rescue a block of one of the two pathways. Thus, the

specific inhibition of MVA-derived isoprenoid biosynthesis with mevinolin (Fig. 1) in radish (*Raphanus sativus*) seedlings cannot be overcome by the delivery of common isoprenoid intermediates from the plastidial MEP pathway (Schindler et al., 1985). Arabidopsis mutant seedlings defective in MEP pathway genes (Mandel et al., 1996; Araki et al., 2000; Estévez et al., 2000; Budziszewski et al., 2001) similarly show an albino phenotype likely because the block in the synthesis of plastid isoprenoids required for photosynthesis and photoprotection (such as chlorophylls, carotenoids, tocopherol, and plastoquinone) cannot be rescued by the import of cytosolic MVA-derived intermediates. The same phenotype is observed when seeds from Arabidopsis (Fig. 4) or tomato (Rodríguez-Concepción et al., 2001) are germinated in the presence of fosmidomycin, a specific DXR inhibitor that causes a general block in plastid isoprenoid biosynthesis (Zeidler et al., 1998). However, the dynamics and the regulation of the crossflow of common intermediates between cell compartments may vary dramatically in different species, cell types, and/or developmental stages. This is an area of intensive research that will benefit from the availability of specific inhibitors such as mevinolin and fosmidomycin (Fig. 1) to block any of the two pathways for isoprenoid synthesis in a given plant, organ, or stage of development. For instance, treatment of tomato mature green fruit with fosmidomycin inhibited subsequent carotenoid accumulation (Zeidler et al., 1998; Rodríguez-Concepción et al., 2001), resulting in fruit of yellow-orange color instead of red when ripe (Fig. 4C). These results and previous experiments of treatment with mevinolin (Rodríguez-Concepción and Gruissem, 1999) support that the MVA pathway does not contribute significantly to carotenoid biosynthesis in tomato fruit. Future experiments should establish how the crossflow of MEP- or MVA-derived isoprenoid intermediates is modulated under physiological conditions and the nature of the transport system for prenyl diphosphate compounds between cytoplasm and plastids.

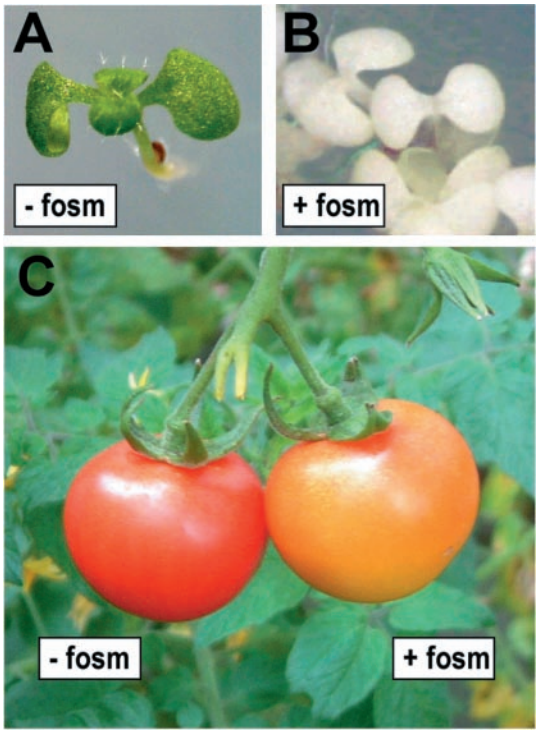

Figure 4. Inhibition of plastid isoprenoid biosynthesis with fosmidomycin (fosm). A, Arabidopsis seedling grown for 10 d on Murashige and Skoog medium. B, Arabidopsis seedlings grown for 10 d on Murashige and Skoog medium supplemented with 100 μM fosmidomycin. C, Tomato fruit 2 weeks after injection at the mature green stage with a 10 mM Tris, pH 8.5, solution (left fruit) or the same solution containing fosmidomycin to a final concentration of 200 μM (right fruit). Fruit volume was estimated from the diameter.

CONCLUDING REMARKS

The joint contribution of genomics integrated with traditional biochemical and genetic approaches has led to the impressively fast elucidation of the MEP pathway for the biosynthesis of plastid isoprenoids, a metabolic milestone that represents a huge step forward toward understanding (and manipulating) isoprenoid biosynthesis in plants. Nevertheless, we still lack fundamental knowledge on the regulatory mechanisms that control the flow of intermediates through the pathway and the coordination with related metabolic pathways. The benefits that the characterization of the MEP pathway can represent go beyond metabolic engineering. The MEP pathway, which is absent from humans but is present in pathogenic bacteria (many of which are acquiring resistance to currently available antibiotics) and in the malaria parasite *Plasmodium falciparum*, constitutes an ideal target for the development of novel antimalarial and antibacterial agents (Jomaa et al., 1999; Altincicek et al., 2001c; Hintz et al., 2001). Plants are promising test systems for the development of such inhibitors of the MEP pathway, which could also serve as herbicides (Zeidler et al., 2000; Lange et al., 2001).

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