Cellular Localization of Isoprenoid Biosynthetic Enzymes in *Marchantia polymorpha*. Uncovering a New Role of Oil Bodies

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Like seed plants, liverworts synthesize and accumulate a myriad of isoprenoid compounds. Using antibodies raised against several isoprenoid biosynthetic enzymes, we investigated their intracellular compartmentation by in situ immunolocalization from *Marchantia polymorpha*. The enzymes examined were deoxy-xylulose phosphate synthase, geranyl diphosphate synthase, farnesyl diphosphate synthase, geranylgeranyl diphosphate synthase, monoterpene synthase, geranylgeranyl diphosphate reductase, phytoene synthase, and phytoene desaturase. Our results show that liverwort oil bodies, which are organelles bound by a single unit membrane, possess isoprenoid biosynthetic enzymes similar to those found in plastids and the cytosol. We postulate that oil bodies play a dynamic role in cell metabolism in addition to their role as sites of essential oil accumulation and sequestration. The occurrence of such enzymes in different cellular compartments might be due to multiple targeting of gene products to various organelles.

Among the Bryophytes, only the liverworts (Hepatics) contain oil bodies (Schuster, 1966) and synthesize large quantities of essential oils. A link between these two features has long been recognized (Lohmann, 1903) and chemical analyses of leafy species belonging to the genus *Calypogeia*, which contain blue-colored oil bodies, indicated that the blue color was due to the extensive accumulation of blue azulene derivatives (Meuche and Huneck, 1966; Takeda and Katoh, 1981; Siegel et al., 1992). More recently, it has been shown by micromanipulation that 3-methoxy-bibenzyl is the main constituent of *Radula complanata* oil bodies (Flegel and Becker, 2000). Further investigations on hundreds of liverwort species showed that these plants contained highly diversified mixtures of terpenoids and/or aromatic constituents (Asakawa, 1995) that most probably accumulated in the oil bodies.

Liverwort oil bodies are intracytoplasmic secretory structures bound by single membranes that originate from the dilatation of endoplasmic reticulum cisternae (Suire, 1970; Duckett and Ligrone, 1995). They have no subcellular equivalent in spermaphytes and are unlike plant seed oil bodies that accumulate mostly acyl lipids surrounded by a monolayer of phospholipids containing basic proteins, oleosins (Tzen and Huang, 1992).

It has been suggested that part of the proteins disintegrated within oil bodies by pronase could be enzymes involved in the biosynthesis of essential oil components (Suire, 1976). In vivo labeling using ^{2}H and ^{13}C precursors (Takeda and Katoh, 1983; Nabeta et al., 1994, 1995b, 1995c; Tazaki et al., 1995; Adam et al., 1998) suggested that isoprenoid biosynthetic pathways in liverworts were similar to those of seed plants (Asakawa, 1995). At the enzyme level, limonene synthase (Adam et al., 1996), bornyl pyrophosphate synthase, and sabinene synthase (Adam and Croteau, 1998) from liverworts displayed similar physical and kinetic characteristics to those of seed plants. However, sites of intracellular isoprenoid synthesis in liverworts have not been identified. For instance, the diterpenoids heteroscyphic acid A (Nabeta et al., 1995a) and phytol (Nabeta et al., 1995b, 1998), as well as β-carotene (Nabeta et al., 1997), are apparently formed by the mevalonic and nonmevalonic pathways of isopentenyl diphosphate synthesis in the leafy liverwort *Heteroscyphus planus*. The isolation and purification of intact liverwort oil bodies is difficult and precludes direct analysis of their enzyme components. To circumvent this difficulty, we have used cyto-immunological approaches based on antibodies available for key isoprenoid enzymes. The immunolocalization of these enzymes from *Marchantia polymorpha* indicated the occurrence in oil bodies of immunoreactive material related to plastid and cytosolic enzymes of isoprenoid biosynthesis in seed plants. Our data suggest that liverwort oil bodies define a new metabolically reactive compartment functionally equivalent to hydrophobic secretory structures of spermaphytes.

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RESULTS AND DISCUSSION

Morphology and Structure of the Secretory System in *M. polymorpha*

M. polymorpha can reproduce asexually from the gemmae growing inside cups that differentiate at the upper side of the thallus (Fig. 1). Oil bodies (oil cells) are restricted to idioblasts that are scattered among vegetative cells of the thallus (Fig. 1). At the electron microscope level, differentiated oil body appears as a central organelle surrounded by an oil body envelope membrane sequestering essential oil globules (Fig. 2A). These globules react with osmium tetroxide giving deposits that artifactually appear as a membrane-like structure (Fig. 2A). Based on earlier studies (Schuster, 1966), it has been assumed and frequently reported that oil cells are devoid of plastids. In contrast to this generally held view, one could note that oil cells of the Marchantiales and especially those of *M. polymorpha* contain plastids (Fig. 2). However, the inner thylakoid membranes of oil cell plastids are usually less developed than those observed in vegetative cells (Fig. 2B). This characteristic coupled with their high starch content suggest that oil cell plastids are metabolically equivalent to chloro-amyloplasts.

Low-M_r Isoprenoids from *M. polymorpha*

The crude ether extract from air-dried liverwort thalli was analyzed by gas chromatography-mass spectrometry (GC-MS) and isoprenoid compounds were identified by comparison of their retention time and mass spectra with published reference spectra (Joulain and König, 1998). As shown by the GC analysis, no monoterpenoid was detected (Fig. 3A). The low-M_r isoprenoid fraction contained mainly sesquiterpene hydrocarbons (peaks 1–7) corresponding to thujopsene, acoradiene, β-chamigrene, cuparene, β-himachalene, γ-cuprenene, and α-chamigren-9-one (Fig. 3B), in addition to unidentified sesquiterpene alcohols and phytol (Fig. 3A). Although monoterpenes were not detected under our experimental conditions this does not preclude the presence of monoterpene biosynthetic genes and enzymes in *M. polymorpha*.

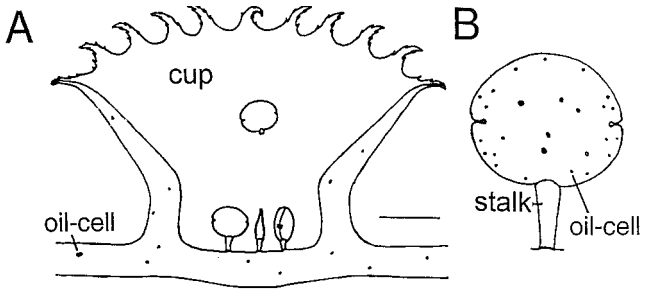

Figure 1. Schematic cross section of the thallus of *M. polymorpha* at the level of a gemma cup. A, Several gemmae are shown inside the cup. Idioblasts (oil cell) containing oil bodies are scattered among vegetative cells. B, Free gemma separated from the thallus.

Compartmentation of Isoprenoid Biosynthetic Enzymes in *M. polymorpha*

Before in situ immuno-analysis for the compartmentation of individual enzymes, we verified by western blotting that deoxy-xylulose phosphate synthase (DXPS), geranyl diphosphate synthase (GPPS), farnesyl diphosphate synthase (FPPS), and geranylgeranyl diphosphate synthase (GGPPS), as well as geranylgeranyl diphosphate reductase (GGPPR), phytoene synthase (PS), and phytoene desaturase (PD) cross-reacted with putative liverwort homologs in total protein extracts from *M. polymorpha* gemmae (Fig. 4). Following this initial observation, tissue sections were probed with the different antibodies. Due to the prominent volume of the oil body and the scarcity of oil cell plastids, we exclusively compared the immunoreactivity of the oil body to that observed in vegetative cell chloroplasts. Anti-GPPS gave a typical green fluorescein isothiocyanate (FITC) labeling of chloroplasts from vegetative cells and oil bodies from oil cells (Fig. 5A). The corresponding control section (Fig. 5B) showed only a faint yellowish background of fluorescence due to the use of glutaraldehyde for tissue fixation. Anti-FPPS labeled only oil bodies and not chloroplasts and barely the cytosol, as usual, due to the limitations of the technique used (Fig. 5C). For anti-GGPPS we noted a labeling pattern (Fig. 5D) similar to that of anti-GPPS (Fig. 4A), except that oil bodies were more strongly labeled than plastids. Although oil bodies are not known to synthesize carotenoids, we noted that antibodies directed against the two carotenogenic enzymes PS and PD gave a strong fluorescence labeling with oil bodies and plastids (Fig. 5, E and G).

Following this observation, the sections were probed with antibodies to LSU and only plastids were strongly labeled in contrast to the oil bodies (Fig. 5H). This behavior argues for the specificity of our immunolocalization analysis and is further confirmed using antibodies against DXPS, one of the first enzymes of the non-mevalonic pathway of isoprenoid biosynthesis, which gave an exclusive and strong labeling with chloroplasts and no reaction with oil bodies (Fig. 5I), according to a pattern similar to that of anti-Rubisco antibodies. Antibodies against monoterpene synthase and GGPPR conversely strongly labeled oil bodies and chloroplasts (Fig. 5, J and K). The selective and strong labeling of the oil body by monoterpene synthase antibodies was observed in the absence of monoterpene accumulation as verified by GC-MS analysis. Further analysis of the FITC fluorescence of oil bodies reveals that the labeling of antibodies raised against the different prenyltransferases (GPPS, FPPS, and GGPPS) was found preferentially peripheral (Fig. 5, A, C, and D), but the

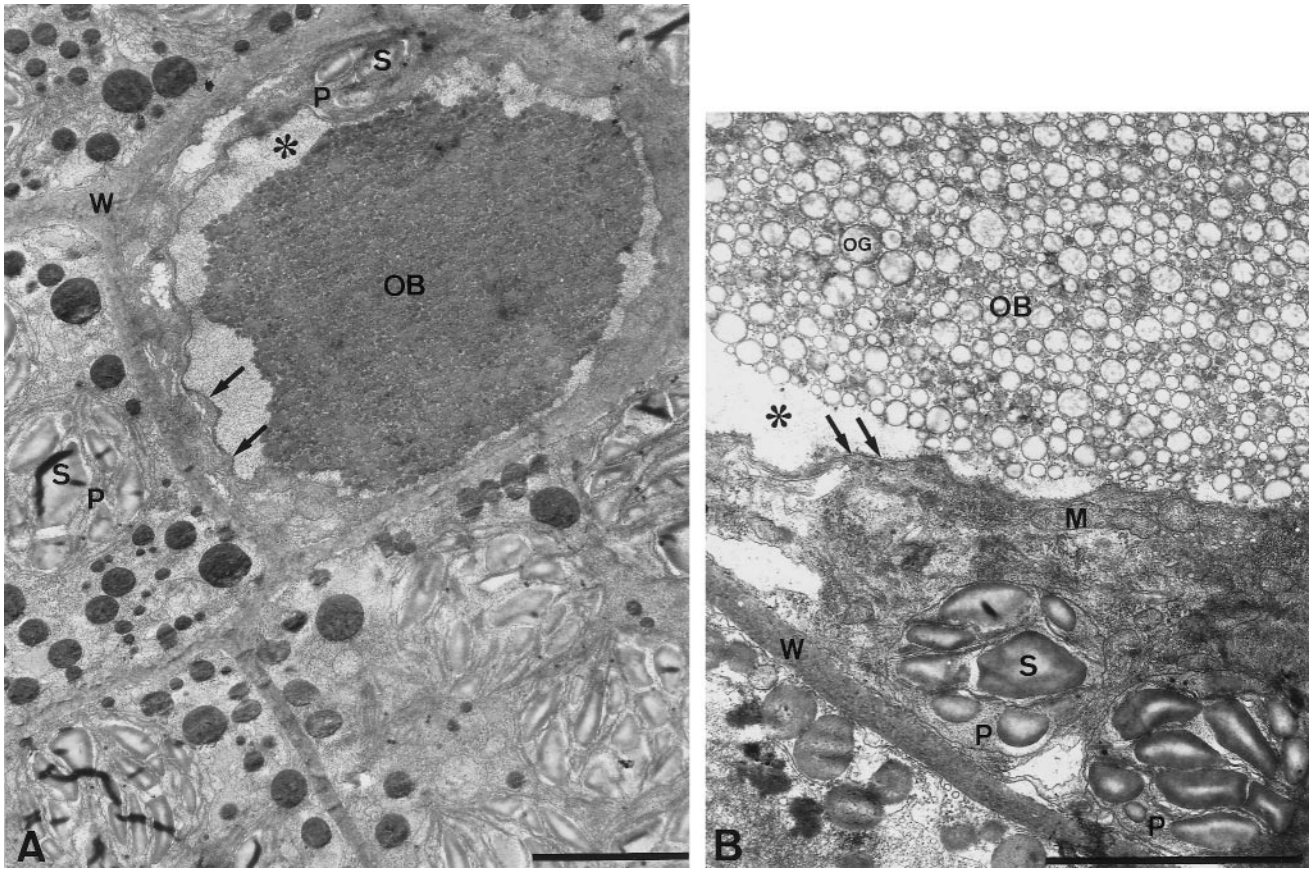

Figure 2. Fine structure of the two cell types in the gemma. A, The gemma is composed of vegetative and oil cells according to a ratio of approximately 150:1. Vegetative cells are devoid of oil bodies and display plastids accumulating starch. The oil cell possesses a large oil body surrounded by the oil body envelope membrane. The oil body content shrinks during the fixation and an artifactual space appears between the oil body envelope membrane and the oil body stroma. Note the occurrence of plastid containing starch in the oil cell. B, Detailed structure of an oil cell. The oil body contains numerous essential oil globules. Note the presence of two plastids and mitochondria between the oil body and the cell wall. w, Cell wall; m, mitochondrion; p, plastid; ob, oil body; og, oil globules; s, starch; and the artifactual space between the oil body matrix and the oil body envelope membrane (arrows) is indicated by an asterisk. Bar = 5 μm.

labeling of antibodies against PS, PD, and monoterpene synthase was more uniform (Fig. 5, E, G, and K). This finding would suggest that the prenyltransferases and the later enzymes of isoprenoid are partitioned respectively in the two sub-compartments, i.e. the oil body envelope membrane and the oil body envelope membrane plus the oil body matrix. These observations, coupled with the negative reactions to different control sera, indicate that the fluorescence labeling is not due to a non-specific binding and define the liverwort oil body as an oil cell compartment whose protein equipment shares antigenic determinants with plastid and cytosolic enzymes of isoprenoid biosynthesis (Fig. 6).

Relationships between Compartmentation and Genetic Sorting of Enzymes

The proposed subcellular compartmentation (Fig. 6), based on the data described above, raises the question of how different enzymes might be directed to the oil body. Isolation of oil bodies would be useful to identify their enzyme equipment, but this does not appear to be a readily accessible experiment. The technical difficulty is linked to the fact that a tiny amount of material can be expected for an oil body fraction from the thalli because there is approximatively only one oil cell per 150 oil-body-free vegetative cells in *M. polymorpha.*

The appearance of identical gene products in different cellular compartments can be explained by a multitargeting process using different transcription starts. This explanation has been demonstrated in Arabidopsis where the same alanyl-tRNA synthetase is targeted to the cytosol and the mitochondria (Mireau et al., 1996) and for histidyl-tRNA synthetase, which is targeted to the chloroplasts and the mitochondria (Akashi et al., 1998). In watermelon, the same 70-kD heat shock protein similarly is targeted to plastids and glyoxysomes (Wimmer et al., 1997). Concerning isoprenoid metabolism, FPPS in Arabidopsis is targeted to both the cytosol and the mito-

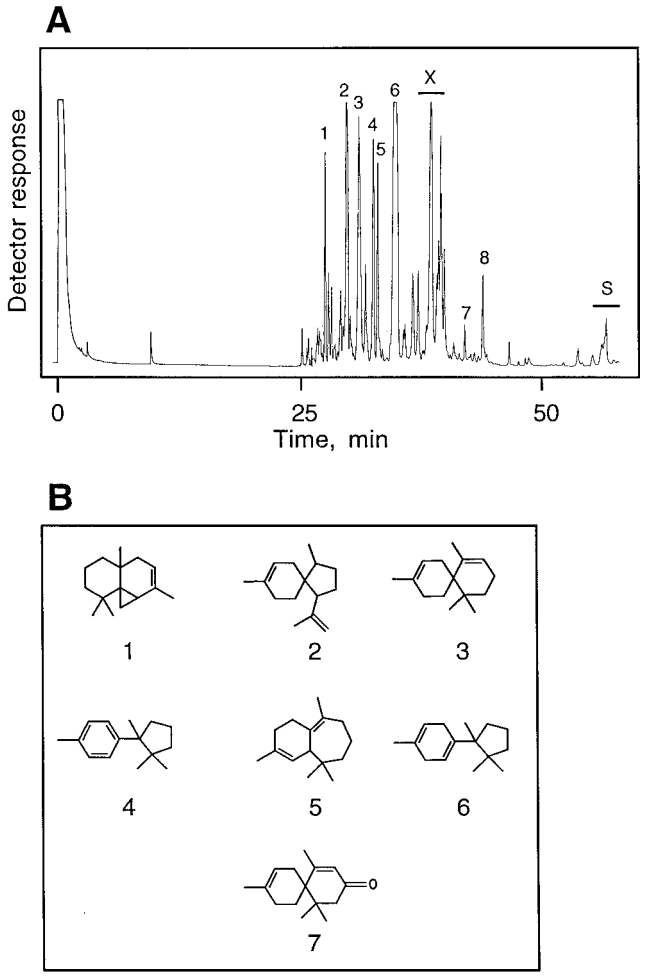

Figure 3. GC and identification of isoprenoid compounds from *M. polymorpha* extract. A, GC profile of *M. polymorpha* lipid extract. B, Structures of characterized isoprenoid compounds. 1, Thujopsene; 2, acoradiene; 3, β-chamigrene; 4, cuparene; 5, β-himachalene; 6, γ-cuprenene; 7, α-chamigren-9-one; 8, phytol; X, unidentified sesquiterpene alcohols; and S, mixture of campesterol, stigmasterol, and sitosterol. oct-1-en-3-yl acetate was used as an internal standard.

chondria (Cunillera et al., 1997). Also, at least two genes encoding putative monoterpene synthases have been released from the large sequencing program of Arabidopsis (accession nos. AAD03382 and CAB10448). The deduced peptide sequences of these genes display the characteristic twin Arg motif of other monoterpene synthases (Williams et al., 1998). Using the ChloroP program (Emanuelsson et al., 1999), one could note that the peptide sequence deduced from AAD03382 has a putative N-terminal transit peptide for targeting to chloroplasts, but that deduced from CAB10448 is apparently devoid of this extension and thus is likely directed to a different compartment.

In conclusion, our data indicate that in addition to their sequestery role for in situ-formed isoprenoids, oil bodies contain protein equipment immunologically related to plastid and cytosolic enzymes of isoprenoid synthesis. These data suggest that liverwort oil bodies define a new metabolic compartment functionally equivalent to spermaphyte secretory structures involved in the synthesis and accumulation of essential oil constituents.

MATERIALS AND METHODS

Plant Materials

Marchantia polymorpha thalli with gemma cups were collected in the greenhouses of the Station de Recherches Forestières (Institut National de la Recherche Agronomique, Pierroton, France).

Preparation of Antibodies

The antibodies were raised in rabbits, either from enzymes purified to homogeneity or from a synthetic peptide. Polyclonal antibodies to ribulose 1,5-bisphosphate carboxylase were obtained using the ribulose 1,5-bisphosphate carboxylase holoenzyme or its LSU isolated from tobacco leaves (Suire et al., 1988). Several antibodies were raised against pepper fruit isoprenoid biosynthetic enzymes including GGPPS (Dogbo and Camara, 1987), FPPS (Hugueney and Camara, 1990), PS (Dogbo et al., 1988), PD (Hugueney et al., 1992), and DXPS (Bouvier et al., 1998). Antibodies against GGPPR antibodies were raised against recombinant Arabidopsis GGPPR (Keller et al., 1998). GPPS antibodies were obtained from previous work (Sommer et al., 1995). Antibodies against monoterpene synthases synthase were obtained from the 14-amino acid peptide DDIYDVYGTLEELE corresponding to the highly conserved sequence of purified and putative monoterpene cyclases (Colby et al., 1993; Yuba et al., 1996; Bohlmann et al., 1997; Bevan et al., 1998). The peptide was coupled with the carrier-activated protein, keyhole limpet hemocyanin, before injection. Immune rabbit sera were desalted on Trisacryl GF-O5 (Sepracor, Villeneuve-la-Garenne, France) and IgG were separated on DEAE-Trisacryl M (Corthier et al., 1984). The same method was used to purify preimmune sera.

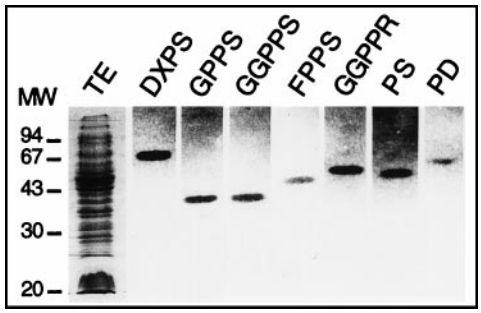

Figure 4. SDS-PAGE and immunoblot analysis of isoprenoid biosynthetic enzymes. From left to right, Coomassie Blue-stained gel of proteins isolated from *M. polymorpha* thalli separated by SDS-PAGE and subjected to immunoblot analysis using different antibodies raised against different isoprenoid biosynthetic enzymes as shown. In each case 50 μg of protein was used and the antibodies were used at 1:5,000 dilution.

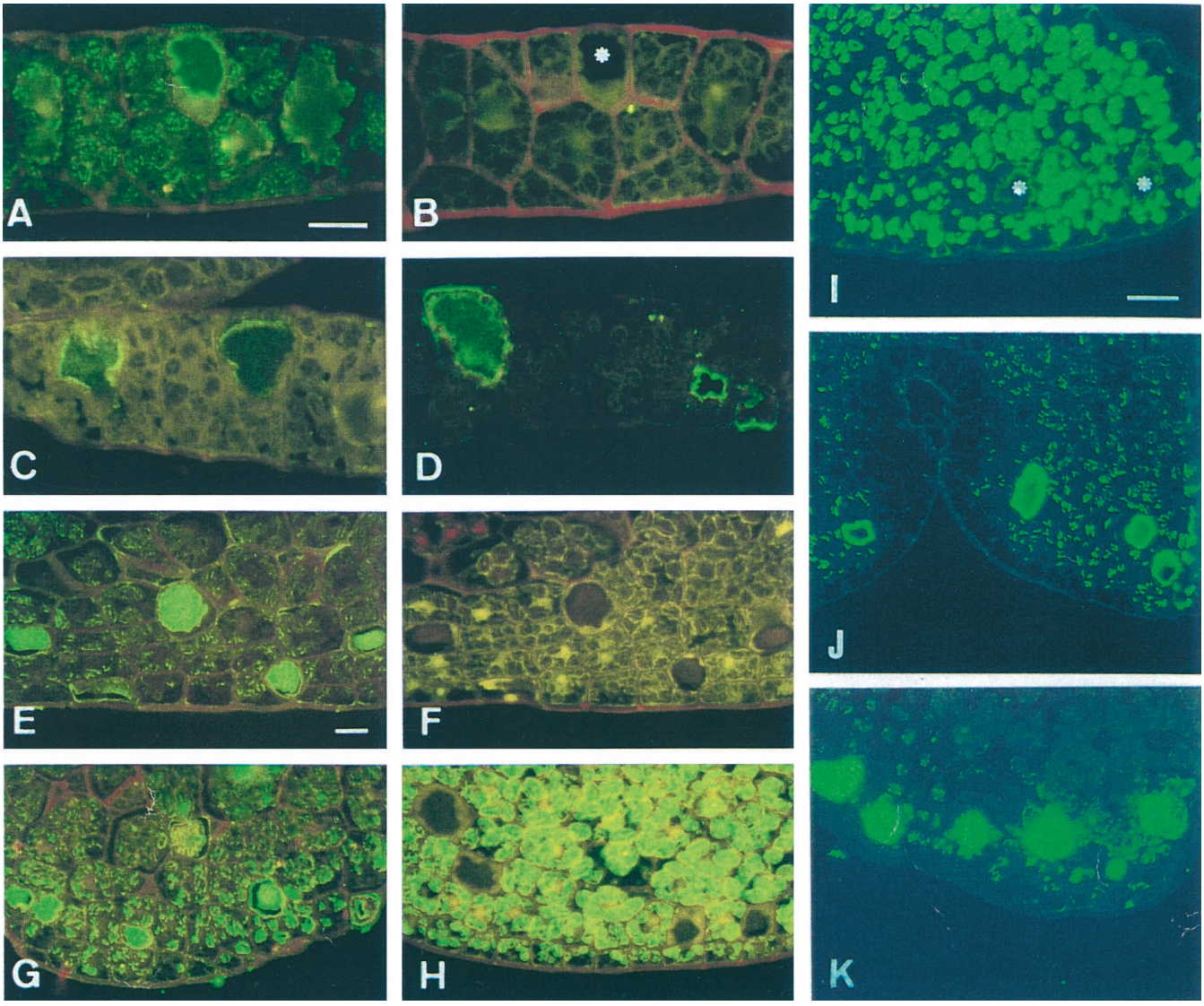

Figure 5. Localization of isoprenoid biosynthetic enzymes and Rubisco in *M. polymorpha* gemmae by FITC-immunofluorescence. A, Cross-section treated with anti-GPPS antibodies. Note the strong labeling of plastidial grana and the oil body. B, Control section corresponding to A. The nucleus and the cytosol display a faint yellowish fluorescence due to glutaraldehyde, the dark oil body is indicated by an asterisk, and cell walls are counterstained into red. C, Test with anti-FPPS antibodies. Note that oil bodies are labeled in contrast to plastids. D, Cross-section treated with anti-GGPPS antibodies. Oil bodies are labeled as in A. E, Longitudinal section probed with anti-PD antibodies showing the labeling of plastids in vegetative cells and oil bodies in idioblasts. F, Control section corresponding to E, devoid of any FITC labeling. G, Test with anti-PS indicating the labeling plastids and oil-bodies. H, Test with anti-large subunit of Rubisco (LSU) antibodies. Note that only plastids are labeled. I, Test with anti-DXPS antibodies showing the labeling of plastids. J, Test with anti-monoterpene synthase. Note that proplastids from the meristematic area (Ω-like dark belt) are unlabeled while chloroplasts of vegetative cells and oil-bodies are labeled. K, Section treated with anti-GGPPR showing the labeling of chloroplasts and oil-bodies. Sections A through H were counterstained with 0.2% (v/v) Evans blue and viewed using an epifluoresence microscope (Axiophot, Zeiss). Sections I through K were examined using an epifluoresence microscope (DMRXA, Leica Microsystems, Wetzlar, Germany). Sections have the same magnification. Bars = 10 μm.

Electron Microscopy and Cyto-Immunolocalization of Proteins

For structural studies, the gemmae were prefixed with 3% (v/v) glutaraldehyde for 30 min at 4°C in 0.1 M NaH_2PO_4-Na_2HPO_4 buffer (pH 7.2) containing 5% (w/v) Suc. Following washing in the same buffer devoid of Suc, the tissues were fixed with 2% (w/v) osmium tetroxide in the same buffer. Thin sections were contrasted successively with uranyl acetate and lead citrate (Suire, 1970). For immunochemistry, thin sections were fixed with 4% (w/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde in 0.1 M NaH_2PO_4-Na_2HPO_4 buffer (pH 7.2) for 4 h at 23°C. The fixed material was embedded in Epon and sections of

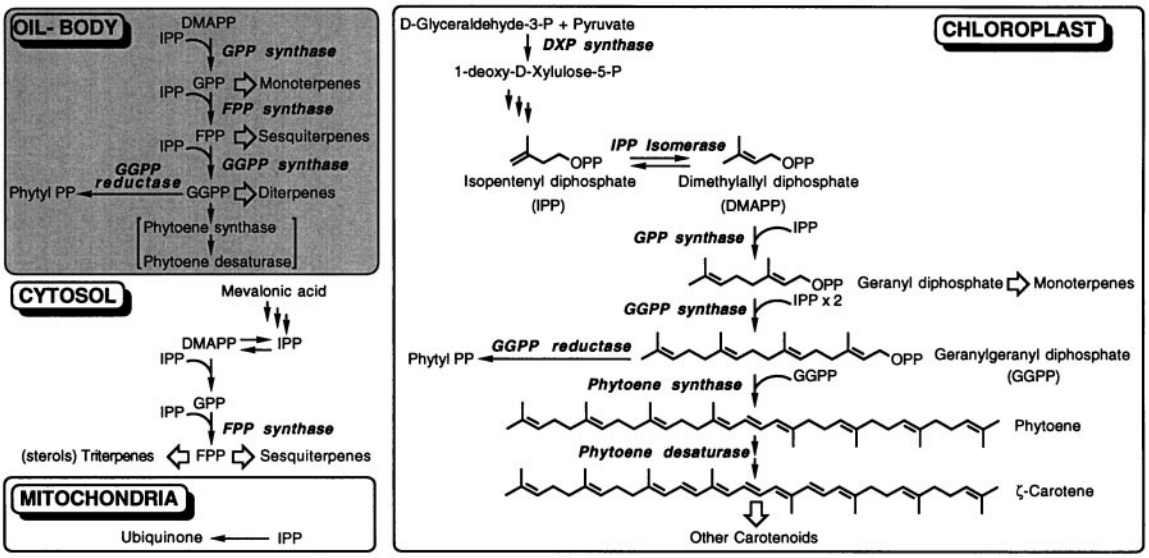

Figure 6. Pathway and subcellular compartmentation of isoprenoid biosynthesis in *M. polymorpha*. In addition to plastidial, cytosolic, endoplasmic reticulum-cytosol, and mitochondrial sites of isoprenoid biosynthesis, we propose that in *M. polymorpha* and in other liverworts, the oil body is a new cellular site of isoprenoid metabolism in addition to its known sequestering role.

approximately 1.5 μm were processed for indirect immunofluorescence (Camara, 1993; Suire, 1993). FITC-conjugated goat anti-rabbit IgG (Sanofi, Marnes-la-Coquette, France) was used as the secondary antibody. Nonimmune, heat-inactivated rabbit sera (R-14123, Bio-Cell, Helsinki, Finland) or purified preimmune rabbit sera were used for control sections. All sections were mounted in Citifluor (R-1320, Agar Aids, Pelco International, Redding, CA). Some were observed with a microscope (DM-RXA, Leica Microsystems) equipped with epifluorescence and fitted with an FITC set (excitation filter at 450–490 nm, dichroic mirror at 510 nm, and barrier filter at 515–560 nm). Pictures were recorded with a cooled Charge Coupled Device Micromax camera (Princeton Instruments, Trenton, NJ), processed by the Metamorph II program (Universal Imaging, West Chester, PA), and printed on a UP-D8800 (Sony, Tokyo) printer using the Corel Xara 2 program (Corel, Ottawa). Other sections were counterstained with Evans blue (3169, Merck, Rahway, NJ) before mounting and observed with an epifluorescence microscope (Axiophot, Zeiss, Jena, Germany) fitted with the same FITC set, except with a less selective barrier filter (from 520 nm). Color slides were prepared on Agfachrome 100 RS+ (Photo Station, Paris) or Fujichrome Sensia 100 ISO film (Fuji Photo Film, Tokyo).

Chemical Analysis of Low-M_r Weight Isoprenoids

Fresh thalli were air dried, then extracted with diethyl ether as described previously (Asakawa, 1988). The resulting lipid extract was analyzed by capillary GC on a 25-m × 0. 25-mm i.d. fused silica column (Cp Sil 5 CB, Chrompack, Raritan, NJ). The gas chromatograph was operated under the following conditions: injector 230°C, detector 295°C, oven temperature programmed from 60°C to 300°C at 6°C min^{-1} and nitrogen as a carrier gas. GC-MS analysis was performed on-line with a VG AutoSpec-Q mass spectrometer (Fisons Instruments, Paris). Mass spectra were recorded at 70 eV using an ionizing current of 200 μA and a source temperature of 200°C. Isoprenoid compounds were identified by comparison of their mass spectra with previously published reference spectra (Joulain and König, 1998).

Other Techniques

Total proteins were extracted from fresh thalli using the hot phenol procedure (Van Etten et al., 1979) and quantified (Smith et al., 1985) before SDS-PAGE and immunoblot analysis and visualization by chemiluminescence using enhanced chemiluminescence reagents (Amersham, Buckinghamshire, UK).

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