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Chlorophyta exclusively use the 1-deoxyxylulose 5-phosphate/2-*C*-methylerythritol 4-phosphate pathway for the biosynthesis of isoprenoids

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Abstract. The biosynthesis of the C_5 building block of isoprenoids, isopentenyl diphosphate (IPP), proceeds in higher plants via two basically different pathways: in the cytosolic compartment sterols are formed via mevalonate (MVA), whereas in the plastids the isoprenoids are formed via the 1-deoxyxylulose 5-phosphate/2-*C*-methylerythritol 4-phosphate pathway (DOXP/MEP pathway). In the present investigation, we found for the Charophyceae, being close relatives to land plants, and in the original green flagellate *Mesostigma viride* the same IPP biosynthesis pattern as in higher plants: sterols are formed via MVA, and the phytol-moiety of chlorophylls via the DOXP/MEP pathway. In contrast, representatives of four classes of the Chlorophyta (Chlorophyceae, Ulvophyceae, Trebouxiophyceae, Prasinophyceae) did not incorporate MVA into sterols or phytol. Instead, they incorporated [1-$^2H_1$]-1-deoxy-D-xylulose into phytol and sterols. The results indicate that the entire Chlorophyta lineage, which is well separated from the land plant/Charophyceae lineage, is devoid of the acetate/MVA pathway and uses the DOXP/MEP pathway not only for plastidic, but also for cytosolic isoprenoid formation.

Key words: Charophyta – 1-Deoxy-D-xylulose – Green alga – Isopentenyl diphosphate – Isoprenoid biosynthesis – Mevalonate

Introduction

Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) represent the common C_5 precursors for the biosynthesis of isoprenoids. The biosynthesis of IPP was first established in animals and yeast by Conrad Bloch and Feodor Lynen in 1958 [for original literature of the acetate/mevalonate (Ac/MVA) pathway see the reviews of Spurgeon and Porter 1981; Lichtenthaler et al. 1997a; Lichtenthaler 1999]. In these organisms IPP derives from mevalonic acid, which is formed from three molecules of acetyl-CoA. The occurrence of an alternative non-mevalonate biosynthetic route for IPP biosynthesis was found much later by ^{13}C-incorporation studies first in eubacteria (Rohmer et al. 1993), then in different photosynthetic organisms such as green algae (Schwender et al. 1995, 1996; Disch et al. 1998a) and higher plants (Lichtenthaler et al. 1997a,b; Arigoni et al. 1997) as well as in a cyanobacterium, a red alga, a Chrysophyte (Schwender et al. 1997; Disch et al. 1998a) and in diatoms (Cvejic and Rohmer 2000) as summarized by Lichtenthaler (1999). The DOXP/MEP pathway also occurs in the apicoplast of the malaria parasite *Plasmodium falciparum* (Jomaa et al. 1999).

The first step of the alternative IPP pathway is the formation of 1-deoxy-D-xylulose-5-phosphate (DOXP) from pyruvate and glyceraldehyde-3-phosphate (Rohmer et al. 1996). In the next step, DOXP is transformed to 2-*C*-methyl-D-erythritol 4-phosphate (MEP), which represents the branched C_5-carbon skeleton of IPP as reviewed in Lichtenthaler (1999). Currently this pathway has been termed the DOXP/MEP pathway. The MEP is further transformed via 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol (Rohdich et al. 1999; Kuzuyama et al. 2000a) to 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol 2-phosphate (Lüttgen et al. 2000; Kuzuyama et al. 2000b) and to 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (Herz et al. 2000; Takagi et al. 2000). The final biosynthetic steps between 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate and IPP have not yet been established, but presumably include two dehydrogenases and

Dedicated to Prof. Dr. Stanislav Procházka, Mendel University of Brno, on the occasion of his 60th anniversary.

Abbreviations: Ac/MVA pathway = acetate/mevalonate pathway; DOX = 1-deoxy-D-xylulose; DOXP = 1-deoxy-D-xylulose 5-phosphate; HMG-CoA = hydroxymethylglutaryl coenzyme A; IPP = isopentenyl diphosphate; MEP = 2-*C*-methyl-D-erythritol 4-phosphate; MVA = mevalonic acid; MVL = mevalonolactone

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two NADPH reductases as proposed by Lichtenthaler et al. (2000).

In higher plants the two different IPP pathways are found in different subcellular compartments as deduced, for example, from the fact that the plastidic isoprenoids such as β-carotene, the phytyl side-chain of chlorophylls and the nona-prenyl chain of plastoquinone-9 are formed via the DOXP/MEP pathway, whereas the cytosolic sterols are synthesized via the classical Ac/MVA pathway of IPP formation (Lichtenthaler et al. 1997a,b; Fig. 1). In addition to sterol biosynthesis, the biosynthesis of the prenyl side-chain of the mitochondrial ubiquinones of higher plants seems to be generally fed by the Ac/MVA pathway (Disch et al. 1998b, Lichtenthaler 1999). Further evidence for the compartmental separation of the two IPP pathways are the observations that for the enzymes of the Ac/MVA pathway in higher plants only cytosolic forms are known, whereas the DOXP synthases possess putative N-terminal chloroplast transit peptides (Lichtenthaler 1999). Moreover, the targeting of DOXP synthases into the chloroplast has now been shown in *Arabidopsis thaliana* (Araki et al. 2000).

It appears that in the evolution of eukaryotic photosynthetic organisms, after the endosymbiotic engulfment of a plastid precursor, the eukaryotic host kept the Ac/MVA pathway in its cytosol, whereas the plastid maintained the operation of the DOXP/MEP pathway (Lichtenthaler 1999). However, three exceptions from this rule have been found among several unicellular green algae: All investigated isoprenoids of *Scenedesmus obliquus*, including the cytosolic sterols and the mitochondrial ubiquinone, are formed via the DOXP/MEP pathway (Schwender et al. 1996, 1997; Disch et al. 1998a). This also applies to *Chlamydomonas reinhardtii* and *Chlorella fusca* (Disch et al. 1998a). These green algae obviously do not possess the Ac/MVA pathway. As green algae and land plants (higher plants, mosses, ferns) form a monophyletic group, also termed "green plants" (Chapman et al. 1998), the question arises where in the evolution of *Scenedesmus obliquus*, *Chlamydomonas reinhardtii*, *Chlorella fusca* and perhaps of other green algae the loss of the cytosolic Ac/MVA pathway of IPP formation occurred. For this reason we have investigated in the present study the IPP-biosynthesis pattern in species of all major classes of green algae, including the Chlorophyta and Charophyceae.

Materials and methods

Chemicals and labeled precursors

[1-$^2H_1$]-1-Deoxy-D-xylulose (DOX) was obtained from [1-$^2H_1$]-1-deoxy-2,3-*O*-isopropyliden-β-D-xylulose (Zeidler et al. 1997) by hydrolysis (40 mM HCl, 2 h at 50 °C), followed by a careful neutralization with 5% $NaHCO_3$. DL-[2-^{13}C]Mevalonic acid lactone (MVL) was purchased from IC Chemikalien (Ismaning, Germany), DL-[5-^{3}H]MVL (2220 GBq/mmol) from American Radiolabeled Chemicals Inc., USA, and [2-^{14}C]DOX (629 MBq/mmol) was synthesized enzymatically (Schwender et al. 1999). Cerivastatin was a gift from Bayer AG (Leverkusen, Germany).

Algal strains

The algal strains applied in this study were obtained from the Sammlung von Algenkulturen (SAG, Göttingen, Germany): *Scenedesmus obliquus* Kützing (strain 276-3c), *Chlamydomonas reinhardtii* Dangeard (strain 83.81), *Gloeotilopsis planctonica* Lyengar et Philipose (strain 29.93), *Klebsormidium flaccidum* Kützing (strain 335-2a), *Spirogyra* sp. Link (strain B169.80), *Trebouxia asymmetrica* Friedl et Gärtner (strain 48.88) *Tetraselmis striata* Butcher (strain 41.85), *Chlorella saccharophila* Krüger (strain 211-9a), *Mesostigma viride* Lauterborn (strain 50-1).

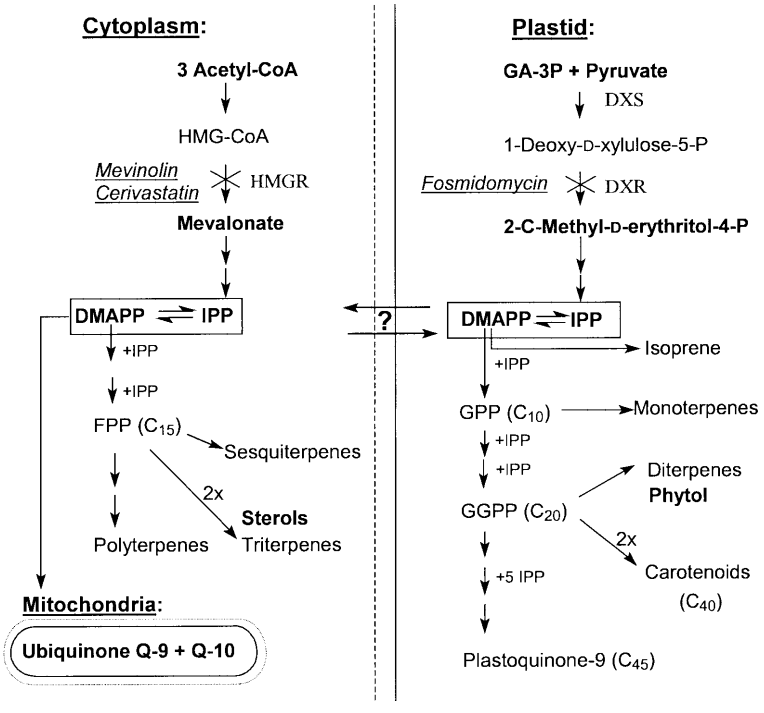

Fig. 1. Supposed compartmentation of the two pathways of IPP and isoprenoid biosynthesis in higher plants, red algae and chrysophytes. The possible exchange of prenyldiphosphates between the cytosol and the plastids is indicated (*arrows*). The cytosolic pathway for IPP formation can specifically be inhibited by the antibiotics mevinolin and cerivastatin and the DOXP/MEP pathway by the antibiotic and herbicide fosmidomycin. *DXS* 1-Deoxy-D-xylulose-5-phosphate synthase; *DXR* 1-deoxy-D-xylulose-5-phosphate reductoisomerase; *DMAPP* dimethylallyl diphosphate; *FPP* farnesyl diphosphate; *GA-3P* glyceraldehyde 3–phosphate; *GPP* geranyl diphosphate; *GGPP* geranylgeranyl diphosphate; *HMGR* hydroxymethylglutaryl-CoA reductase (scheme modified after Lichtenthaler 1999)

Mesostigma viride Lauterborn (strain NIES-296) was purchased from the Microbial Culture Collection of the National Institute for Environmental Studies, Ibakari, Japan.

Growth of green algae

The algal strains were received and grown as axenic cultures. The different algae were grown in Erlenmeyer flasks on a rotary shaker at 22 °C in the light (100 μmol photons m^{-2} s^{-1}) under aseptic conditions. *Chlamydomonas reinhardtii* and *Chlorella saccharophila* were grown on the following mineral medium: 20 mM Tris-HCl (pH 7.0), 8 mM NH_4Cl, 0.54 mM K_2HPO_4, 0.46 mM KH_2PO_4, 1.5 mM $MgSO_4$, 0.4 mM $CaCl_2$ (Surzycki 1971). For *S. obliquus*, *G. planctonica*, *Tetraselmis striata* and *K. flaccidum* the following medium was used: 8 mM KNO_3, 1 mM Na_2HPO_4, 3 mM NaH_2PO_4 (pH 6.8), 1 mM $MgSO_4$, 0.1 mM $CaCl_2$ (Bishop and Senger 1971). *Spirogyra* sp. was grown on the medium: 0.1 mM KNO_3, 0.2 mM K_2HPO_4, 0.1 mM $MgSO_4$, 30 ml/l soil extract (Schlösser 1994). *Trebouxia asymmetrica* was cultivated as *Spirogyra* sp. with addition of 0.1% proteose-peptone. *Mesostigma viride* was grown on the mineral medium: 0.6 mM $Ca(NO_3)_2$, 1 mM KNO_3, 0.2 mM NaH_2PO_4, 0.2 mM $MgSO_4$, 4 mM Tris-HCl (pH 7.5), 1 ng/l vitamin B_{12}, 1 ng/l biotin, 0.1 μg/l thiamine.

Microelements used for all species were: 36 μM $FeSO_4$/Titriplex III, 8 μM H_3BO_3, 2.5 μM $MnCl_2$, 0.7 μM $ZnSO_4$, 0.4 μM $CuSO_4$, 16 nM $(NH_4)_6Mo_7O_{24}$.

Labeling with [1-$^2H_1$]DOX and [2-^{13}C]MVL

At the beginning of growth [1-$^2H_1$]DOX or DL-[2-^{13}C]MVL were added in a concentration of 0.05% (w/v). After 10–20 d of growth in the light the cells had doubled in number several times and were harvested by centrifugation.

Labelling with radioactive precursors

[2-^{14}C]-1-Deoxy-D-xylulose (DOX) (74 kBq, 629 MBq/mmol) and DL-[5-^{3}H]mevalonolactone (MVL) (148 kBq, 629 MBq/mmol) were added to cultures of *K. flaccidum* (50 ml) and *Lemna gibba* (50 ml, using a growth medium as described in Lichtenthaler et al. 1997b). After 48 h of growth in the light (500 μmol photons m^{-2} s^{-1}) the cells were harvested. To a 10 ml volume of a culture of *M. viride* (SAG 50-1) [2-^{14}C]DOX (18.5 kBq, 629 MBq/mmol) and DL-[5-^{3}H]MVL (37 kBq, 629 MBq/mmol) were added for 24 h (500 μmol photons m^{-2} s^{-1}). After isolation of phytol and sterols (see below), the radioactivity was measured with a Tri-Carb 2000CA Liquid Scintillation Analyser (Packard Instrument Company). Dual label measurements were used to determine ^{3}H- and ^{14}C-activities.

Extraction and purification of phytol and sterols of algae

The algal cells were extracted several times with methanol. The methanolic extracts were saponified, the unsaponifiable lipids transferred to hexane and separated by TLC on silica gel (Schwender et al. 1997). Finally, phytol and sterols were recovered and acetylated.

Analysis of phytyl- and steryl-acetates

The lipid fractions containing the acetates of phytol and sterols were analyzed by GC-MS: Hewlett-Packard 5890 Series II gas chromatograph (cross-linked methylsilicone; 20 m long, 0.32 mm i.d.), coupled with a Hewlett-Packard 5971A mass selective detector. Temperature programming was 80 °C for 3 min, heating to 280 °C (20 °C/min), and 20 min at 280 °C. The incorporation of ^{13}C and ^{2}H into phytol and different sterols was determined from the mass spectra, which were obtained by GC-MS. By using single ion monitoring (SIM), ions representing phytol (phytyl$^+$, m/e = 278.3) and sterols (e.g. ergost-7-enyl-acetate$^+$, m/e = 442.4) were analyzed. The intensity of the isotope peaks of these ions results from the natural ^{13}C abundance and from incorporation of ^{2}H or ^{13}C from the labeled substrates. After application of a subtraction method to eliminate the contribution of the natural ^{13}C abundance (Rauschenbach et al. 1974), the resulting relative intensities of the isotope peaks M + 1, M + 2, M + 3 etc. denote molar fractions of molecules that were labeled with ^{2}H or ^{13}C at one, two, three or more positions, respectively. By taking into account the number of isoprenic units, which can be labeled in phytol- and in sterol-molecules, the percentage of isotope-labeled isoprenic units was calculated.

Inhibition tests

Mesostigma viride (SAG 50-1) was grown in 24-well tissue culture plates (1 ml culture volume; Becton Dickinson Labware, USA) with addition of cerivastatin at 10^{-6}, 10^{-5} and 10^{-4} M. After 5 d of growth, the cells were harvested by centrifugation. After extraction with acetone, the concentration of chlorophylls (*a* + *b*) and total carotenoids were determined spectrophotometrically using re-determined extinction coefficients (Lichtenthaler 1987).

Identification of phytol and sterols

Phytylacetate was identified by GC-MS, using an authentic standard which was obtained from chlorophyll *a* by saponification. The sterylacetates were analyzed by GC-MS and identified by the fragmentation pattern found in their mass spectra (Goad 1991). The sterols were also identified by comparison with the fragmentation pattern and the retention times of the sterol mixtures of *Scenedesmus obliquus*, *Lemna gibba* and *Ochromonas danica,* which had been analyzed previously using different analytical methods (Schwender et al. 1996; Lichtenthaler et al. 1997b; Disch et al. 1998a).

Results

Incorporation of labeled precursors into Chlorophyta

After growth of *Gloeotilopsis planctonica* with [1-$^2H_1$]DOX, the mass spectrum of phytol showed, as expected from its diterpenic structure, that up to four positions per molecule were deuterated (Fig. 2). By considering the four isoprenic units that can be labeled, it was calculated that 74% of the isoprenic units of phytol were labeled, indicating that DOX is a good isoprenoid precursor (Table 1). The mass spectrum of the main sterol component of *G. planctonica*, a Δ^5 24-ethyl-sterol, also showed deuterium-label at four positions with nearly the same labeling degree (73%) as found for phytol[1]

[1] In sterol molecules, although consisting of six isoprenic units, only four positions can be labeled from [1-$^2H_1$]DOX because in sterol biosynthesis three methyl groups of the original triterpenic carbon skeleton are removed. Two of these derive from C-5 of IPP carrying label from [1-$^2H_1$]DOX.

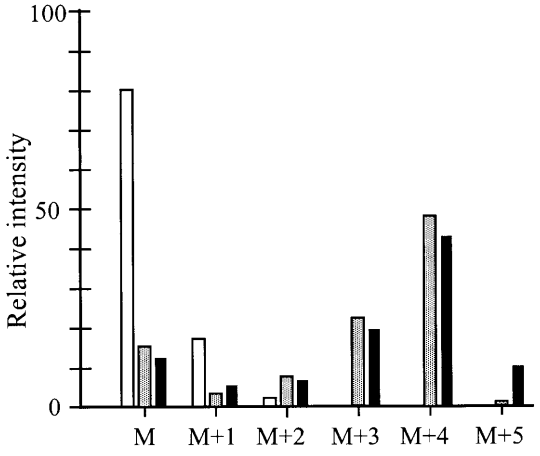

Fig. 2. Labeling of phytol of *Gloeotilopsis planctonica* from [1-$^2H_1$]DOX as shown by a section of the mass spectrum of phytylacetate (*black bars*, fragment [phytylacetate-AcOH]$^+$; M = 278.3 m/e). In comparison to unlabeled phytylacetate with natural ^{13}C abundance (*white bars*) a shift to M + 4 was observed, which becomes more obvious after application of the subtraction method for the elimination of the contribution of the natural ^{13}C abundance (*grey bars*) (Rauschenbach et al. 1974). The subtracted spectrum shows that phytol is deuterium-labeled at four positions, revealing that 74% of the isoprenic units have been formed from the applied [1-$^2H_1$]DOX

(Table 1). After growth of *G. planctonica* with [2-^{13}C]MVL, label could neither be detected in phytol nor in Δ^5 24-ethyl-sterol. In a similar way, *Chlamydomonas reinhardtii*, *Scenedesmus obliquus*, *Tetraselmis striata*, *Trebouxia asymmetrica* and *Chlorella saccharophila* incorporated [1-$^2H_1$]DOX to equal degrees into phytol and the sterols, respectively, whereas label from [2-^{13}C]MVL was not found (Table 1).

Incorporation of labeled precursors into Charophyceae and Mesostigma

In *Spirogyra* sp., [1-$^2H_1$]DOX was incorporated better into phytol (58% labeled isoprenic units) than into the two sterols (26% and 27%), while [2-^{13}C]MVL was preferentially incorporated into the sterols (18% and 31%) and hardly into phytol (2%) (Table 1). In *Mesostigma viride* (NIES-296), [2-^{13}C]MVL was preferentially incorporated into the sterols (Table 1), whereas [1-$^2H_1$]DOX was incorporated into phytol and into sterols (32% and 22%, respectively). In *Klebsormidium flaccidum*, [1-$^2H_1$]DOX was incorporated to the same extent into phytol and into sterols, whereas [2-^{13}C]MVL was not incorporated into phytol but only labeled the sterols to about 25% (Table 1).

Additional incorporation experiments were performed with *K. flaccidum*, *Mesostigma viride* (SAG 50-1) and the higher plant *Lemna gibba*, using [2-^{14}C]DOX and [5-^{3}H]MVL. In *K. flaccidum* and in *M. viride*, the incorporation ratio of $^{14}C/^3H$ in phytol was 95 and 108, respectively, indicating that phytol was predominantly labeled from [2-^{14}C]DOX. For the sterols this ratio was 0.6 and 1.2, respectively, indicating that both IPP pathways significantly contribute to the sterol biosynthesis in both organisms (Table 2). Similar differences in the incorporation characteristics of [2-^{14}C]MVL and [5-^{3}H]MVL into phytol and sterols were also found in duckweed (*Lemna gibba* L.) (Table 2), for which the existence of a cytosolic Ac/MVA pathway and a plastidic DOXP/MEP pathway is well established (Lichtenthaler et al. 1997; Schwender et al. 1997).

Table 1. Percentage incorporation of the applied deuterium and ^{13}C-labeled precursors into the plastidic isoprenoid phytol and into cytosolic sterols of different green algae. The algae were grown in liquid media for 10–20 d with addition of [1-$^2H_1$]DOX (0.05%, w/v) or [2-^{13}C]MVL (0.05%, w/v). In each case a lipid fraction containing the acetates of phytol and sterols was analyzed by GC-MS. The incorporation rates were estimated from the relative intensities of the isotope peaks in the mass spectra (see *Material and methods*). Nomenclature of the sterols – examples: $\Delta^{5,7,22E}$ 24-Ethyl-sterol is an abbreviation for (22E)-24-ethylcholesta-5,7,22-trien-3β-ol; $\Delta^{5,24(24')}$ 24-Ethyliden-sterol is an abbreviation for [24(24^1)E]-24-ethyliden-cholesta-5,24(24^1)-dien-3β-ol (IUPAC-IUB 1989). A distinction between the 24-α and 24-β configurations is not possible by GC-MS analysis (Goad 1991), and is therefore not given

Organism/Isoprenoid	[1-$^2H_1$]-1-deoxy -D-xylulose	DL-[2-^{13}C]-mevalonolactone
Gloeotilopsis planctonica		
Phytol	74%	0%
Δ^5 24-Ethyl-sterol	73%	0%
Chlamydomonas reinhardtii		
Phytol	11%	0%
$\Delta^{5,7,22E}$ 24-Ethyl-sterol	10%	0%
$\Delta^{5,7,22E}$ 24-Methyl-sterol	8%	–
Scenedesmus obliquus		
Phytol	37%	0%
$\Delta^{7,22E}$ 24-Ethyl-sterol	39%	0%
Δ^7 24-Methyl-sterol	43%	0%
Tetraselmis striata		
Phytol	47%	0%
Δ^5 24-Methyl-sterol	44%	0%
$\Delta^{5,24}$ 24-Methyl-sterol	54%	0%
Trebouxia asymmetrica		
Phytol	3%	0%
Δ^5 24-Methyl-sterol	2%	0%
$\Delta^{5,22E}$ 24-Ethyl-sterol	3%	0%
Δ^5 24-Ethyl-sterol	3%	0%
Chlorella saccharophila		
Phytol	21%	0%
$\Delta^{5,24(24')}$ 24-Ethyliden-sterol	19%	0%
$\Delta^{5,24(24')}$ 24-Methylen-sterol	19%	0%
Spirogyra sp.		
Phytol	58%	2%
$\Delta^{5,24(24')}$ 24-Ethyliden-sterol	27%	18%
$\Delta^{5,24(24')}$ 24-Methylen-sterol	26%	31%
Klebsormidium flaccidum		
Phytol	4%	0%
Δ^5 24-Methyl-sterol	4%	26%
$\Delta^{5,22E}$ 24-Ethyl-sterol	4%	22%
Mesostigma viride (NIES-296)		
Phytol	32%	6%
$\Delta^{5,7}$-Sterol	22%	29%

Table 2. Contribution of the DOXP/MEP pathway and of the Ac/MVA pathway to isoprenoid biosynthesis in the two green algae *Klebsormidium flaccidum* (Charophyceae) and *Mesostigma viride* (Prasinophyceae) and in the higher plant *Lemna gibba* L. [2-^{14}C]-1-Deoxy-D-xylulose (^{14}C-DOX) and [5-^{3}H]-mevalonolactone (^{3}H-MVL) were applied to the cultures (see *Material and methods*). The radioactivity of phytol and sterols is given in decays per minute. The ratio $^{14}C/^{3}H$ is a measure of the contribution of the DOXP/MEP pathway and the Ac/MVA pathway to isoprenoid biosynthesis. The incorporation pattern found was similar in both the two green algae and the higher plant

Organism/ Isoprenoid	Applied precursor		Ratio $^{14}C/^{3}H$
	^{14}C-DOX	^{3}H-MVL	
Klebsormidium flaccidum			
Phytol	257.7	2.7	95.4
Sterols	114.2	174.9	0.7
Mesostigma viride (SAG 50-1)			
Phytol	1600.0	14.9	107.7
Sterols	618.9	535.7	1.2
Lemna gibba			
Phytol	359.6	15.4	23.4
Sterols	28.3	46.5	0.6

Inhibition of growth by cerivastatin

Mesostigma viride (SAG 50-1) was grown autotrophically in the presence of cerivastatin, like mevinolin a specific and efficient inhibitor of the HMG-CoA reductase (Bach and Lichtenthaler 1987), the key enzyme of the Ac/MVA pathway. At a concentration of 10^{-6} M and higher, cerivastatin had a significant inhibition effect on the growth of *Mesostigma viride*, as seen by the inhibition of chlorophyll and carotenoid accumulation (Table 3). This demonstrates that growth and multiplication of *M. viride* is dependent on a functional Ac/MVA pathway, which cannot be compensated by the DOXP/MEP pathway.

Discussion

The algal species investigated in this study belong to different taxa within the green algae. Based on the ultrastructural traits of motile cells and of cytokinesis, as well as on gene sequence data, there is general agreement today that green plants are separated into two distinct evolutionary lineages (Friedl 1997; Bhattacharya and Medlin 1998; Chapman et al. 1998). These are (i) the Chlorophyta, including the Chlorophyceae, the Trebouxiophyceae, the Ulvophyceae and a part of the Prasinophyceae, and (ii) the Streptophyta, including the land plants and the Charophyceae (Klebsormidiales, Zygnematales, Charales, Fig. 3).

For the Streptophyta species *Klebsormidium flaccidum* and *Spirogyra* sp., as well as for the prasinophyte *Mesostigma viride* (strains NIES-296 and SAG 50-1), we found that sterols are formed via the Ac/MVA pathway (with a considerable contribution from the DOXP/MEP pathway), whereas the plastidic phytol-chain of chlorophylls was formed via the DOXP/MEP pathway. These green algae obviously use the Ac/MVA pathway for the sterol biosynthesis in the cytosolic compartment, whereas the DOXP/MEP pathway is utilized for the synthesis of plastidic isoprenoids such as phytol. This IPP biosynthesis pattern had also been found before in higher plants like *Lemna gibba* (Fig. 1; Lichtenthaler et al. 1997a,b; Schwender et al. 1997) and can therefore be attributed to the entire Streptophyta lineage (Fig. 3).

The Prasinophyceae, comprising primitive green flagellates, are regarded as the earliest offshoots of green plants. Although they are polyphyletic, the taxon "Prasinophyceae" is still in use by most authors (Friedl 1997; Bhattacharya and Medlin 1998; Bhattacharya et al. 1998; Chapman et al. 1998; Lemieux et al. 2000). The prasinophyte *Mesostigma viride* has a special position within the pedigree shown in Fig. 3. While earlier it had been grouped into the Streptophyta lineage (Bhattacharya et al. 1998; Marin and Melkonian 1999), strong evidence has recently emerged that *M. viride* is an "early offshoot", branching from the base of the green-plant pedigree (Lemieux et al. 2000, see Fig. 3). Our labeling experiments with both strains of *M. viride,* and also the inhibition results obtained with cerivastatin, demonstrate that *M. viride* uses both pathways of IPP formation in the manner shown for the Streptophyta. From this, it follows that the ancestors of all green plants must have used in the cytosol the MVA pathway and in the plastid the DOXP/MEP pathway of IPP formation (Fig. 3). This was to be expected, since the isoprenoid biosynthesis pattern of higher plants also exists in other eukaryotic photosynthetic organisms such as red algae, Chrysophytes (Schwender et al. 1997; Disch et al. 1998a) and diatoms (Cvejic and Rohmer 2000).

Table 3. Inhibitory effect of cerivastatin on the growth of *Mesostigma viride* (SAG 50-1) determined here as an inhibition of the accumulation of chlorophylls and total carotenoids (μg per ml culture suspension). The initial values for chlorophylls and carotenoids of the suspensions were 1.79 and 0.5 μg/ml, respectively. Cells were grown for 5 d autotrophically. Data represent the mean of six determinations (±SD) from six separate cultivations. Significantly lower levels than in control (*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$)

Condition	Chlorophyll (μg/ml)	Inhibition (%)	Carotenoids (μg/ml)	Inhibition (%)
Control	8.12 ± 0.38	0	2.62 ± 0.15	0
10^{-6} M Cerivastatin	7.50 ± 0.33**	10	2.45 ± 0.15*	8
10^{-5} M Cerivastatin	6.46 ± 0.30***	26	2.16 ± 0.21***	22
10^{-4} M Cerivastatin	2.57 ± 0.22***	88	0.84 ± 0.07***	84

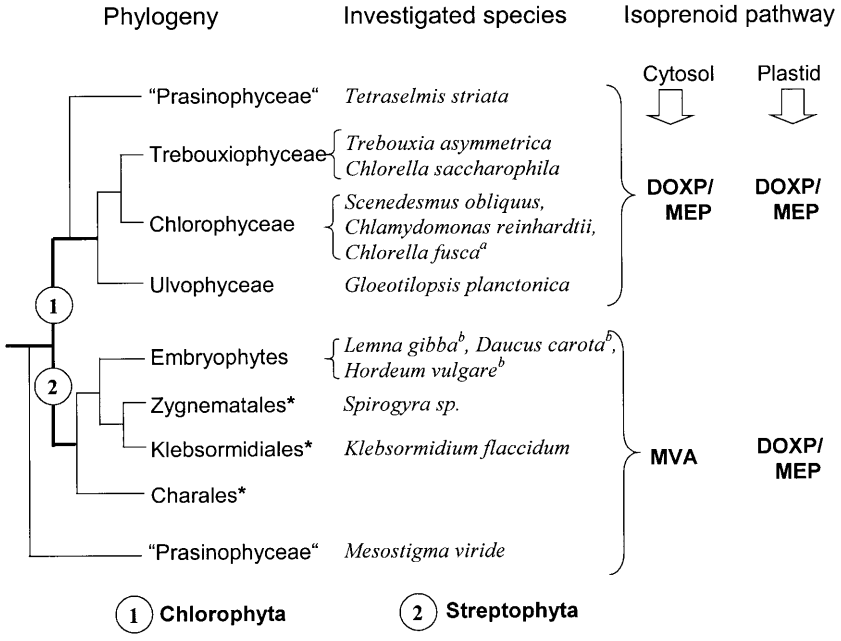

Fig. 3. Distribution of the DOXP/MEP pathway and the Ac/MVA pathway of IPP biosynthesis in Chlorophyta (*1*) and Streptophyta, including higher plants (*2*). The distribution correlates with the current phylogeny of green algae and higher plants (Friedl 1997) based on 18S-rRNA sequences, ultrastructural characteristics of flagellate cells and of cell mitosis. That the "Prasinophyceae" are not a monophyletic group (Friedl 1997) is underlined by the occurrence of the DOXP/MEP and Ac/MVA pathways in one member (*M. viride*), whereas the other one (*T. striata*) only contains the DOXP/MEP pathway. *, These clades of green algae are summed up by most of the recent authors as "Charophyceae", but they are not monophyletic. [a,b] IPP and isoprenoid biosynthesis of these organisms had been studied before: [a]Disch et al. 1998a; [b]Lichtenthaler et al. 1997b

In contrast to the Streptophyta species and to *M. viride*, none of the seven Chlorophyta species investigated used MVA as an isoprenoid precursor, whereas DOX was incorporated into both sterols and phytol to the same labeling degree. This particularly applies to different classes of the Chlorophyta – to Chlorophyceae (*Scenedesmus obliquus*, *Chlamydomonas reinhardtii*), to Ulvophyceae (*Gloeotilopsis planctonica*), to Trebouxiophyceae (*Trebouxia asymmetrica*, *Chlorella saccharophila*) as well as to the Prasinophyte *Tetraselmis striata* (Fig. 3) being placed at the basis of the Chlorophyta pedigree (Friedl 1997). Thus the lack of incorporation of MVA into sterols and into phytol can be attributed to the Chlorophyta in general. In addition to the investigation of phytol and the major sterol components of this study, it has been shown before that in Chlorophyta the DOXP/MEP pathway is used for the biosynthesis of β-carotene (*Scenedesmus obliquus*, *Chlamydomonas reinhardtii*, *Chlorella fusca*), lutein and the isoprenoid side chains of plastquinone-9 and ubiquinone (*S. obliquus*) (Schwender et al. 1996; Disch et al. 1998a). This strongly suggests, that in all Chlorophyta species exclusively the DOXP/MEP pathway is used for the biosynthesis of the different cellular isoprenoid components.

From isotopic incorporation experiments alone one cannot exclude a very minimal contribution of the Ac/MVA pathway to the biosynthesis of any isoprenoid in Chlorophyta species. Thus, in an incorporation experiment with *Chlorella saccharophila*, using [2-^{14}C]DOX and [5-^{3}H]MVA, we detected a low tritium activity in phytol and the sterols, which amounted to about 3–5% of the incorporated radioactivity of [2-^{14}C]DOX. Since in the same experiment we also found very low amounts of ^{3}H activity in the glycerolipids, this suggests an unspecific incorporation of [^{3}H]MVL into lipids and, as a consequence, that [^{3}H]MVA does *not* serve as a direct precursor of IPP in *C. saccharophila* (via MVA 5-phosphate and MVA 5-pyrophosphate). The question of whether the Ac/MVA pathway is totally absent in the Chlorophyta can be addressed as follows: If the Ac/MVA pathway of IPP formation were present in Chlorophyta, making only a minimal contribution to isoprenoid biosynthesis, it would be reasonable to assume that the degree of this contribution is species-dependent. However, in none of the seven Chlorophyta species investigated here could we detect a significant incorporation of ^{13}C-labeled MVL into isoprenoids. This observation is in favour of the total absence of the Ac/MVA pathway in the Chlorophyta. Furthermore, in colourless green algae, one could expect that the overall metabolic activity of plastids is reduced and that the cytosolic Ac/MVA pathway, if it really existed, should be more active than in their green counterparts. In contrast to this assumption, it was recently shown, that in the pigment-less green alga *Prototheca wickerhamii* (Chlorophyta, Trebouxiophyceae) the main sterol component ergosterol is formed via the DOXP/MEP pathway (Zhou and Nes 2000). The apparent absence of the Ac/MVA pathway in the Chlorophyta is further supported by the following facts: With mevinolin and cerivastatin, highly specific inhibitors of HMG-CoA-reductase (Bach and Lichtenthaler 1987), we could not find inhibitory effects on the growth of *Scenedesmus obliquus* (mevinolin, Schwender et al. 1996) or *Chlamydomonas reinhardtii* (cerivastatin, data not shown). Also, our attempts to detect gene sequences that are homologous to HMG-CoA reductase failed in several Chlorophyta species such as *Chlamydomonas*.

The phenomenon of the apparent loss of the cytosolic IPP pathway in the Chlorophyta parallels the loss of the cytosolic enzyme activities of sugar-phosphate metabolism (Schnarrenberger et al. 1990). In *Chlamydomonas reinhardtii*, *Dunaliella bioculata* (Chlorophyceae) and in

Acetabularia mediterranea (Ulvophyceae), only plastidic enzyme activities of fructose 1,6-bisphosphate aldolase and glucose 6-phosphate isomerase were detected (Schnarrenberger et al. 1990). In addition, in *Chlamydomonas* there appears to be only one gene for aldolase, which codes for a protein with high sequence homology to plastidic aldolases of higher plants and with a putative plastid transit peptide (Schnarrenberger et al. 1994). Thus, in addition to the loss of the Ac/MVA pathway in Chlorophyta, a reduction or even a total loss of cytosolic enzyme activities of sugar phosphate metabolism can be stated. In contrast, members of the Charophyta (*Chara foetida*, *Klebsormidium flaccidum*), as well as higher plants, contain plastidic and cytosolic isoforms of fructose 1,6-bisphosphate aldolase and glucose 6-phosphate isomerase (Schnarrenberger et al. 1990) and, in addition, they also possess the cytosolic Ac/MVA pathway. Thus, the Chlorophyta seem to have lost several cytosolic pathways.

In *Chlamydomonas* we found only one gene for a DOXP-synthase that is obviously targeted via a transit peptide into the plastid (Schwender 1999). This observation excludes the theoretical possibility that a second DOXP/MEP pathway might operate in the cytosol.

In summary, there is ample evidence that the Chlorophyta in general do not possess the Ac/MVA pathway. Like other photosynthetic eukaryotes, the flagellate ancestors of the Chlorophyta and Streptophyta exhibited the cytosolic Ac/MVA pathway and in parallel the plastidic DOXP/MEP pathway. Since today the Ac/MVA pathway cannot be found in any of the representatives of the Chlorophyta, the pathway was apparently lost early in the evolution of this green alga group, in the context of the separation of the Chlorophyta lineage from the Streptophyta lineage.

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