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Isoprenoid biosynthesis in plant chloroplasts via the MEP pathway: Direct thylakoid/ferredoxin-dependent photoreduction of GcpE/IspG

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Abstract In the methylerythritol phosphate pathway for isoprenoid biosynthesis, the GcpE/IspG enzyme catalyzes the conversion of 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate into (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate. This reaction requires a double one-electron transfer involving a [4Fe–4S] cluster. A thylakoid preparation from spinach chloroplasts was capable in the presence of light to act as sole electron donor for the plant GcpE *Arabidopsis thaliana* in the absence of any pyridine nucleotide. This is in sharp contrast with the bacterial *Escherichia coli* GcpE, which requires flavodoxin/flavodoxin reductase and NADPH as reducing system and represents the first proof that the electron flow from photosynthesis can directly act in phototrophic organisms as reducer in the 2-*C*-methyl-D-erythritol 4-phosphate pathway, most probably via ferredoxin, in the absence of any reducing cofactor. In the dark, the plant GcpE catalysis requires in addition of ferredoxin NADP$^+$/ferredoxin oxido-reductase and NADPH as electron shuttle.

Keywords: Ferredoxin; GcpE; Isoprenoid biosynthesis; IspG; Photosynthesis

1. Introduction

Isoprenoids represent the most diverse natural product family. They are found in all living organisms, including essential metabolites such as sterols regulating plasma membrane fluidity and permeability in eukaryotes and precursor of steroid hormones in animals, dolichols and long chain polyprenols acting as carbohydrate carriers, the prenyl chains of quinones from electron transport chains (ubiquinones, menaquinones, plastoquinone), carotenoids and phytol from chloroplasts, as well as a multitude of secondary metabolites of less obvious function. Highest structural diversity is certainly found in phototrophic organisms (mosses, liverworts, ferns, higher plants). Isopentenyl diphosphate (IPP) (**9**) and dimethylallyl diphosphate (DMAPP) (**10**) are the universal precursors for all isoprenoids in all living organisms (Fig. 1). Two biosynthetic pathways are leading to the isoprene unit. In the firstly elucidated mevalonate (MVA) pathway, IPP is synthesized from acetyl-CoA much like fatty acids, via MVA, which is the key intermediate of this metabolic route [1]. In the more recently disclosed methylerythritol phosphate (MEP) pathway [2–4], IPP (**9**) and DMAPP (**10**) are carbohydrate derivatives, their synthesis starting from pyruvate (**1**) and D-glyceraldehyde phosphate (**2**) via 1-deoxy-D-xylulose 5-phosphate (**3**), 2-*C*-methyl-D-erythritol 4-phosphate (**4**), 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (**5**), 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate (**6**), 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (MEcDP) (**7**) and 4-hydroxy-2-methylbut-2-enyl 1-phosphate (HMBPP) (**8**) (Fig. 1). The MVA pathway is the only pathway found in animals and fungi as well as in the cytoplasm of phototrophic organisms, whereas the MEP pathway is present in most bacteria [5,6] and in plant chloroplasts [7–9].

The last two enzymes of the MEP pathway, encoded by the *gcpE* and *lytB* genes, respectively, catalyze the conversion of MEcDP (**7**) into HMBPP (**8**) (Fig. 1) [10–13] and the conversion of HMBPP into IPP and DMAPP [14–17] by eliminations coupled with reduction steps (Fig. 1). Both enzymes are characterized upon reconstitution by a [4Fe–4S] prosthetic group [18–20], acting in its reduced $[4Fe–4S]^{1+}$ form as one electron donor. For catalytic activity, both enzymes have to be coupled with a system allowing the reduction of the oxidized $[4Fe–4S]^{2+}$ cluster. This can be completed in the bacterium *Escherichia coli* by the natural flavodoxin/flavodoxin reductase/NADPH system [18,21]. The *fldA* gene encoding flavodoxin I was found to be essential in *E. coli* and supposed to be mainly involved in the reduction of the GcpE and LytB Fe/S clusters [22]. Reduction can also be performed by chemical means: e.g., with the semiquinone radical of 5-deazaflavin for the recombinant GcpE enzyme from *E. coli* [18] and *Arabidopsis thaliana* [19] or even dithionite

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Abbreviations: DMAPP, dimethylallyl diphosphate; GcpE, IspG, HMBPP synthase; HMBPP, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate; IPP, isopentenyl diphosphate; LytB, IspH, HMBPP reductase; MEcDP, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate

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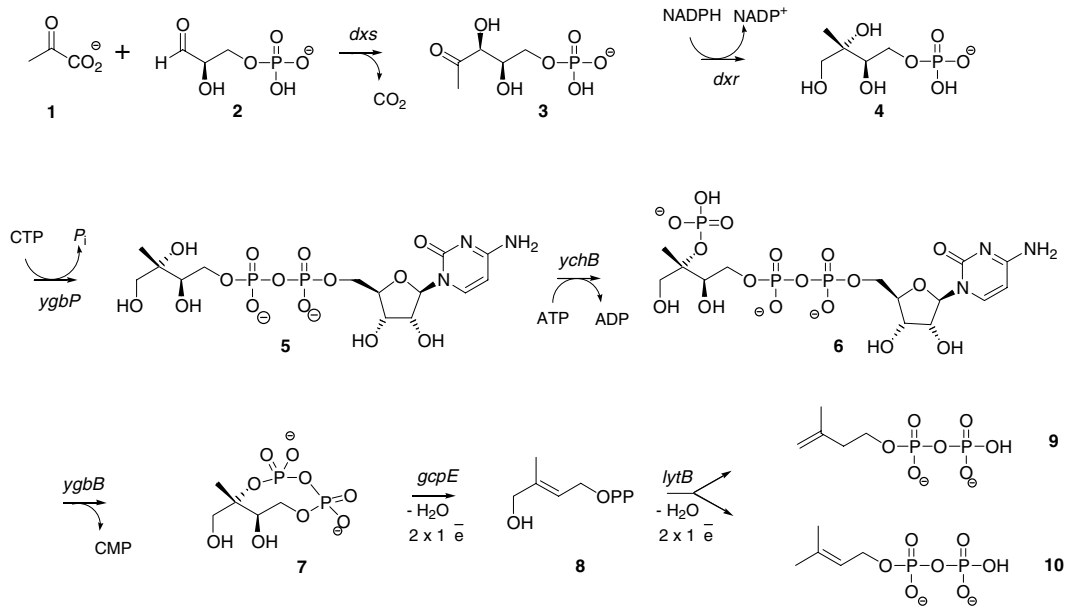

Fig. 1. MEP pathway for isoprenoid biosynthesis.

for GcpE from the bacterium *Thermus thermophilus* [23] and LytB from *Aquifex aeolicus* [15]. The plant enzyme showed, however, no activity in the presence of the flavodoxin/flavodoxin reductase from *E. coli* and NADPH [19]. In addition, flavodoxin is absent in the plastids of phototrophic organisms and in the plastid-like apicoplasts of *Plasmodium* spp. [24,25]. As the MEP pathway is closely linked to photosynthesis by its chloroplast localization and the supply of the first C_3 precursors, it seemed tempting to check whether the electron transport chain involved in photosynthesis was capable of transferring electrons to the Fe/S clusters of the last two enzymes via ferredoxin. The electron transfer from ferredoxin onto GcpE is apparently possible in the cyanobacterium *Thermosynechococcus elongatus* lacking flavodoxin [26]. LytB from *E. coli* displays some reducing activity in the presence of spinach ferredoxin/ferredoxin–$NADP^+$ reductase/NADPH [27], whereas the same enzyme from *Plasmodium falciparum* was shown to be catalytically active in the presence of the same system [25]. In the present study, we show that the electron flow from photosynthesis can directly provide in the presence of light and via ferredoxin the electrons required for the GcpE catalyzed reaction without any contribution of the ferredoxin–$NADP^+$ reductase and NADPH, the latter system being only required in the dark.

2. Materials and methods

2.1. Purification of the enzymes

The GcpE enzyme from *A. thaliana* (At-GcpE) was purified and reconstituted as already described [19]. Spinach (*Spinacea oleracea*) was purchased from a local market. Ferredoxin was purified from spinach leaves using the purification procedure described for the *Clostridium pasteurianum* ferredoxin [28]. Ferredoxin–NADP reductase from spinach leaves was purified on a ferredoxin–Sepharose affinity column as described for ferredoxin–thioredoxin reductase [29]. A final purification step was carried out on a 2′–5′ ADP-Sepharose column [30].

2.2. Preparation of thylakoids

All preparative steps were performed at 4 °C. Spinach leaves (50 g) were washed with water, deribbed and homogenized in a Waring blender in 50 mM MES buffer (pH 6.5, 250 ml) containing 330 mM sorbitol and 1 mM $MgCl_2$. The homogenate was filtered through several layers of surgical gauze, and the residue was discarded. After centrifugation of the filtrate ($3000 \times g$, 1 min), the pellet was used for the preparation of spinach thylakoids as described for the preparation of pea thylakoids [31]. This final thylakoid pellet was resuspended in 50 mM HEPES (pH 7.6, 2 ml), 330 mM sorbitol and 1 mM $MgCl_2$ to reach a ca. 3.4 mg ml^{-1} chlorophyll concentration. Chlorophyll was determined by the method of Arnon [32].

2.3. Enzymatic assays using thylakoids

[2-^{14}C]MEcDP was synthesized [12] and purified by high-performance liquid chromatography [33] as previously reported. The reaction medium (97 μl) containing 920 μM [2-^{14}C]MEcDP (0.015 μCi) and various amounts of thylakoid preparation in 50 mM Tris–HCl (pH 8) was degassed for 25 min at 4 °C in the dark under a stream of argon before adding with a gas-tight syringe the At-GcpE solution (3 μl, 1.05 μM final concentration). Each assay was incubated under irradiation with a white fluorescent tube (Osram L 18W/765, 250 μEinstein m^{-2} s^{-1}) at 30 °C for 10 min. The reaction was stopped by freezing in liquid nitrogen. After thawing, losses occurring during the degassing process were evaluated by measuring the volume of the assays with a syringe. The assays were hydrolyzed using alkaline phosphatase (5 μl, 0.16 U) at 25 °C for 15 h. Two different methods were used for determining the GcpE enzyme activity. In Method 1, an aliquot (40 μl) was separated on a thin-layer chromatography silica plate. After elution with isopropanol/water/ethyl acetate (6:3:1), the radioactivity was monitored using a PhosphorImager. After scrapping off the bands corresponding to (*E*)-2-methylbut-2-ene-1,4-diol ($R_f = 0.79$) and [2-^{14}C]MEcDP ($R_f = 0.50$), the silica was directly introduced into counting vials, and the radioactivity was quantified by liquid scintillation.

In Method 2, another aliquot (30 μl) was passed through a QAE-A25 Sephadex column (0.7 cm × 1.5 cm) equilibrated with water. [2-^{14}C]-(*E*)-2-methylbut-2-ene-1,4-diol was recovered by washing the column with water (4 ml), and radioactivity was measured by liquid scintillation after lyophilization of the sample. The background corresponding to the radioactivity of the water eluate of an aliquot (30 μl) from a similar blank experiment performed without At-GcpE was deduced from the former value. [2-^{14}C]MEcDP was eluted with 1.5 M ammonium acetate (2 ml) and counted separately.

2.4. Enzymatic assays using ferredoxin, ferredoxin reductase and NADPH

A reaction medium (70 μl final volume) containing 23 μM [2-^{14}C]MEcDP (0.025 μCi), 5 mM DTT, 2.5 mM NADPH, 40 μM ferredoxin, 9.2 nM ferredoxin reductase in 50 mM Tris–HCl (pH 8) was degassed for 45 min at room temperature under a stream of wet argon before adding 2.5 μM of AtGcpE. The incubation was performed anaerobically at 30 °C for 1 h.

3. Results

The GcpE enzyme from *A. thaliana* (At-GcpE) was purified and reconstituted as already described [19]. As this enzyme contains an oxygen sensitive $[4Fe–4S]^{2+}$ prosthetic group, all tests were performed under an inert argon atmosphere.

A novel assay was developed for At-GcpE. It relies on the non-sensitivity of MEcDP (**7**) towards alkaline phosphatase [12], which, in contrast, hydrolyses HMBPP (**8**) into (*E*)-2-methylbut-2-ene-1,4-diol, which is more easily purified on TLC silica gel plates than HMBPP. Furthermore, MEcDP is retained on anionic ion exchange column, whereas (*E*)-2-methylbut-2-ene-1,4-diol is not. These chromatographic properties were used to quantify the GcpE activity by two different analytical methods that led to the same specific activity (Table 1). According to the blank experiment of Method 1, MEcDP was not totally recovered from the silica, most probably because of its high polarity. Method 2 using a separation of the diol and MEcDP on an ion exchange column seemed thus more efficient for the recovery of the cyclodiphosphate. In Method 2, the water eluate contained (*E*)-2-methylbut-2-ene-1,4-diol as well as some free methylerythritol (less than 4%) that arose from the slow degradation of MEcDP. Corrections were accordingly introduced.

In the presence of NADPH, ferredoxin reductase (9.2 nM) and ferredoxin (40 μM), At-GcpE (2.5 μM) converted quantitatively [2-^{14}C]MEcDP (**7**) (23 μM) into HMBPP (**8**) after 1 h. Accordingly, no specific activity was calculated. No activity was detected when At-GcpE was tested under the same conditions, but in the absence of ferredoxin and in the sole presence of NADPH and ferredoxin reductase as reducing system, indicating that ferredoxin is essential in the reduction process. This shows for the first time that the plant GcpE can use ferredoxin as an electron donor, and that ferredoxin might represent the reducing system coupled to GcpE in vivo. The reducing power of ferredoxin in chloroplasts is however linked to photosynthesis, ferredoxin being the final electron donor. At-GcpE was accordingly tested in the absence of NADPH and added ferredoxin reductase, but in the light and in the presence of spinach thylakoids containing some endogenous ferredoxin in order to mimic what may happen in vivo and to check whether the photosynthetic electron may reduce the Fe/S cluster flow.

The specific activity of reconstituted At-GcpE was determined upon photoactivation in the presence of increasing amounts of the thylakoid preparation. Thylakoids were quantified by their chlorophyll content determined by the method of Arnon [39]. A 160 nmol min^{-1} mg^{-1} constant maximal activity was obtained in a 300–600 μg ml^{-1} chlorophyll concentration range, already indicating saturation at a ca. 300 μg ml^{-1} chlorophyll concentration (Fig. 2). No activity was detected when the assays were run in the dark. As plant GcpE is a plastidial enzyme, the possible presence of endogenous spinach GcpE was checked in the thylakoid preparation. A test was therefore performed without adding At-GcpE, resulting in no activity. This indicated that the thylakoid preparation was not contaminated with significant amounts of endogenous GcpE enzyme from spinach. At-GcpE was only active in the light and required the presence of thylakoids, showing the involvement of the photosynthetic electron transfer, most probably via ferredoxin, in the MEP pathway.

The bacterial GcpE is known to use flavodoxin/flavodoxin reductase/NADPH as reducing system [18]. It was therefore tempting to check whether the At-GcpE enzyme test with thylakoids applies for the *E. coli* enzyme. A thylakoid preparation (corresponding to 600 μg ml^{-1} chlorophyll) was tested in the presence of light and in the absence of NADPH on the bacterial GcpE from *E. coli*: a ca. 30 nmol min^{-1} mg^{-1} activity was recorded. The spinach thylakoids are thus also capable in the

Table 1
Conversion of MEcDP (79 000 dpm) by GcpE from *A. thaliana* and *E. coli* in the presence of thylakoids (600 μg ml^{-1} chlorophyll) and light

		Ec-GcpE	At-GcpE	Blank
Final volume + phosphatase (5 μl)		95 μl	95 μl	90 μl
Method 1: TLC	(*E*)-2-methylbut-2-ene-1,4-diol	13 900 dpm in 40 μl 33 000 dpm total	10 280 dpm in 40 μl 24 400 dpm total	0 0
	MEcDP	15 300 dpm in 40 μl 36 300 dpm total	20 900 dpm in 40 μl 49 600 dpm total	23 200 dpm 52 200 dpm total
Method 2: ion exchange column	Water elution: diol	12 700 dpm in 30 μl 40 200 dpm total	8800 dpm in 30 μl 28 000 dpm total	1000 dpm in 30 μl 2950 dpm total
	NH_4OAc elution: MEcDP	8250 dpm in 30 μl 26 100 dpm in total	15 200 dpm in 30 μl 48 200 dpm total	19 900 dpm in 30 μl 59 700 dpm total
GcpE		0.0225 mg	0.0083 mg	0
Time (min)		45	15	45
Substrate (μmol)		0.061	0.061	0.061
Specific activity (nmol min^{-1} mg^{-1}, Method 1)		25	152	–
Specific activity (nmol min^{-1} mg^{-1}, Method 2)		28	155	–

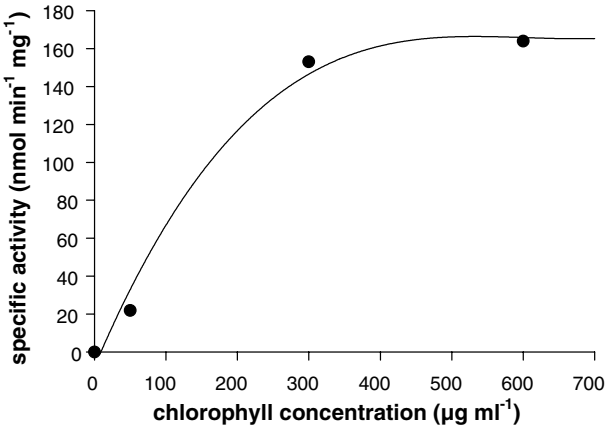

Fig. 2. Activity of At-GcpE upon illumination in the presence of increasing amounts of thylakoid under an inert N_2 atmosphere. 250 µEinstein m^{-2} s^{-1}, 30 °C, 10 min, At-GcpE (1.05 µM), [2-^{14}C]MEcDP (920 µM, 0.015 µCi).

presence of light of acting as reducing system for the bacterial enzyme. It was previously shown that ferredoxin from spinach in the presence of ferredoxin reductase and NADPH could be used as reducing system in the absence of light on the bacterial GcpE [34].

4. Discussion

The GcpE catalyzed reaction is dependent on an electron donating system. In *E. coli*, this can be completed by the flavodoxin/flavodoxin reductase/NADPH system already present in the bacterium [18]. This bacterial redox shuttle is, however, ineffective with the *A. thaliana* GcpE [19]. In addition, flavodoxin is absent in plant chloroplasts, suggesting that another electron transfer shuttle is operating in these organelles.

In the present study, we show that electron flow from photosynthesis can directly provide in the presence of light and via ferredoxin the electrons required for the GcpE catalyzed reaction without any contribution of ferredoxin–NADP$^+$ reductase and NADPH (Fig. 3, Part A). Indeed, the GcpE activity in the light did not require the addition of pyridine nucleotides, and no activity could be detected in the dark, suggesting that the reducing cofactors had been eliminated by washing the thylakoids. In contrast, in the absence of light, GcpE requires an electron shuttle, as shown by the activity in the presence of ferredoxin/ferredoxin reductase/NADPH. This situation prevails in the dark, and especially in non-phototrophic tissues such as roots (Fig. 3, Part B). Indeed, this reducing system has been characterized in pea roots [35], and an active MEP pathway has been pointed out in roots of *Glycyrrhiza glabra* [36], *Catharanthus roseus* [37], *Ophiorrhiza pumila* [38] and *Daucus carota* [39], mycorrhized roots of *Hordeum vulgare* [40], many species of the Poaceae family [41,42] and *Salvia miltiorrhiza* [43]. The *dxs* and *dxr* genes are expressed in the roots of *A. thaliana* [44] and *Artemisia annua* [45]. In *Medicago truncatula*, two distantly related genes have been found: the deoxyxylulose phosphate synthase gene, DXS1, is preferentially expressed in many developing tissues excepting roots, whereas the DXS2 transcript levels are highly expressed in roots upon mycorrhizal colonization and are low in other tissues [46]. A mycorrhiza-inducible DXR has been identified by immunolocalization in arbuscule-containing cells of *Zea mays* [47].

Two biosynthetic pathways for isoprenoid biosynthesis are present in plants. The mevalonate pathway is found in the cytoplasm, whereas the MEP pathway is only present in the plastids. The latter biosynthetic route is strongly linked to photosynthesis. Of its first two precursors, glyceraldehyde 3-phos-

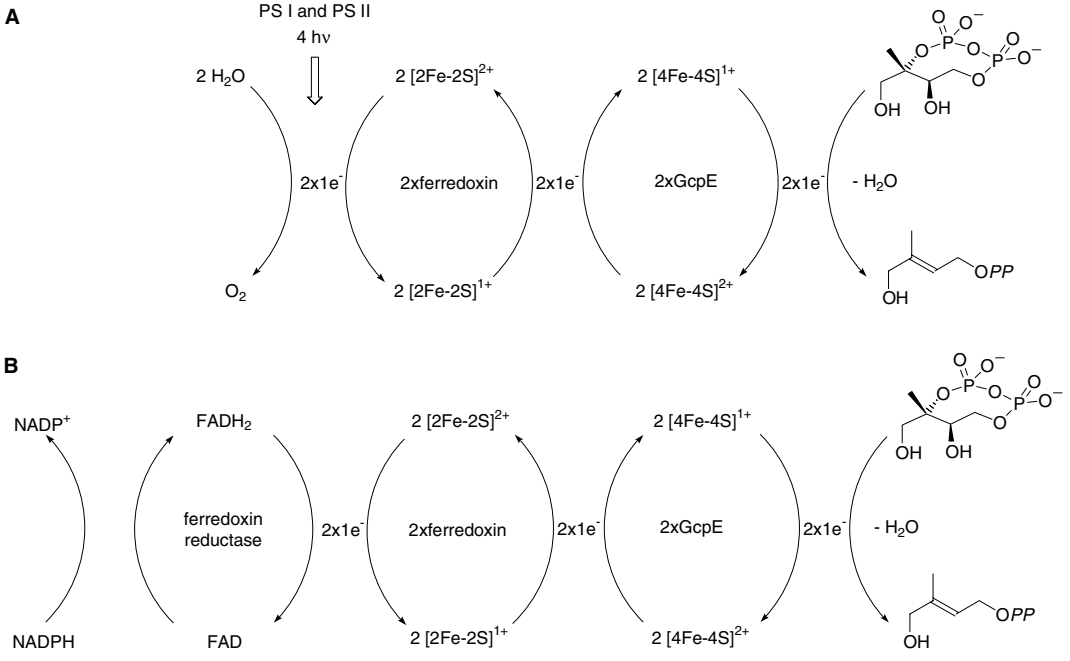

Fig. 3. MEP pathway for isoprenoid biosynthesis: hypothetical biogenetic scheme for plant GcpE activity: (A) in photosynthetic tissues in the light; (B) in photosynthetic tissues in the dark and in non-photosynthetic tissues. PS I and PS II: photosystems I and II.

phate belongs to the Calvin cycle, and pyruvate is derived from hexoses directly issued from photosynthesis. Activation of the MEP pathway by light is also documented by the rapid emission of isoprenoids known to be derived from MEP pathway upon irradiation of green tissues [48]: isoprene [49], 2-methyl-3-buten-2-ol [50] and monoterpenes [51]. $^{13}CO_2$ feeding to *Quercus rubra* leaves indicated a close linkage between the photosynthetic carbon reducing pathway and the carbon source required for isoprene biosynthesis [52]. Illumination of *Quercus ilex* leaves induced emission of α-pinene and other monoterpenes, which depended on photosynthetic carbon assimilation products. In the latter study, de novo synthesis, and not mobilization of endogenous terpenes or terpene precursor pools, is responsible for this quick production [53,54]. Light dependence of isoprene and methylbutenol emission in cottonwood (*Populus deltoides*) is in part due to controls over DMAPP biosynthesis [55]. In the petals of snapdragon flowers (*Antirrhinum majus*), the MEP pathway provides IPP and DMAPP for both the plastidial monoterpenes and the cytosolic sesquiterpenes biosynthesis and operates only in the light in a rhythmic manner controlled by the circadian clock [56]. All this data strongly suggests a close linkage between photosynthesis and the MEP pathway for isoprenoid biosynthesis, not only for providing the carbon source, but also the required reducing electron flow. Light may thus directly interfere with the regulation of the MEP pathway and with the cross-talk between the cytoplasmic mevalonate and the plastidial MEP pathways [57].

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References

[1] Bloch, K. (1992) Sterol molecule: structure, biosynthesis, function. Steroids 57, 378–383.
[2] Rohmer, M., Knani, M., Simonin, P., Sutter, B. and Sahm, H. (1993) Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. Biochem. J. 295, 517–524.
[3] Rohmer, M. (1999) The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Nat. Prod. Rep. 16, 565–573.
[4] Eisenreich, W., Rohdich, F. and Bacher, A. (2001) Deoxyxylulose phosphate pathway to terpenoids. Trends Plant Sci. 6, 78–84.
[5] Boucher, Y. and Doolittle, W.F. (2000) The role of the lateral gene transfer in the evolution of isoprenoid biosynthesis pathways. Mol. Microbiol. 37, 703–716.
[6] Laupitz, R., Hecht, S., Amslinger, S., Zepeck, F., Kaiser, J., Richter, G., Schramek, N., Steinbacher, S., Huber, R., Arigoni, D., Bacher, A., Eisenreich, W. and Rohdich, F. (2004) Biochemical characterization of *Bacillus subtilis* type II isopentenyl diphosphate isomerase, and phylogenetic distribution of isoprenoid biosynthesis pathways. Eur. J. Biochem. 271, 2658–2669.
[7] Schwarz, M. (1994) Terpen-Biosynthese in *Ginkgo biloba*: eine überraschende Geschichte. Ph.D. Thesis, Eidgenössische Technische Hochschule, Zürich.
[8] Eisenreich, W., Menhard, B., Hylands, P.J., Zenk, M.H. and Bacher, A. (1996) Studies on the biosynthesis of taxol: the taxane skeleton is not of mevalonoid origin. Proc. Natl. Acad. Sci. USA 93, 6431–6436.
[9] Lichtenthaler, H.K., Schwender, J., Disch, A. and Rohmer, M. (1997) Biosynthesis of isoprenoids in higher plants proceeds via a mevalonate independent pathway. FEBS Lett. 400, 271–274.
[10] Hecht, S., Eisenreich, W., Adam, P., Amslinger, S., Kis, K., Bacher, A., Arigoni, D. and Rohdich, F. (2001) Studies on the nonmevalonate pathway to terpenes: the role of the GcpE (IspG) protein. Proc. Natl. Acad. Sci. USA 98, 14837–17842.
[11] Seemann, M., Campos, N., Rodriguez-Concepción, M., Hoeffler, J.-F., Grosdemange-Billiard, C., Boronat, A. and Rohmer, M. (2002) Isoprenoid biosynthesis via the methylerythritol phosphate pathway: accumulation of 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate in a gcpE deficient mutant of *Escherichia coli*. Tetrahedron Lett. 43, 775–778.
[12] Seemann, M., Campos, N., Rodriguez-Concepción, M., Ibanez, E., Duvold, T., Tritsch, D., Boronat, A. and Rohmer, M. (2002) Isoprenoid biosynthesis in *Escherichia coli* via the methylerythritol phosphate pathway: enzymatic conversion of methylerythritol cyclodiphosphate into a phosphorylated derivative of (*E*)-methylbut-2-ene-1,4-diol. Tetrahedron Lett. 43, 1413–1415.
[13] Wolff, M., Seemann, M., Grosdemange-Billiard, C., Tritsch, D., Campos, N., Rodriguez-Concepción, M., Boronat, A. and Rohmer, M. (2002) Isoprenoid biosynthesis via the methylerythritol phosphate pathway. (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate: chemical synthesis and formation from methylerythritol cyclodiphosphate by a cell-free system from *Escherichia coli*. Tetrahedron Lett. 43, 2555–2559.
[14] Hintz, M., Reichenberg, A., Altincicek, B., Bahr, U., Gschwind, R.M., Kollas, A.-K., Beck, E., Wiesner, J., Eberl, M. and Jomaa, H. (2001) Identification of (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as major activator for human γδ T cells in *Escherichia coli*. FEBS Lett. 509, 317–322.
[15] Altincicek, B., Duin, E.C., Reichenberg, A., Hedderich, R., Kollas, A.-K., Hintz, M., Wagner, S., Wiesner, J., Beck, E. and Jomaa, H. (2002) LytB catalyzes the terminal step of the 2-*C*-methyl-D-erythritol-4-phosphate pathway for isoprenoid biosynthesis. FEBS Lett. 532, 437–440.
[16] Rohdich, F., Hecht, S., Gärtner, K., Adam, P., Krieger, C., Amslinger, S., Arigoni, D., Bacher, A. and Eisenreich, W. (2002) Studies on the nonmevalonate terpene biosynthetic pathway: metabolic role of IspH (LytB) protein. Proc. Natl. Acad. Sci. USA 99, 1158–1163.
[17] Adam, P., Hecht, S., Eisenreich, W., Kaiser, J., Gräwert, T., Arigoni, D., Bacher, A. and Rohdich, F. (2002) Biosynthesis of terpenes: studies on 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate reductase. Proc. Natl. Acad. Sci. USA 99, 12108–12113.
[18] Seemann, M., Tse Sum Bui, B., Wolff, M., Tritsch, D., Campos, N., Boronat, A., Marquet, A. and Rohmer, M. (2002) Isoprenoid biosynthesis through the methylerythritol phosphate pathway: the (*E*)-4-hydroxy-3-methylbut-2-enyl synthase (GcpE) is a [4Fe–4S] protein. Angew. Chem., Int. Ed. 41, 4337–4339.
[19] Seemann, M., Wegner, P., Schünemann, V., Tse Sum Bui, B., Wolff, M., Marquet, A., Trautwein, A.X. and Rohmer, M. (2005) Isoprenoid biosynthesis in chloroplasts via the methylerythritol phosphate pathway: the (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (GcpE) from *Arabidopsis thaliana* is a [4Fe–4S] protein. J. Biol. Inorg. Chem. 10, 20672–20679.
[20] Wolff, M., Seemann, M., Tse Sum Bui, B., Frapart, Y., Tritsch, D., Garcia-Estrabot, A., Rodriguez-Concepción, M., Boronat, A., Marquet, A. and Rohmer, M. (2003) Isoprenoid biosynthesis via the methylerythritol phosphate pathway: the (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (LytB/IspH) from *Escherichia coli* is a [4Fe–4S] protein. FEBS Lett. 541, 115–120.
[21] Rohdich, F., Zepeck, F., Adam, P., Hecht, S., Kaiser, J., Laupitz, R., Gräwert, T., Amslinger, S., Eisenreich, W., Bacher, A. and Arigoni, D. (2003) The deoxyxylulose phosphate pathway of isoprenoid biosynthesis: studies on the mechanisms of the reactions catalyzed by IspG and IspH protein. Proc. Natl. Acad. Sci. USA 100, 1586–1591.
[22] Puan, K.J., Wang, H., Dairi, T., Kuzuyama, T. and Morita, C.T. (2005) *fldA* is an essential gene required in the 2-*C*-methylerythritol 4-phosphate pathway for isoprenoid biosynthesis. FEBS Lett. 579, 3802–3806.
[23] Kollas, A.-K., Duin, E.C., Eberl, M., Altincicek, B., Hintz, M., Reichenberg, A., Henschker, D., Henne, A., Steinbrecher, I., Ostrovsky, D.N., Hedderich, R., Beck, E., Jomaa, H. and

Wiesner, T.J. (2003) Functional characterization of GcpE, an essential enzyme of the non-mevalonate pathway for isoprenoid biosynthesis. FEBS Lett. 532, 432–436.
[24] Medina, M. and Gomez-Moreno, C. (2004) Interaction of ferredoxin–NADP(+) reductase with its substrate: optimal interaction for efficient electron transfer. Photosynth. Res. 79, 113–131.
[25] Röhrich, R.C., Englert, N., Trotschke, K., Reichenberg, A., Hintz, M., Seeber, F., Balconi, E., Aliverti, A., Zanetti, G., Köhler, U., Pfeiffer, M., Beck, E., Jomaa, H. and Wiesner, J. (2005) Reconstitution of an apicoplast-localised electron transfer pathway involved in the isoprenoid biosynthesis in *Plasmodium falciparum*. FEBS Lett. 579, 6433–6438.
[26] Okada, K. and Hase, T. (2005) Cyanobacterial non-mevalonate pathway: (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase interacts with ferredoxin from *Thermosynechococcus elongatus*. J. Biol. Chem. 276, 5483–5490.
[27] Gräwert, T., Kaiser, J., Zepeck, F., Laupitz, R., Hecht, S., Amslinger, S., Schramek, N., Schleicher, E., Weber, S., Haslbeck, M., Buchner, J., Rieder, C., Arigoni, D., Bacher, A. and Rohdich, F. (2004) IspH protein of *Escherichia coli*: studies on iron sulfur cluster implementation and catalysis. J. Am. Chem. Soc. 126, 12847–12855.
[28] Mayhew, S.T. (1971) Nondenaturing procedure for rapid preparation of ferredoxin from *Clostridium pasteurianum*. Anal. Biochem. 42, 191–194.
[29] Droux, M., Jacquot, J.-P., Miginiac-Maslow, M., Gadal, P., Huet, J.C., Crawford, N.A., Yee, B.C. and Buchanan, B.B. (1987) Ferredoxin–thioredoxin reductase, an iron–sulfur linking light to enzyme regulation in oxygenic photosynthesis: purification and properties of the enzymes from C_3, C_4 and cyanobacterial species. Arch. Biochem. Biophys. 252, 426–439.
[30] Hodges, M., Miginiac-Maslow, M., Le Maréchal, P. and Rémy, R. (1990) The ATP-dependent post translational modification of ferredoxin: $NADP^+$ oxidoreductase. Biochim. Biophys. Acta 1052, 446–452.
[31] Miginiac-Maslow, M., Jacquot, J.P. and Droux, M. (1985) Energetic aspects of the light activation of two chloroplast enzymes: fructose-1,6-biphosphatase and NADP-malate dehydrogenase. Photosynth. Res. 6, 201–213.
[32] Arnon, D.I. (1949) Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol. 24, 1–15.
[33] Herz, S., Wungsintaweekul, J., Schuhr, C.A., Hecht, S., Lüttgen, H., Sagner, S., Fellermeier, M., Eisenreich, W., Zenk, M., Bacher, A. and Rohdich, F. (2000) Biosynthesis of terpenoids: YgbP protein converts 4-diphosphocytidy-2*C*-methyl-D-erythritol 2-phosphate to 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate. Proc. Natl. Acad. Sci. USA 97, 2486–2490.
[34] Zepeck, F., Gräwert, T., Kaiser, J., Schramek, N., Eisenreich, W., Bacher, A. and Rohdich, F. (2005) Biosynthesis of isoprenoids. Purification and properties of IspG protein from *Escherichia coli*. J. Org. Chem. 70, 9168–9174.
[35] Bowsher, C.G. and Knight, J.S. (1996) The electronic plant gene register. The isolation of a pea root ferredoxin–$NADP^+$ oxidoreductase (FNR) cDNA (Accession No. X99419). Plant Physiol. 112, 861–864.
[36] Asada, Y., Li, W. and Yoshikawa, T. (2000) Biosynthesis of the dimethylallyl moiety of glabrol in *Glycyrrhiza glabra* hairy root cultures via a non-mevalonate pathway. Phytochemistry 55, 323–326.
[37] Hong, S.B., Hughes, E.H., Shanks, J.V., San, K.Y. and Gibson, S.I. (2003) Role of the non-mevalonate pathway in indole alkaloid production by *Catharanthus roseus* hairy roots. Biotechnol. Prog. 19, 1105–1108.
[38] Yamazaki, Y., Kitajima, M., Arita, M., Takayama, H., Sudo, H., Yamazaki, M., Aimi, N. and Sato, K. (2004) Biosynthesis of camptothecin in silico and in vivo tracer study from [1-^{13}C]glucose. Plant Physiol. 134, 161–170.
[39] Hampel, D., Mosandl, A. and Wüst, M. (2005) Biosynthesis of mono- and sesquiterpenes in carrot roots and leaves (*Daucus carota* L.): metabolic cross talk of cytosolic mevalonate and plastidial methylerythritol phosphate pathways. Phytochemistry 66, 305–311.
[40] Maier, W., Schneider, B. and Strack, D. (1998) Biosynthesis of sesquiterpenoid cyclohexenone derivatives in mycorrhizal barley roots proceeds via the glyceraldehyde 3-phosphate/pyruvate pathway. Tetrahedron Lett. 39, 521–534.
[41] Walter, M.H., Fester, T. and Strack, D. (2000) Arbuscular mycorrhizal fungi induce the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis correlated with accumulation of the 'yellow pigment' and other apocarotenoids. Plant J. 21, 571–578.
[42] Fester, T., Schmidt, D., Lohse, S., Walter, M.H., Giuliano, G., Bramley, P.M., Fraser, P.D., Hause, B. and Strack, D. (2002) Stimulation of carotenoid metabolism in arbuscular mycorrhizal roots. Planta 216, 148–154.
[43] Ge, X. and Wu, J. (2005) Tanshinone production and isoprenoid pathways in *Salvia miltiorrhiza* hairy roots induced by Ag^+ and yeast elicitor. Plant Sci. 168, 487–491.
[44] Carretero-Paulet, L., Ahulada, I., Cunillera, N., Rodriguez-Concepcion, M., Ferrer, A., Boronat, A. and Campos, N. (2002) Expression and molecular analysis of the *Arabidopsis* DXR gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase, the first committed enzyme of the 2-*C*-methyl-D-erythritol 4-phosphate pathway. Plant Physiol. 129, 1581–1591.
[45] Souret, F., Kim, Y., Wyslouzil, B.E., Wobbe, K.K. and Weathers, P.J. (2003) Scale-up of *Artemisia annua* L. hairy root cultures produces complex patterns of terpenoid gene expression. Biotechnol. Bioeng. 83, 653–667.
[46] Walter, M.H., Hans, J. and Strack, D. (2002) Two distantly related genes encoding 1-deoxyxylulose 5-phosphate synthases: differential regulation in shoots and apocarotenoid-accumulating mycorrhizal roots. Plant J. 31, 243–254.
[47] Hans, J., Hause, B., Strack, D. and Walter, M.H. (2004) cloning, characterization, and immunolocalization of a mycorrhiza-inducible 1-deoxy-D-xylulose 5-phosphate reductoisomerase in arbuscule-containing cells of maize. Plant Physiol. 134, 614–624.
[48] Lerdau, M. and Gray, D. (2003) Ecology and evolution of light-dependent and light-independent phytogenic volatile organic carbon. New Phytol. 157, 199–211.
[49] Zeidler, J.G., Lichtenthaler, H.K., May, H.U. and Lichtenthaler, F.W. (1997) Is isoprene emitted by plants synthesized via the novel isopentenyl pyrophosphate pathway? Z. Naturforsch. 52c, 15–23.
[50] Zeidler, J. and Lichtenthaler, H.K. (2001) Biosynthesis of 2-methyl-3-buten-2-ol from needles of *Pinus ponderosa* via the non-mevalonate DOXP/MEP pathway for isoprenoid formation. Planta 213, 323–326.
[51] Eisenreich, W., Sagner, S., Zenk, M.H. and Bacher, A. (1997) Monoterpenoid essential oils are not of mevalonoid origin. Tetrahedron Lett. 38, 3889–3892.
[52] Delwiche, C.F. and Sharkey, T.D. (1993) Rapid appearance of ^{13}C in biogenic isoprene when $^{13}CO_2$ is fed to intact leaves. Plant Cell Environ. 16, 587–591.
[53] Loreto, F., Ciccioli, P., Cecinato, A., Brancaleoni, E., Frattoni, M. and Tricoli, D. (1996) Influence of environmental factors and air composition on the emission of α-pinene from *Quercus ilex* leaves. Plant Physiol. 110, 267–275.
[54] Loreto, F., Ciccioli, P., Cecinato, A., Brancaleoni, E., Frattoni, M., Fabozzi, C. and Tricoli, D. (1996) Evidence of the photosynthetic origin of monoterpenes emitted by *Quercus ilex* L. leaves by ^{13}C labeling. Plant Physiol. 110, 1317–1322.
[55] Rosenstiel, T.N., Fisher, A.J., Fall, R. and Monson, R.K. (2002) Differential accumulation of dimethylallyl diphosphate in leaves and needles of isoprene- and methylbutenol-emitting and nonemitting species. Plant Physiol. 129, 1276–1284.
[56] Dudareva, N., Andersson, S., Orlova, I., Gatto, N., Reichelt, M., Rhodes, D., Boland, W. and Gershenzon, J. (2005) The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. Proc. Natl. Acad. Sci. USA 102, 933–938.
[57] Hemmerlin, A., Hoeffler, J.-F., Meyer, O., Tritsch, D., Kagan, I., Grosdemange-Billiard, C., Rohmer, M. and Bach, T.J. (2003) Cross-talk between the cytosolic mevalonate and the plastidial methylerythritol phosphate pathways in tobacco bright yellow-2 cells. J. Biol. Chem. 278, 2666–26676, and references cited therein.