Methyl erythritol 4-phosphate (MEP) pathway metabolic regulation

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Methyl Erythritol 4-phosphate (MEP) Pathway Metabolic Regulation

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The methylerythritol 4-phosphate (MEP) pathway is the recently discovered source of isoprenoid precursors isopentenyl diphosphate and dimethylallyl diphosphate in most bacteria, some eukaryotic parasites, and the plastids of plant cells. The precursors lead to the formation of various isoprenoids having diverse roles in different biological processes. Some isoprenoids have important commercial uses. Isoprene, which is made in surprising abundance by some trees, plays a significant role in atmospheric chemistry. The genetic regulation of this pathway has been discussed but information about metabolic regulation is just now becoming available. This review covers metabolic regulation of the MEP pathway starting from the inputs of carbon, ATP, and reducing power. A number of different regulatory mechanisms involving intermediate metabolites and/or enzymes are discussed. Some recent data indicate that methylerythritol cyclodiphosphate, the fifth intermediate of this pathway, is a key metabolite. It has been found to play diverse roles in regulation within the pathway as well as coordinating other biological processes by acting as a stress regulator in bacteria and possibly a retrograde signal from plastids to nucleus in plants. In this review we focus on the role of the MEP pathway in photosynthetic leaves during isoprene emission and more generally the metabolic regulation of the MEP pathway in both plants and bacteria.

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1 Introduction

Isoprenoids, also known as terpenoids, constitute the largest group of natural products. They are the most abundant secondary metabolites present in all living organisms including both prokaryotes and eukaryotes.[1, 2] More than 35,000 different isoprenoids have been reported so far.[3] Some of them include carotenoids, chlorophylls, plastoquinones, ubiquinones, sterols, dolichols, cytokinins, brassinosteroids, gibberellic acid, abscisic acid, and prenylated proteins.[2] Some isoprenoids have significant roles in primary metabolism like photosynthesis, respiration, and regulation of growth and development.[2, 4] Various other biological processes like defence mechanisms of plants against different biotic and abiotic stresses, attracting pollinators and seed dispersers for reproductive processes in plants, intracellular signal transduction, vesicular transport within the cell, and construction of cellular and organelle membrane are mediated by different isoprenoids.[2-6]

In addition to various biological roles, some isoprenoids have commercial applications as pigments, fragrance and flavours, drugs, and polymers.[7] A large number of natural products used as therapeutic agents are terpenoids. A wide variety of therapeutic properties of this group of natural products include anticancer, antiparasitic, antimicrobial, antiallergenic, antispasmodic, antihyperglycemic, anti-inflammatory, and immunomodulatory properties.[8] A well-known anticancer drug,

paclitaxel, is a complex diterpenoid obtained from the bark of Pacific yew.[8] A wide variety of monoterpenes and sequiterpenes contribute to various odours ranging from fruity and flowery smell to woody and balsamic smell.[9] Different terpenoids like menthol (minty odour), D-carvone (spicy odour), D-limonene (orange peel odour), citral (lemon peel odour), and 1,8-cineole (Eucalyptus odour) are extensively used in flavor and fragrance industries.[9] Different terpenoids like β-carotene, lutein, zeaxanthin, lycopene, phytoene are widely used as pigments in food industries.[10] Rubber, the most abundant polymer used in various industries, is chemically composed of linearly arranged polyterpenoids.[11]

In terms of total production, the most important isoprenoid is isoprene, the smallest member of isoprenoid family. Isoprene is emitted by many organisms including bacteria, plants, and humans. The global annual production of isoprene from plants is estimated to be 600 Tg (teragrams), which is about one third of the global non-methane hydrocarbon emission.[12] A few plants, mainly certain species of pine trees (e.g., lodgepole, ponderosa) in the western part of North America[13-16] also produce the related compound 2-methyl-3-buten-2-ol (methylbutenol or MBO).[17] Atmospheric chemistry is strongly affected by these hemiterpenes emitted by vegetation. In the presence of nitric oxide, isoprene catalyses the formation of ozone, which can cause atmospheric pollution and is detrimental to both humans and plants.[18] There have been many attempts to build mechanistic models of isoprene emission from leaves, which should help identify gaps in our understanding and may better predict isoprene emission under future conditions[19] but it has been difficult to determine the correct molecular basis for these models. A major limitation is understanding the metabolic regulation of the MEP pathway in isoprene-emitting leaves.

Isoprenoids are derived from two isomeric five-carbon units called isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP).[20, 21] (These compounds are also known as pyrophosphates but diphosphate is the preferred term when pyrophosphate is esterified.) Isoprene itself is made from DMADP.[22, 23] It was known for a long time that IDP (only) is synthesized by the acetate/mevalonate (MVA) pathway followed by isomerization by one of two different isomerases (IDI1 and IDI2).[20, 24, 25] Studies involving labelling of polyprenoids by feeding ^{13}C-labelled precursors indicated that an alternative pathway exists for the biosynthesis of isoprenoids.[20] In the early 1990s, an alternative pathway, now known as the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, was discovered in bacteria, that leads to the biosynthesis of both IDP and DMADP.[20, 26, 27] Because the last step in the pathway makes both IDP and DMADP, bacteria do

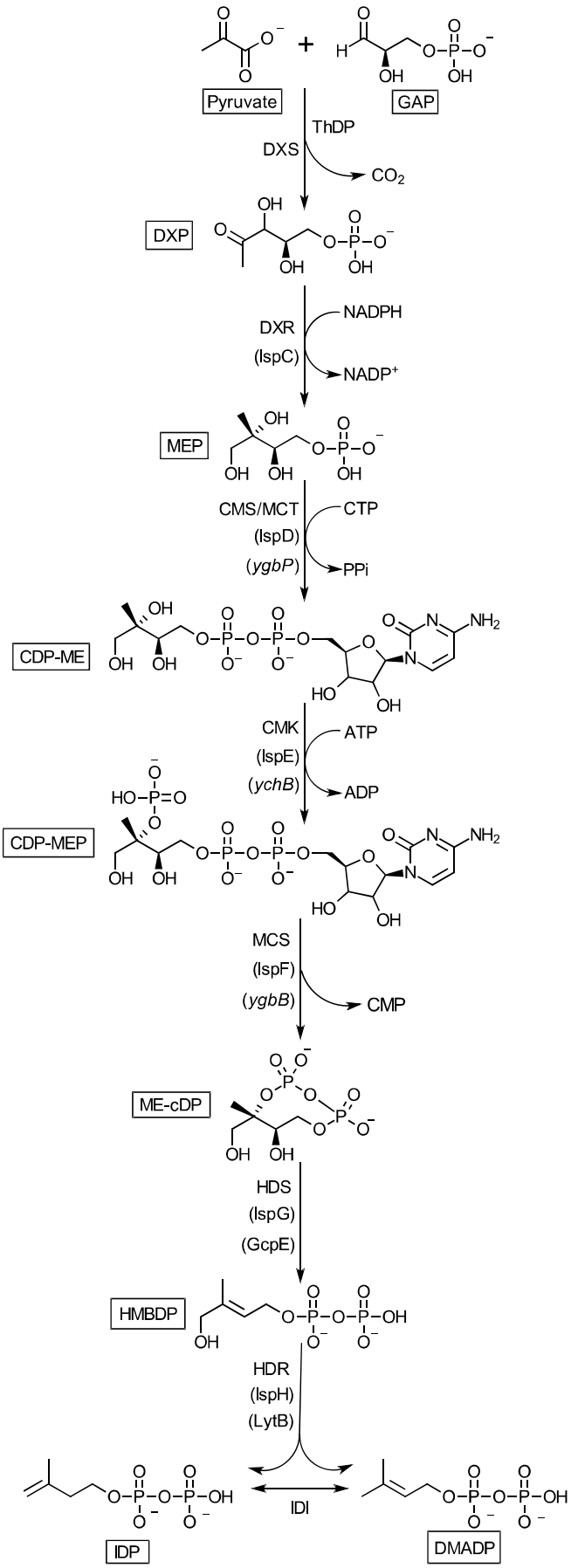

Fig. 1. The metabolites and the enzymes involved in the MEP pathway. DXS = 1-deoxy-D-xylulose 5-phosphate synthase, DXR = 1-deoxy-D-xylulose 5-phosphate reductoisomerase, CMS/MCT = 4-diphosphocytidyl-2-*C*-methylerythritol synthase/2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase, CMK = 4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol kinase, MCS = 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase, HDS = 4-hydroxy-3-methylbut-2-enyl diphosphate synthase, HDR = 4-hydroxy-3-methylbut-2-enyl diphosphate reductase.

not require IDI and some do not have IDI. Subsequent studies have demonstrated the presence of the MEP pathway in plastids of green algae and higher plants.[28-34] Both the MEP and the MVA pathway are present in higher plants and are localized in the chloroplast and cytoplasm respectively.[5] However, the MEP pathway is not present in humans.

The MEP pathway comprises seven enzymatic steps (Fig. 1).[18] It starts with the biosynthesis of 1-deoxy-D-xylulose 5-phosphate (DXP) from pyruvate and D-glyceraldehyde 3-phosphate (GAP) catalysed by the enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXS).[27, 35] In the next step, DXP is converted to MEP by the enzyme 1-deoxy-D-xylulose- 5-phosphate reductoisomerase (DXR). MEP is then converted to the cyclic intermediate methylerythritol 2,4-cyclodiphosphate (MEcDP) through three consecutive enzymatic steps involving cytidylation (CTP-dependent), phosphorylation (ATP-dependent), and cyclization. In the sixth step, MEcDP is converted into hydroxymethylbutenyl diphosphate (HMBDP) catalyzed by HMBDP synthase (HDS). In the last step, HMDBP is reduced to IDP and DMADP by HMBDP reductase (HDR). IDP and DMADP are also isomerized by isopentenyl diphosphate isomerase (IDI).

The MEP pathway was originally known as non-mevanolate pathway or Rohmer pathway.[27, 35-37] The discovery of the first step of this pathway involving the formation DXP from pyruvate and glyceraldehyde-3-phosphate led to the name of DXP pathway or pyruvate/glyceraldehyde-3-phosphate pathway.[35] DXP is, however, found to be a precursor for the biosynthesis of thiamin and pyridoxol in certain bacteria.[35, 38, 39] The second intermediate MEP, on the other hand, contains the characteristic branched C5 skeleton for all isoprenoids and is so far not known to be involved in other biochemical pathways.[40] Thus MEP is considered to be the first committed intermediate of this pathway and the name of this pathway is widely accepted as MEP pathway.[35, 40]

The absence of the MEP pathway in humans and its presence in eubacteria, apicomplexa parasites, and photosynthetic eukaryotes make it a target for development of new antibiotics, antiparasitic drugs, and herbicides.[21, 41] Various terpenoids with potential therapeutic activities are available in limited quantity from natural sources;[8] metabolic engineering leading to improved biosynthetic production of these important terpenoids has commercial potential. A sesquiterpene antimalarial drug, artemisinin, comes from *Artemisia annua* but now has been engineered in yeast.[42, 43] Some isoprenoids including carotenoids, tocopherols, and antimicrobial drugs are important targets for biotechnological manipulation.[21, 44]

The genetic regulation of the MEP pathway has been reviewed extensively.[7, 21, 45] A recent review has discussed the mechanistic details of the enzymes involved in this pathway.[46] A short account of the regulation involved in the metabolomics of the MEP pathway has also been recently discussed.[47] Here we emphasize discoveries that have been made in the past ten years regarding the regulation of MEP pathway based on enzymatic activity and metabolites involved in the pathway. Insights into the metabolic regulation of the MEP pathway can be beneficial for the biomedical and biotechnological purposes. In this review we provide some examples of how improved understanding of the MEP pathway may improve models of global isoprene emission.

2 Regulation of inputs into the pathway

Metabolic regulation of the MEP pathway is dictated by the source of carbon and energetic cofactors. Intensive research has been carried out in the past few years to understand how the carbon flux contributes to the regulation of this pathway. Earlier studies were mainly done by observing the pattern of labelled isoprene emission after feeding $^{13}CO_2$ or deuterated deoxyxylulose, the isotopic composition of isoprene being an indicator of the carbon source of DMADP and hence the MEP pathway.[48-55] The effect of the availability of carbon, ATP, and reducing power equivalents on the metabolomics of the MEP pathway are summarized here.

2.1 Carbon supply

Results from early studies involving the incorporation of ^{13}C-labelled precursors into terpenoids was inconsistent with acetate as the starting compound.[20, 26, 56] Later studies showed that the MEP pathway starts with the synthesis of DXP from GAP and pyruvate catalysed by DXS.[27, 38, 39, 57-60] The supply of GAP and pyruvate for the MEP pathway in bacteria can be maintained through primary metabolism and so will not be considered here. The source of GAP and pyruvate for the MEP pathway in chloroplasts of plants is potentially more complex (Fig. 2).

When $^{13}CO_2$ is fed to photosynthesizing plant leaves, isoprene rapidly becomes labelled confirming the close relationship between isoprene synthesis and the Calvin-Benson cycle.[48, 50, 61-63] Sugars transported in the xylem can provide additional carbon for leaf isoprene biosynthesis through MEP pathway.[53, 54] Isoprene does not become completely labelled when $^{13}CO_2$ is fed, but, for reasons not yet known, the intermediates of the Calvin-Benson cycle also do not become fully labelled over short time frames,[64] thus isoprene labelling kinetics may be fully consistent with all of the carbon for isoprene coming from the Calvin-Benson cycle.[48] However, analysis of the fragments of isoprene in mass spectrometry studies have been interpreted to indicate a slightly slower labelling of carbon atoms derived from pyruvate.[50, 65]

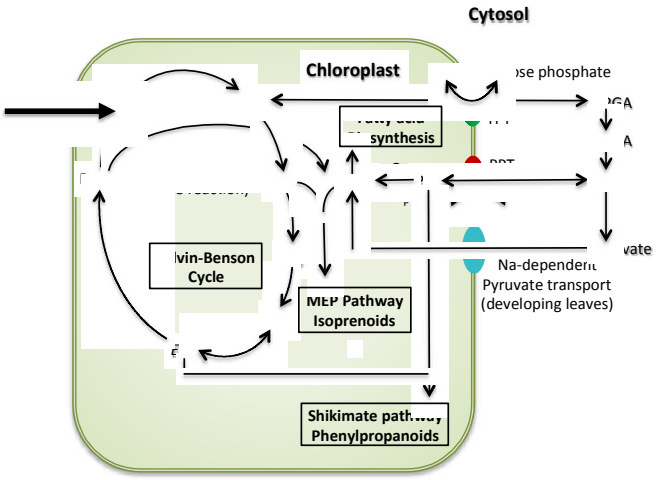

Fig. 2. Possible sources of pyruvate required for feeding into the MEP pathway. Abbreviations RuBP = ribulose 1,5-bisphosphate, 3-PGA = 3-phosphoglyceric acid, FBP = fructose 1,6-bisphosphate, F6P = fructose 6-phosphate, E4P = erythrose 4-phosphate, PEP = phosphoenolpyruvate, GAP = glyceraldehyde 3-phosphate, 2-PGA = 2-phosphoglyceric acid, TPT = triose phosphate/phosphate transporter, PPT = phospho*enol*pyruvate/phosphate translocator, Pi = inorganic phosphate, PK_p = pyruvate kinase (plastidic).

There are several sources of chloroplastic pyruvate for the MEP pathway. A small amount of pyruvate is produced by Rubisco through β-elimination of phosphate from a carbocation intermediate of the Rubisco reaction.[66] The ratio of pyruvate produced by carboxylation of ribulose bisphosphate is 0.7% at 25 °C. One pyruvate leads to the loss of five carbons as isoprene. Therefore, Rubisco production of pyruvate could support carbon loss as isoprene at a rate of 3.5% (0.7% times five carbons in isoprene) of carbon assimilation and as much as 4.3% if photorespiration, which makes the rate of CO_2 assimilation smaller than the rate of carboxylation. Carbon loss as a result of isoprene emission in excess of 3.5 to 4.3% of photosynthetically fixed carbon would require pyruvate from other carbon sources.

Pyruvate cannot be directly synthesized from 3-phosphoglycerate inside the chloroplast of mesophyll cells mainly because of the absence of the glycolytic enzymes phosphoglyceromutase and enolase.[67-69] Activity of these enzymes inside plastids are observed only in the developing embryos in *Arabidopsis*.[70] A feasible route could be the transport of phospho*enol*pyruvate (PEP) produced by glycolysis in the cytosol into the chloroplast involving a phospho*enol*pyruvate/phosphate translocator (PPT) followed by the synthesis of pyruvate from PEP by pyruvate kinase inside the chloroplast.[71, 72] It is known that the chloroplast of photosynthesizing leaves is dependent on the cytosol for PEP (but not necessarily pyruvate).[73]

There is evidence for the presence of plastidic pyruvate kinase (PK_p) in different heterotrophic tissues, e.g. leucoplast pyruvate kinase has been purified and characterized from developing castor bean (*Ricinus communis*) endosperm, *Brassica napus* (Rapeseed) suspension cells, and plastidic pyruvate kinase complex has been purified and characterized from the developing seeds of *Arabidopsis*.[74-77] Isoenzymes of pyruvate kinase from green leaves of castor bean and etiolated leaves of pea plants have been separated by ion filtration chromatography and one of the isoenzymes is located in the plastid.[78] Considering the use of pyruvate in other metabolic pathways inside the chloroplast (e.g. fatty acid biosynthesis), it is highly likely that a plastidic pyruvate kinase exists.

Recently, a plastidial sodium-dependent pyruvate transporter, BASS2, has been discovered.[79] It has been observed abundantly in C_4 plant species and in considerable amount in C_3/C_4 intermediate species. The authors showed that an *Arabidopsis thaliana* BASS2 orthologue is mainly observed in developing leaves and is thought to provide pyruvate for the MEP pathway in developing leaves.[79] Chloroplastic pyruvate obtained from imported cytosolic PEP is important for the MEP pathway in a fully expanded leaf when the isoprene emission occurs in its full capacity.[18, 80]

The suppression of isoprene emission and DMADP content under high CO_2 concentration has been hypothesized to be due to the competition for PEP by cytosolic PEP carboxylase over the transport of PEP from cytosol to chloroplast.[81] However, this hypothesis has been challenged.[82, 83] Rasulov *et al.* concluded that the variation of isoprene emission with CO_2 concentration depends on the regulation of the synthesis of DMADP by energetic cofactors instead of the carbon availability. In addition, there is now evidence that CO_2-suppression is eliminated at 30 °C and above.[84-87]

2.2 Input of reducing power

Several enzymatic steps of the MEP pathway need reducing power. DXR, the second enzyme of the MEP pathway uses NADPH for reducing power.[88] It is likely that NADPH is obtained from the photosynthetic electron transport chain in phototrophic organisms. This helps explain the lack of isoprene emission in the darkness, when NADPH from photosynthesis is not available. A post-illumination isoprene burst is often observed in oak and poplar leaves.[89] It has been suggested that this burst is made possible by NADPH supplied by the oxidative branch of the pentose phosphate pathway in darkness[90] but other mechanisms are possible. It has also been suggested that the dark isoprene emission from the aspen leaves could arise from the pool of phosphorylated intermediates of the MEP pathway when the required energetics (ATP and NADPH) are available through the chloroplastic glycolysis or chlororespiration.[91]

Both HDS and HDR, the last two enzymes of the MEP pathway, have [4Fe-4S] clusters and involve double one-electron transfers in their catalytic reaction mechanism.[92] It has been observed that in presence of light the HDS/GcpE from *Arabidopsis thaliana* obtain the required electrons from the photosynthetic electron transport chain through ferredoxin whereas the bacterial HDS enzyme requires flavodoxin/flavodoxin reductase and NADPH as the reducing system.[93, 94] It has however, been suggested that an electron shuttle is required for plant HDS in darkness and a ferredoxin/ferredoxin reductase/NADPH system can provide

the required electron shuttle in the darkness.[94] The bacterial HDR enzyme is also found to be dependent on the flavodoxin/flavodoxin reductase/NADPH system for the shuttle of electrons for its reducing activity.[95]

Reducing power can affect the MEP pathway in a complex fashion. The carbon flow through the pathway can be limited at DXR by low NADPH/NADP ratio as DXR is dependent on NADPH. Recent measurements of metabolites has shown that the ratio of DXP to MEP is high, indicating a potential limitation at DXR.[90] NADPH can also indirectly restrict the supply of GAP to the MEP pathway during photosynthesis by modifying the ratio of PGA to GAP in the Calvin-Benson cycle.

The consumption of ferredoxin instead of NADPH by the last two iron-sulphur containing enzymes introduces another reducing-power-mediated regulation of the MEP pathway.[92-94] Ferredoxin has significantly more reduced midpoint potential than NADPH.[96] This means that even though the NADPH/NADP ratio may be very high,[97] the effective redox potential of the NADP/NADPH redox pair is likely to be well below that of ferredoxin.[98] This allows the ferredoxin supply to be limiting even when NADPH supply is favourable. HDS[99] and HDR[100] require a very negative reducing potential that could be supplied by ferredoxin but not NADPH. Plant enzymes appear to use ferredoxin directly even though some bacteria use an NADPH/flavodoxin system.[94] The importance of reducing power is supported by the finding that MEcDP can accumulate to very high concentration.[90, 101, 102] In chloroplasts, it is likely that maximal MEcDP concentrations are limited by the amount of available phosphate. As much as 3 mM phosphate[90] and even 20 mM phosphate[101] in MEcDP has been reported while typical chloroplasts normally contain just 2 mM free phosphate.[103]

2.3 Input of ATP (CTP)

The conversion of MEP into diphosphocytidylyl methylerythritol (CDP-ME) followed by its conversion to diphosphocytidylyl methylerythritol 2-phosphate (CDP-MEP) by CDP-ME synthase (CMS) and CDP-ME kinase (CMK) respectively involves the consumption of CTP and ATP. The MEP pathway uses one CTP and one ATP molecule for the synthesis of each DMADP molecule. However, CTP loses a diphosphate moiety in the course of conversion of MEP to CDP-ME. It is assumed that the regeneration of CTP requires two more ATP molecules. Therefore, the overall cost becomes three molecules of ATP for the synthesis of each DMADP molecule.

The control of the MEP pathway by ATP is evident from the study demonstrating that isoprene emission is best correlated with ATP among all the other metabolites when monitored under a range of environmental factors with the condition of non-limiting carbon availability.[104] Dependence of CMS and CMK on ATP can explain the effect of ATP content on the MEP pathway and isoprene emission. ATP can also have an indirect effect on the MEP pathway by affecting the availability of GAP. This is due to the higher affinity of phosphoribulokinase for ATP than PGA kinase.[105] In the presence of a low level of ATP during darkness, it can preferentially be used by phosphoribulokinase rather than PGA kinase, leading to a high amount of PGA and low availability of GAP. Another study shows that an initial increase in the rate of isoprene emission is observed with the feeding of methyl viologen to oak leaves.[106] Methyl viologen leads to the reduction of oxygen to superoxide by diverting the electron flow from photosystem I. The superoxide is further converted to water by the consumption of NADPH. This results in a significant disturbance in the balance of reducing power to ATP availability inside the chloroplast. It was originally interpreted that the initial increase in the rate of isoprene emission in the presence of methyl viologen demonstrates that ATP control is more important than the reducing power control of the carbon flux through the MEP pathway. Recently, it has been shown that methyl viologen can facilitate the transfer of electrons to HDS and HDR.[99, 100] This would provide an alternative explanation for the initial increase of isoprene emission in presence of methyl viologen.

3 Regulation of DXS

Several gene expression studies have demonstrated indirect evidence supporting the regulatory role for DXS.[21] The first evidence from the metabolic stand-point came from the observation of labelled isoprene emission when leaves were fed dideuterated deoxyxylulose (DOX-d_2).[107, 108] Feeding eucalyptus leaves with DOX-d_2 results in the displacement of the endogenous, unlabelled isoprene by labelled isoprene derived from exogenous DOX, keeping the overall rate of isoprene emission almost constant. This indicates that the concentration of DMADP remains constant inside the chloroplast even during feeding. Maintenance of a constant level of DMADP would require reduced activity of some enzyme upstream of the entry point of exogenous DOX resulting in a tight regulation of the flow of carbon through this pathway. Therefore, it is possible that a negative feedback loop from any metabolite downstream of DXP affects the activity of DXS.

Recently, we found that the recombinant DXS enzyme from *Populus trichocarpa* (*Pt*DXS) is inhibited by IDP and DMADP.[109] This can explain the observation of Wolfertz *et al.* Feedback inhibition of DXS by IDP and DMADP can control the carbon flow through the MEP pathway and therefore, constitutes a significant regulatory mechanism of the MEP pathway (Fig. 3). Overall, this inhibitory mechanism allows DMADP and IDP, the last metabolites of the MEP pathway, to limit their pool size by controlling the activity of the very first enzyme of the pathway. Feedback from the last metabolite on the activity of the first enzyme in a pathway is a common regulatory mechanism. Another group has also recently confirmed the feedback regulation of DMADP on poplar DXS and this potentially contributes to the *in vivo* regulation of the MEP pathway.[110]

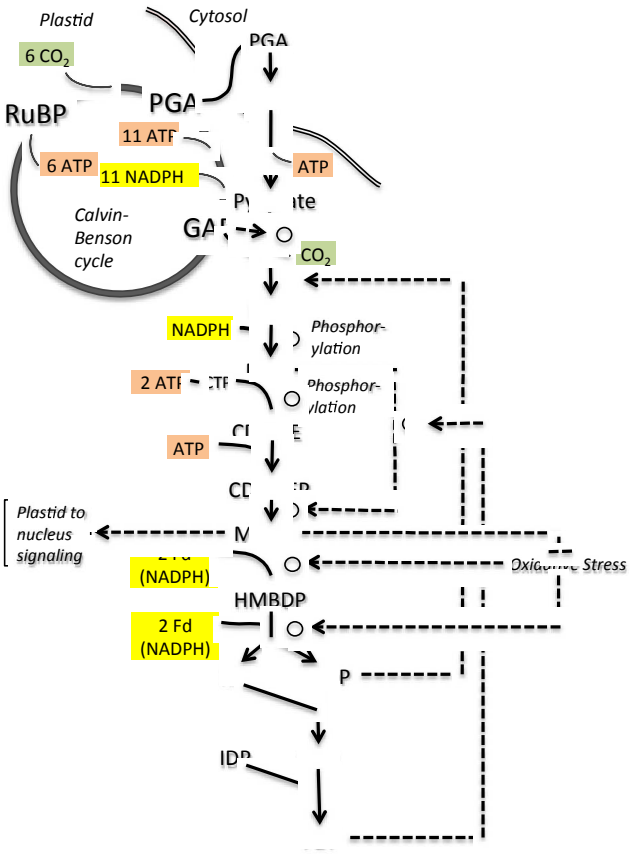

Fig. 3. MEP pathway and related metabolism showing the major metabolic regulatory points discussed. Abbreviations GDP = geranyl diphosphate, FDP = farnesyl diphosphate, Fd = ferredoxin.

It was observed that IDP and DMADP compete with thiamin diphosphate (ThDP) for binding with *Pt*DXS.[109] This was unexpected, as ThDP is considered to act as a cofactor tightly embedded in the active site of the enzyme. The K_i of IDP and DMADP are in the low micromolar range (60 - 80 μM) indicating their significant binding ability relative to ThDP under physiological conditions. DXP, the product of DXS, serves as a precursor for thiamin and pyridoxol biosynthesis in *E. coli*.[35, 38, 39] Therefore, inhibition of DXS by IDP and DMADP might have some regulatory effects on thiamin biosynthesis in bacteria. The involvement of DXP in thiamin biosynthesis in eukaryotes, including yeasts and plants, has not been elucidated clearly.[111-117] Therefore, the modulation of thiamin biosynthesis by IDP and DMADP inhibition of DXS might be restricted to some bacteria.

Another recent study has shown that the rate of pyruvate decarboxylation by DXS is accelerated by the presence of GAP.[118] This constitutes a potential feedforward regulation at DXS by its substrate (Fig. 3). This effect ensures that the initial product of pyruvate and ThDP, lactyl-ThDP, will not be converted to the *C*2α-carbanion or its conjugate acid, hydroxyethyl-ThDP (HEThDP), unless GAP is present so that the reaction can go to completion. Elimination of ThDP from the hydroxyethyl-ThDP intermediate leads to the formation of acetaldehyde and ThDP. Formation of acetaldehyde is specifically catalyzed by pyruvate decarboxylase.[119] HEThDP made by DXS could be the source of a short burst of acetaldehyde observed when a leaf is first put into darkness or subjected to mechanical stress.[120-122] If declining GAP levels inhibit the use of HEThDP before its production, HEThDP could accumulate. In darkness, the pH of the chloroplast stroma declines making release of acetaldehyde more likely. However, once the GAP level declines low enough, production of HEThDP would stop along with the release of acetaldehyde.

4 Regulation of DXR and CMS by phosphorylation

It has been reported that DXR from *Francisella tularensis* has a phosphorylation site at Ser177, which is equivalent to Ser186 in the *E. coli* DXR enzyme.[123] Ser186 of *E. coli* DXR acts as an important residue for binding of substrate. It is positioned near the substrate binding site in such a way that it participates in hydrogen bonding with the phosphate moiety of the substrate.[124] It also causes some conformational changes upon substrate binding which is important for the enzyme activity. Ser177 of *Francisella tularensis* DXR has roles in substrate binding and enzyme activity. It has been shown that mutation of Ser177 by an aspartate or glutamate results in complete abolition of enzyme activity.[123] Both aspartate and glutamate act as mimics of phosphoserine and disrupt the required interaction for substrate binding. This causes the enzyme to be inactive. This indicates that the activity of the enzyme is affected by the phosphorylation of this particular serine residue. This serine residue is conserved in the plant DXR. There is no information at present whether this mechanism plays any role in regulating the MEP pathway. This mechanism has not been demonstrated for plant DXR.

Similar studies on CMS from *Francisella tularensis* has found a phosphorylation site at Thr141, which is equivalent to Thr140 in the *E. coli* CMS enzyme.[125] The crystal structure of *E. coli* CMS complexed with CDP-ME has revealed that Thr140 plays critical role in binding with the substrate. The sidechain hydroxyl and backbone amide groups of Thr140 participate in the hydrogen bonding with the C3 and C1 hydroxyl oxygen atoms of MEP respectively.[126, 127] It has been suggested by mutagenesis studies that Thr141 in the *Francisella tularensis* also involves in substrate binding. Mutagenesis of Thr141 with aspartate or glutamate, both of which mimic phosphothreonine, lead to reduced or abolished activity of the enzyme respectively. As discussed above for DXR, both T141D and T141E can lead to the disruption of important interactions involving substrate binding resulting in the impairment of enzyme activity. This could be another mechanism controlling carbon flux through the MEP pathway in bacteria but this has not yet been demonstrated. This mechanism has also not yet been tested in plants.

There is a need for further investigation of these potential control mechanisms in both bacteria and plants.

5 Regulation of and by MEcDP concentration

Several recent studies have demonstrated that MEcDP is a key intermediate in the MEP pathway. It has been observed that in leaves in the presence of light, more MEcDP is accumulated than all the other MEP pathway intermediates.[90] Here we discuss the various types of regulation inside and outside the MEP pathway that are coordinated by this metabolite.

5.1 A feedforward effect

MEcDP, the cyclo-diphosphate-containing intermediate of the MEP pathway, is synthesized by MEcDP synthase (MCS) from CDP-MEP. The crystal structure of MCS is known from different organisms.[128-131] These structural studies have shown that a hydrophobic cavity is present along the threefold non-crystallographic symmetry axis of the enzyme. Evidence indicates that the cavity is occupied with different isoprenoids containing a diphosphate moiety like IDP/DMADP, geranyl diphosphate (GDP), and farnesyl diphosphate (FDP).[130, 131] Sequence alignment studies of the MCS enzyme from various organisms indicate that the motif involved in the formation of the cavity and the binding of the ligand are well conserved in the protein family suggesting that the simultaneous conservation of both the motifs might have evolved due to a biological function.[130] It has been proposed that MCS could be a significant point of feedback regulation by the downstream isoprenoids.[130, 131]

Recent studies have shown that recombinant MCS enzyme from *E. coli* is stabilized and activated in the presence of IDP, DMADP, GDP, and FDP.[132] Analysis of the effect of different MEP pathway metabolites on MCS stability and activation by the *in vitro* assays has identified MEP as the most effective modulator for MCS. It has also been shown by *in vitro* studies that the methylerythritol scaffold is essential and sufficient for the observed effect of activation and enhancement of stability of MCS by MEP. The 2-*C*-methylerythritol scaffold is unique to the MEP pathway. The feedforward activation of MCS by MEP (Fig. 3) constitutes a regulatory mechanism very specific to the MEP pathway.

It is also observed that FDP inhibits the *E. coli* MCS-MEP complex whereas it activates and stabilizes *E. coli* MCS alone.[132] It has been speculated that the binding of MEP to MCS might cause some conformational changes of MCS and the inhibitory effect of FDP is selective for the MEP-bound conformation of MCS. The feedback inhibition of MCS-MEP complex by FDP (Fig. 3) indicates that the downstream isoprenoids control their biosynthesis by modulating the activity of a key enzyme involved in the biosynthesis of their precursor. There is another significance of this feedback inhibition. It sets a limit on the activated MCS-MEP complex in the presence of high levels of downstream isoprenoids so that the carbon flux through the MEP pathway is controlled. Overall, this observation suggests that MCS plays a key role in the regulation of the MEP pathway.

5.2 Regulation of the biosynthesis and metabolism of MEcDP

It has been observed that MEcDP accumulates in bacteria under oxidative stress.[133-135] Nitrosative stress (caused by the reactive species nitric oxide, NO) is also found to be responsible for the accumulation of MEcDP, to a lesser extent than oxidative stress, in *Corynebacterium ammoniagenes*.[135] Recently, spinach leaves were also found to accumulate MEcDP under high light and high temperature and in the presence of heavy metals like Cd.[101] These external factors can cause oxidative stress *in vivo* leading to the accumulation of MEcDP. HDS contains a [4Fe-4S] cluster susceptible to oxidative stress. Studies have found that ROS generated under oxidative stress damages the reconstitution of the [4Fe-4S]-cluster and thus interferes with the turnover of the holo enzyme.[101] It has been suggested that under oxidative stress, the reconstitution of the apo-HDS with the [4Fe-4S]-cluster functions as the rate limiting step of the MEP pathway and thus is a bottleneck in the MEP pathway.

MEcDP has also been found to act as an effective antioxidant.[136] This property of MEcDP allows the repair of the HDS enzyme to keep it functional by limiting oxidative stress. This protective ability of MEcDP is not sufficient for the reconstitution of the holo-enzyme in the presence of inhibitors like Cd. The accumulation of MEcDP in illuminated leaves may affect the phosphate balance of the chloroplast. Synthesis of high levels of MEcDP could potentially act as a sink for phosphate and disturb the phosphate supply for ATP synthesis. It has been demonstrated that utilization of phosphate to maintain the synthesis of high level of MEcDP can cause phosphate deficiency syndrome in chloroplast.[101] It is possible that the maximal MEcDP concentration is restricted by the amount of chloroplastic phosphate.

Another interesting observation in this context is the accumulation of a very high level of MEcDP and block of isoprene emission from leaves under nitrogen atmosphere (i.e. CO_2- and O_2-free air).[90, 107] Limited availability of carbon through the Calvin-Benson cycle to feed the MEP pathway cannot explain the phenomenon of abolished isoprene emission from leaves held under nitrogen. This is because replenishing the carbon supply of the MEP pathway by feeding the leaves directly with deoxyxylulose in the presence of nitrogen is not able to restore isoprene emission.[107] Accumulation of a high level of MEcDP under nitrogen atmosphere indicates that the downstream enzymes may not be functional, causing isoprene emission to stop. It is likely that under a nitrogen atmosphere the iron-sulphur complexes of HDS and HDR are disrupted. A nitrogen atmosphere can possibly lead to some signals that cause these enzymes to become inactive. The exact mechanisms by which nitrogen atmosphere disrupts the activity of these two enzymes in leaves are yet to be determined.

5.3 Effect of MEcDP accumulation in other biochemical processes

It has been found that MEcDP plays a significant role in various other biochemical pathways unrelated to isoprenoid biosynthesis.

5.3.1 Bacteria

In bacteria, studies showed that oxidative stresses caused by benzyl viologen or other redox mediators lead to the

accumulation of MEcDP, which has been suggested to play an important role as an antistressor in bacteria.[133, 134, 137] It has also been observed that MEcDP prevents DNA from falling apart in the presence of Fenton reagent.[138] This is achieved when a complex is formed between the ferrous ions (present in the Fenton reagent) and MEcDP resulting in their reduced ability to form hydroxyl radicals and hydrogen peroxide.[138] This suggests that MEcDP could act as an endogenous stabilizing agent for bacterial cells subjected to oxidative stress.[138]

MEcDP is also found to modulate chromatin structure by disrupting the chlamydial histone-DNA interaction in the intracellular pathogen *Chlamydia trachomatis*.[139, 140] The chlamydial developmental cycle alternates between the extracellular infectious form called the elementary body (EB) and the intracellular replicative form termed the reticulate body (RB). These two different forms have characteristic chromatin structures. The RB form has condensed nucleoid structure mediated by histone-like DNA binding proteins, Hc1 and Hc2.[139, 140] Within a few hours of infection, the metabolically inert EB form is transformed into the metabolically active RB form. Dispersion of the chromatin structure is required for the differentiation of the EB form into the RB form. It was suggested that MEcDP disrupts the binding between DNA and histone-like proteins leading to the release of Hc1and Hc2 from the DNA causing the dispersion of the chromatin and initiation of transcription. Thus, MEcDP mediates the decondensation of the chromatin allowing the differentiation of the EB form to the RB form. Another example of the role of MEcDP in the regulation of the bacterial genome activity includes its resuscitating effect regulating the transition of the non-culturable form of *Mycobacterium smegmatis* into the state of its active growth.[141]

Recent metabolite profiling studies showed an efflux of MEcDP from genetically engineered *E. coli* cells containing the overexpressed enzymes DXS, IDI, CMS, and MCS.[142] It has been observed that the efflux of MEcDP is accompanied with the simultaneous reduction of the production of lycopene, a downstream isoprenoid. It was possible to reduce the efflux of MEcDP by the overexpression of HDS, which consumes MEcDP, directing more carbon through the last part of the MEP pathway, resulting in the increased production of lycopene. This indicates that the efflux of MEcDP could act as a limiting step in microbial isoprenoid production. Preliminary studies indicate the involvement of a fosmidomycin resistance (fsr) efflux pump[143] for the process of exporting MEcDP out of the cell.[142] The active efflux of MEcDP from the engineered lycopene-producing *E. coli* cells suggests the possibility of a potential MEP pathway branch point which diverts the carbon source of the MEP pathway to another competing pathway.[142] This is also supported by the study of restoration of the complete and active MEP pathway by heterologous expression of HDS and HDR into *Listeria innocua* lacking these enzymes.[144] Bioinformatics analysis has shown that *L. innocua* has lost the genes for HDS and HDR through evolution while the rest of the MEP pathway genes are present.[144] The ability of this organism to have an active MEP pathway with the introduction of the lost enzymes suggests that the rest of the MEP pathway enzymes, which were already present, are functional. Evolution has selectively truncated the MEP pathway in such a way that the existing enzymes could catalyse the biosynthesis of MEcDP, which can further lead to end products of the MEP pathway in the presence of HDS and HDR. This suggests some important yet unidentified biochemical role for MEcDP.[144]

5.3.2 Plants

In plants, recent studies have demonstrated that in addition to its role in the bacterial system, MEcDP has a potential role as a signalling molecule in *Arabidopsis*. Plastidial MEcDP leads to a retrograde signal regulating the expression of nuclear-encoded, stress-responsive genes for plastidial proteins (Fig. 3).[145] Hydroperoxide lyase (HPL) is a stress-inducible plastidial protein in the oxylipin pathway encoded by a nuclear gene. It has been shown that a mutant *ceh1* shows constitutive expression of HPL. *CEH1* encodes for HDS and thus *ceh1* mutant is defective in the utilization of MEcDP resulting in its accumulation. It has also been reported that abiotic stresses including high light or wounding cause a high level of MEcDP to build up. These abiotic stresses causing accumulation of endogenous MEcDP, as well as exogenous MEcDP, lead to the elevated expression of HPL. This indicates that MEcDP is, directly or indirectly, a retrograde signalling molecule. It has been shown that abscisic acid and methyl jasmonate, stress-responsive hormones of plants, increase the activity of DXS.[146] It is tempting to speculate that these stress-responsive hormones lead to the regulation of HPL by accumulating MEcDP through the increased activity of the upstream enzyme DXS. The mode of action of MEcDP in the retrograde signalling is not fully understood. Considering the involvement of MEcDP in the nucleoid decondensation in chlamydia, it may be that MEcDP modulates nuclear gene expression in plants through the remodelling of the nuclear architecture.[139, 140] This mechanistic model would require the transport of plastid-localized MEcDP to the nucleus. No information is available for any such transport of MEcDP in plants but the presence of the fsr efflux pump in bacteria for moving MEcDP out of the cells[142] raises the possibility of such transporter in plants as well. One such candidate is the Arabidopsis gene At3g47450.

Accumulation of MEcDP can cause transient effects in isoprene emission. Upon darkening a leaf, isoprene emission continues long enough to consume the existing DMADP and IDP but not MEcDP.[90, 147] After about five minutes in the dark the leaf regains the ability to consume MEcDP but not to make additional MEcDP. This causes a small post-illumination burst of isoprene between 5 and 10 min after darkening the leaf (Fig. 4). The very high level of MEcDP that builds up in leaves held in a nitrogen atmosphere (Section 5.2) is likely responsible for a large overshoot in isoprene emission when O_2 and CO_2 are added back to the air (Fig. 4).

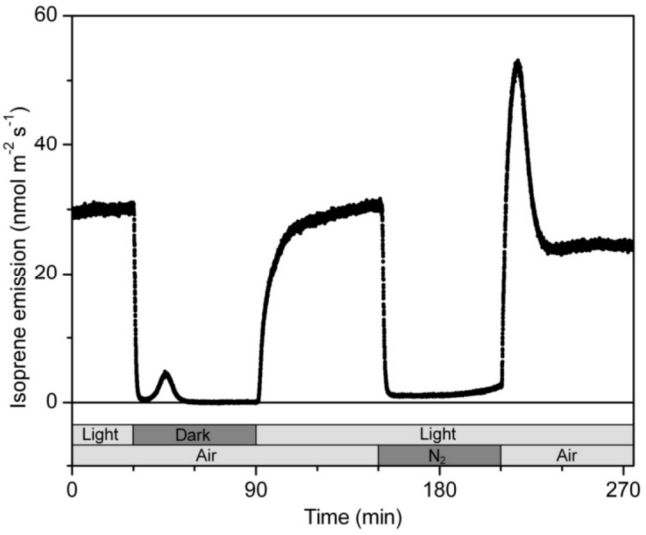

Fig. 4. Effects of MEcDP on isoprene emission after darkness or nitrogen atmosphere. Following darkness MEcDP causes a small second burst of isoprene while a nitrogen atmosphere stops isoprene emission without a burst. Upon adding oxygen and CO_2 isoprene emission overshoots but establishes a steady state slightly lower than before imposing a nitrogen atmosphere. This may result from a very high MEcDP level in the leaf. From Li and Sharkey, 2013 (Ref 90, Copyright American Society of Plant Physiologists, www.plantphysiol.org).

6 Regulation at HDS and HDR

Given the propensity for MEcDP to accumulate in plants and bacteria it is likely that there is significant regulation of HDS. However, less is known about HDS regulation than HDR regulation. It has been shown that nitrosative stress in *Mycobacterium smegmatis* causes the accumulation of HMBDP, the substrate for HDR.[135] This suggests that NO damages the [4Fe-4S]-cluster of HDR resulting in the dysfunctional enzyme, which leads to the accumulation of HMBDP. The gene of HDR in *E. coli* has been found to be involved in penicillin tolerance through its interaction with RelA responsible for the synthesis of guanosine 3',5'-bispyrophosphate (ppGpp), which acts as a nutritional stress alarmone.[148]

It has been shown that a point mutation in *E. coli* HDR ($LytB^{G120D}$) enables it to selectively synthesize DMADP over IDP.[149] This suggests that the structural modification of HDR can potentially regulate the *in vivo* concentration of DMADP and IDP, the end products of the MEP pathway.

It has been seen that engineering an additional HDS gene into *E. coli* without increasing the activity of HDR leads to a reduction in productivity in bacteria engineered to emit isoprene (A.E. Wiberley, E.L. Singsaas, T.D. Sharkey, unpublished).[150] Chotani et al. found that HMBDP accumulated in such bacteria and that this was correlated with reduced isoprene production from engineered bacteria. One explanation for this is that HMBDP is toxic to cells.

Purified HDR is shown to require a very negative redox potential, maximal activity was found at -450 mV, much lower than the midpoint potential of NADPH (-320 mV). The presumed electron source for this enzyme gives an activity less than 2% of maximal. Xiao et al. suggest that HDR might be regulated by modulation of the redox potential of its [4Fe-4S] cluster.[100]

It is likely that both HDS and HDR are highly regulated and this regulation has a strong impact on the carbon flow of the MEP pathway. These are likely to be the steps where light regulation of DMADP in plants occurs, but there is no information on how this occurs. It is also tempting to assume that the metabolites downstream of MEcDP might have some toxic effect in the cell. This might lead the carbon flux of the MEP pathway to be constricted, building up a pool of only MEcDP under the condition of oxidative stresses.

7 Conclusions and perspectives

The MEP pathway is one of the most important biochemical pathways for sustaining life on earth. Understanding the different regulations involved in this pathway is critical for biological, environmental, as well as commercial purposes. Mechanisms of genetic regulation of this pathway have started emerging only in the last decade. We have discussed in this review several different regulatory mechanisms involved in the metabolism of this pathway and these are summarized in Fig. 3. Several questions are still to be answered regarding regulatory mechanisms of the MEP pathway, especially in plants. The source of pyruvate for the MEP pathway is not clearly understood. Understanding the source of pyruvate may explain the discrepancy in the labelling of the isoprenoids derived from the MEP pathway. Several studies have indicated that MEcDP has potential roles in MEP pathway regulation. MEcDP may connect metabolism in the MEP pathway with other cellular metabolism, independent of its role in making precursors for isoprenoids. It has been suggested that MEcDP can act as a stress sensor and can accordingly coordinate stress responses. The exact mode of its action in response to the stress signals has yet to be understood.

It has been suggested that the [4Fe-4S]-cluster containing enzymes, HDS and HDR, can also contribute to the regulatory mechanisms of the MEP pathway. The susceptibility of the [4Fe-4S]-clusters to oxidative stress indicates that the *in vivo* redox status can influence the carbon flow of the MEP pathway through these enzymes. In-depth knowledge of the structural and functional integrity of these enzymes under various redox-sensitive conditions would be helpful in understanding their role in the MEP pathway regulation.

Understanding of the metabolic regulation of the MEP pathway has emerged in the last decade and currently can be considered at its nascent stage. Studies so far have demonstrated that several enzymes and metabolites could have various regulatory roles in this pathway. However, not much is known regarding the primary points of regulation and how the overall regulation of the pathway is finely tuned by both the primary and secondary points of regulation. Future studies in the field

should be aimed at a complete understanding of the metabolic regulation of the MEP pathway. This would be useful in biomedical and biotechnological uses of the MEP pathway and would also help in finding a mechanistic basis for modelling isoprene emission.

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