

Molecular cloning of geranyl diphosphate synthase and compartmentation of monoterpene synthesis in plant cells

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Summary

The nature of isoprenoids synthesized in plants is primarily determined by the specificity of prenyltransferases. Several of these enzymes have been characterized at the molecular level. The compartmentation and molecular regulation of geranyl diphosphate (GPP), the carbon skeleton that is the backbone of myriad monoterpene constituents involved in plant defence, allelopathic interactions and pollination, is poorly understood. We describe here the cloning and functional expression of a GPP synthase (GPPS) from *Arabidopsis thaliana*. Immunohistological analyses of diverse non-secretory and secretory plant tissues reveal that GPPS and its congeners, monoterpene synthase, deoxy-xylulose phosphate synthase and geranylgeranyl diphosphate synthase, are equally compartmentalized and distributed in non-green plastids as well in chloroplasts of photosynthetic cells. This argues that monoterpene synthesis is not solely restricted to specialized secretory structures but can also occur in photosynthetic parenchyma. These data provide new information as to how monoterpene biosynthesis is compartmentalized and induced *de novo* in response to biotic and abiotic stress in diverse plants.

Keywords: geranyl diphosphate synthase, monoterpene compartmentation, plastid, cytosol, secretory tissue.

Introduction

Plants have the capacity to produce diverse isoprenoids which form in plastids, mitochondria and the endoplasmic reticulum–cytosol of their cells. The types of isoprenoids synthesized depends on the carbon skeletons required in each compartment, and this is largely directed by the class of prenyltransferases localized within each organelle. For example, in plastids, where C₂₀ isoprenoids are required for carotenoid and prenyl lipid synthesis, geranylgeranyl diphosphate synthase (GGPPS) is the predominant prenyltransferase. In the endoplasmic reticulum–cytosol, where C₁₅ skeletons are needed for steroid synthesis, farnesyl diphosphate synthase (FPPS) predominates. Efforts at purifying these two enzymes (Dogbo and Camara, 1987; Hugueney and Camara, 1990; Laferrière and Beyer, 1991) resulted in the cloning and molecular characterization of these proteins (Hugueney *et al.*, 1996;

Kuntz *et al.*, 1992). Much less is known about geranyl diphosphate synthase (GPPS), the enzyme responsible for the C₁₀ skeletons of monoterpenes. Monoterpenes are diverse compounds that are crucial for numerous biological actions in plants, including defence against herbivores and pathogens, allelopathic interactions and pollination (Langenheim, 1994). Monoterpenoids are also among the most important constituents of flavourings and fragrances (Mulder-Krieger *et al.*, 1988) for human consumption, and can serve as therapeutic agents in human medicine (Gould, 1997).

The first evidence for the isolation of a *bona fide* GPPS was the partial purification of the enzyme from four plant sources, *Lithospermum* (Heide and Berger, 1989; Sommer *et al.*, 1995), sage (Croteau and Purkett, 1989), *Pelargonium* (Suga and Endo, 1991) and *Vitis vinifera* (Soler *et al.*, 1992).

Although a heterodimeric GPPS has been described from *Mentha trichomes* (Burke *et al.*, 1999), several studies suggest the possible existence of a homodimeric GPPS. For instance, a homodimeric FPPS from yeast in which Lys197 is mutated into Glu predominantly formed GPP in contrast to FPP, the natural product (Blanchard and Karst, 1993). In addition, the conversion of a *Bacillus stearothermophilus* homodimeric FPPS to GPPS has been recently demonstrated by limited site-directed mutagenesis of active amino acid residues (Narita and Ohnuma, 1999).

In this paper, we describe the cloning and functional characterization of a cDNA for a potentially homodimeric GPPS and its *in situ* localization in plants using GPPS-specific antibodies. We also show that GPPS occurs in chloroplasts of photosynthetically active parenchyma cells as well as in non-green plastids of secretory cells, which are widely recognized as sites of monoterpene synthesis in plants (Cheniclet and Carde, 1985; Gleizes *et al.*, 1983; Turner *et al.*, 1999). In chloroplasts, GPPS co-localizes with the congener enzymes of isoprenoid biosynthesis monoterpene synthase, deoxy-xylulose synthase and GGPPS. Collectively, these findings offer novel insights as to how isoprenoid biosynthesis is compartmentalized in plants and triggered *de novo* in response to diverse factors.

Results

Molecular cloning of GPPS

To identify and isolate the DNA for GPPS sequences, a PCR cloning strategy based on two amino acid motifs highly conserved between different prenyltransferases was used. A pair of primers (1S and 4R) described in Experimental procedures permitted amplification of a 0.3 kb cDNA fragment from *Arabidopsis thaliana* seedlings. The sequence of this fragment revealed that it represented a putative member of the prenyltransferase family. This probe was subsequently used to characterize a putative, full-length GPPS clone. The first ATG was preceded by a stop codon. The coding region of this cDNA is 1266 bp with a deduced protein of 422 amino acids (Figure 1). A BLAST sequence similarity search (Altschul *et al.*, 1990) revealed that GPPS has partial sequence identity to an *Arabidopsis* protein, devoid of the N-terminal extension, released after our sequence was submitted (accession number AAC26705) and to a partial GPPS from *Citrus* sp. (accession number AJ243739) (Figure 1). The N-terminus deduced from our cDNA has a characteristic transit peptide sequence (Von Heijne *et al.*, 1991) which was observed in a recently released partial EST clone from cotton (accession number AI726442) which displays significant homology to GPPS (Figure 1). Compared to other *Arabidopsis* prenyltransferases, GPPS shows 24.2% identity to FPPS (acces-

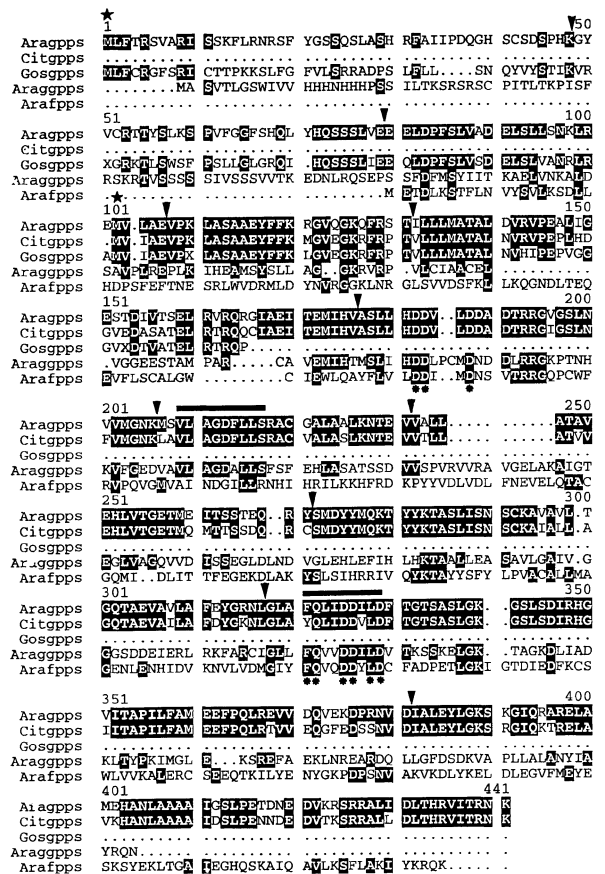


Figure 1. Deduced peptide sequence of GPPS compared to different prenyltransferases.

From top to bottom are shown: Aragpps (GPPS, accession number Y17376, from *Arabidopsis*); Citgpps (GPPS, accession number AJ243739, partial sequence of *Citrus* sp.); Gosgpps (accession number AI726442, partial cotton EST clone); Aragpps (GGPPS, accession number P34802, from *Arabidopsis*) and Arafpps (FPPS, accession number Q0152, from *Arabidopsis*). Identical amino acids are shaded in black and two characteristic prenyltransferase motifs are indicated by asterisks. Domains used for PCR primers are indicated by bold lines. Methionines 1 and 102 are indicated by stars and the positions of introns deduced from the genomic sequence (EMBL accession number AC004077.2) are indicated by arrowheads.

sion number Q0152) and 28.7% identity to GGPPS (accession number P34802).

Alternative generation of two GPPS

In the predicted sequence of the GPPS cDNA, two methionines, Met1 and Met102 (Figure 1), could serve as potential translation initiation sites. We reasoned that these might be clues to the compartmentalization of GPPS in plants. Comparison of GPPS with the EMBL database revealed a genomic sequence (EMBL accession number AC004077.2) in chromosome II of *A. thaliana*. Analysis of the genomic sequence revealed that the mRNA corresponding to GPPS cDNA has 11 exon sequences (indicated

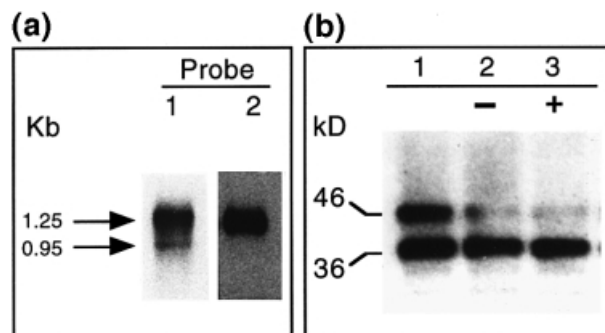


Figure 2. Northern blot analysis and import of ^{35}S -labelled GPPS into pepper leaf chloroplasts.

(a) Total RNA (20 μg) was hybridized with probe 1 (lane 1) corresponding to the internal sequence of GPPS cDNA and probe 2 (lane 2) corresponding to the extreme 5' sequence of GPPS cDNA. (b) *In vitro* transcription and plastid import were performed as described in Experimental procedures. An aliquot of the *in vitro* transcription-translation products (lane 1) was used for plastid import in the absence (lane 2) and the presence (lane 3) of thermolysin before SDS-PAGE analysis and fluorography.

by arrowheads, Figure 1) and the 5' region displayed two TATA elements upstream of the ATG corresponding to Met102 (data not shown) that might be functional in *Arabidopsis* (Mukumoto *et al.*, 1993) for *in vivo* transcription of a shorter GPPS isoform. Indeed, Northern blot analysis using the probe corresponding to the internal region of GPPS cDNA (probe 1) indicated hybridization to 1.26 and 0.95 kb transcripts corresponding to the two putative GPPS isoforms, while probe 2 (corresponding to the extreme 5' region of GPPS cDNA) hybridized to a 1.26 kb transcript only (Figure 2a). *In vitro* transcription-translation of GPPS using wheatgerm lysate yielded two polypeptides with molecular mass of 45 and 36–38 kDa (Figure 2b, lane 1) that might result from premature stop of the translation or initiation from the ATG corresponding to Met102. When the *in vitro* transcription-translation mixture was incubated with intact chloroplasts, only the higher-molecular-mass polypeptide was imported into the chloroplasts, as shown by protection against thermolysin (Figure 2b, lanes 2 and 3). Finally, immunoblot analysis using anti-GPPS synthase antibodies verified that *Arabidopsis* chloroplasts contained GPPS corresponding approximately to a polypeptide of 36–38 kDa (Figure 3), while the mitochondrial proteins did not cross-react with the antibodies (data not shown). The purity of the organelles was further verified by the absence of immunodetectable mitochondrial formate dehydrogenase in the chloroplast fraction in contrast to the mitochondrial fraction, which gave a 40 kDa reactive polypeptide (data not shown).

Functional characterization of GPPS

To evaluate whether authentic GPPS was being encoded, two methods were used. One exploited the fact that the

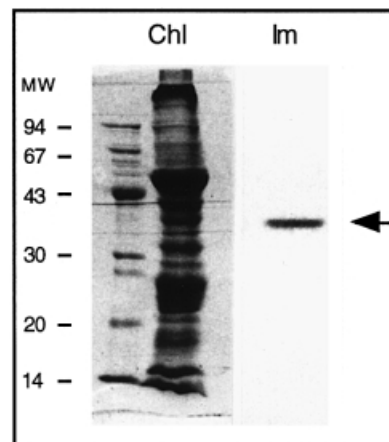


Figure 3. SDS-PAGE and immunoblot analysis of plastid GPPS. Lane 1, total chloroplast protein from *Arabidopsis* (Chl); lane 2, immunoblot analysis of chloroplast protein with anti-GPPS antibodies (Im).

terpenoid side chain length of ubiquinone in yeasts (Okada *et al.*, 1992) or *E. coli* (Okada *et al.*, 1997) is partially governed by the prenyl diphosphate substrate present. To test this, *E. coli* JM109 was transformed with our GPPS cDNA, and the ubiquinone content was analysed by reverse-phase HPLC using methanol:tetrahydrofuran (95:5 v/v) as eluting solvent. Lipid extracts showed that only ubiquinone-8 was formed in control and transformed *E. coli*, suggesting that the cloned prenyltransferase was not involved in high-molecular-weight prenyl diphosphate synthesis. In the other method, a GPPS devoid of the putative transit peptide sequence, as described in Experimental procedures, was expressed in *E. coli* using the pBAD-TOPO TA vector (Invitrogen). After arabinose induction and SDS-PAGE analysis of lysed bacterial cells, a prominent band for the recombinant protein having a molecular mass of 36 kDa was observed only in *E. coli* lysates harbouring GPPS cDNA (Figure 4a). To avoid interference with endogenous *E. coli* prenyltransferases, recombinant GPPS was immunoselected (Figure 4a) prior to incubation using an immunoaffinity Sepharose column coupled to anti-GPPS-specific antibodies as described in Experimental procedures. Following elution and incubation of the active fraction with radioactive isopentenyl diphosphate (IPP) and unlabelled dimethylallyl diphosphate (DMAPP), we noted after TLC and HPLC that DMAPP was accepted and exclusively elongated to GPP (Figure 4b,c). Further HPLC analysis of the reaction products using the same column eluted with methanol:cyclohexane:hexane (80:10:10 v/v) did not reveal any radioactivity in the peak corresponding to solanesol (retention time 7.5 min) or higher prenols. This trend was confirmed by the fact that GPP, FPP or GGPP were not accepted as allylic diphosphate substrates for prenyl condensation. In control experiments, we noted that the

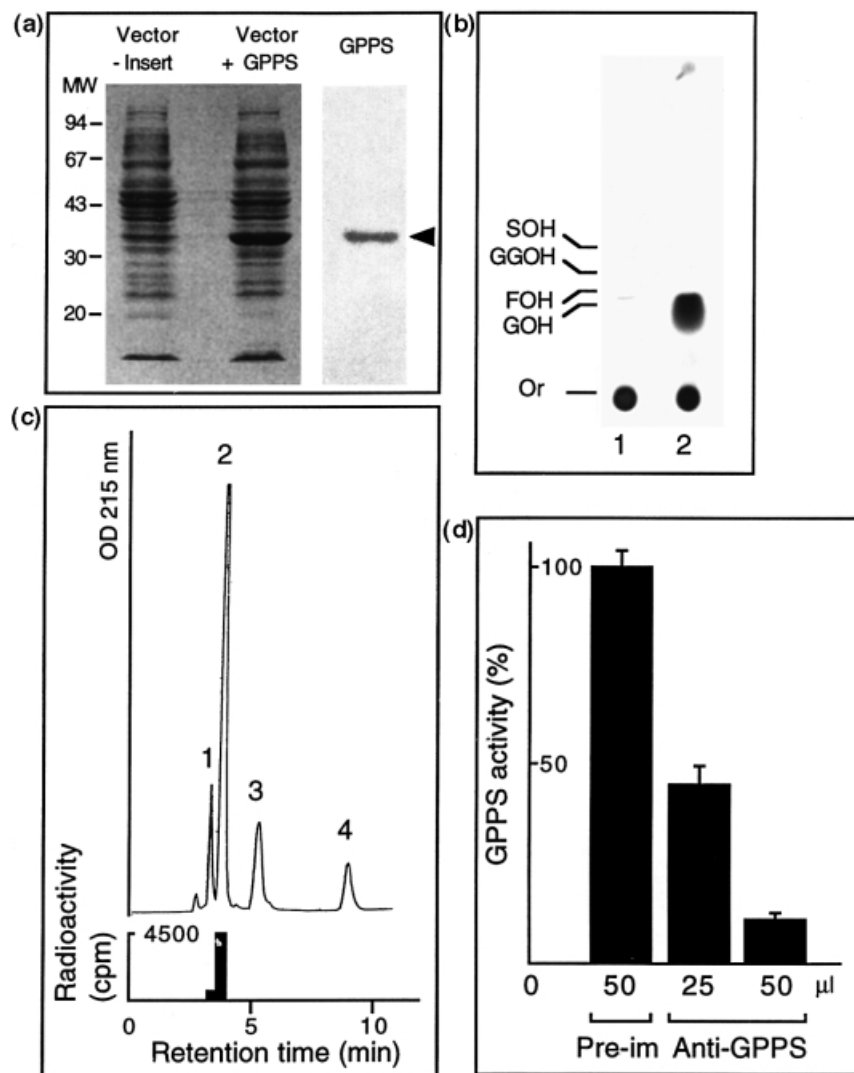


Figure 4. Expression and activity of recombinant GPPS.

(a) *E. coli* expression of GPPS. Lane 1: total proteins from *E. coli* devoid of GPPS cDNA. Lane 2: total proteins from *E. coli* expressing GPPS cDNA. The position of the recombinant protein is indicated. Lane 3: immunoselection of recombinant GPPS using the affinity column described in Experimental procedures. (b) TLC characterization of GPPS products. Following incubation with [^{14}C]IPP in the absence (lane 1) and the presence (lane 2) of DMAPP, the reaction products were dephosphorylated to yield free geraniol before thin-layer chromatography on silica gel plates developed with benzene:ethylacetate (90:10 v/v). Or and GOH refer to the origin and geraniol ($R_f = 0.3$), identified using authentic standards including geraniol, farnesol (FOH, $R_f = 0.35$), geranylgeraniol (GGOH, $R_f = 0.4$) and solanesol (SOH, $R_f = 0.57$). (c) HPLC characterization of GPPS products. Following incubation as in (b), the reaction products were dephosphorylated and subjected to reverse-phase HPLC analysis using methanol:water (90:10, v/v) for elution after addition of authentic standards including isopentenol + dimethylallyl alcohol (peak 1, co-eluting with the solvent peak), geraniol (peak 2), farnesol (peak 3) and geranylgeraniol (peak 4). The radioactivity of labelled reaction products was monitored by liquid scintillation counting. (d) Concentration dependence of GPPS inhibition by anti-GPPS. Following addition of the indicated volumes of anti-GPPS serum (anti-GPPS), the complex was precipitated using protein A-Sepharose beads followed by centrifugation at 12 000 g for 30 min before testing the activity for 30 min in the supernatant as indicated in (b) and (c). The values are expressed as a percentage of the control experiment which was treated with 50 μl of pre-immune serum (Pre-im). Each value is the mean of three experiments.

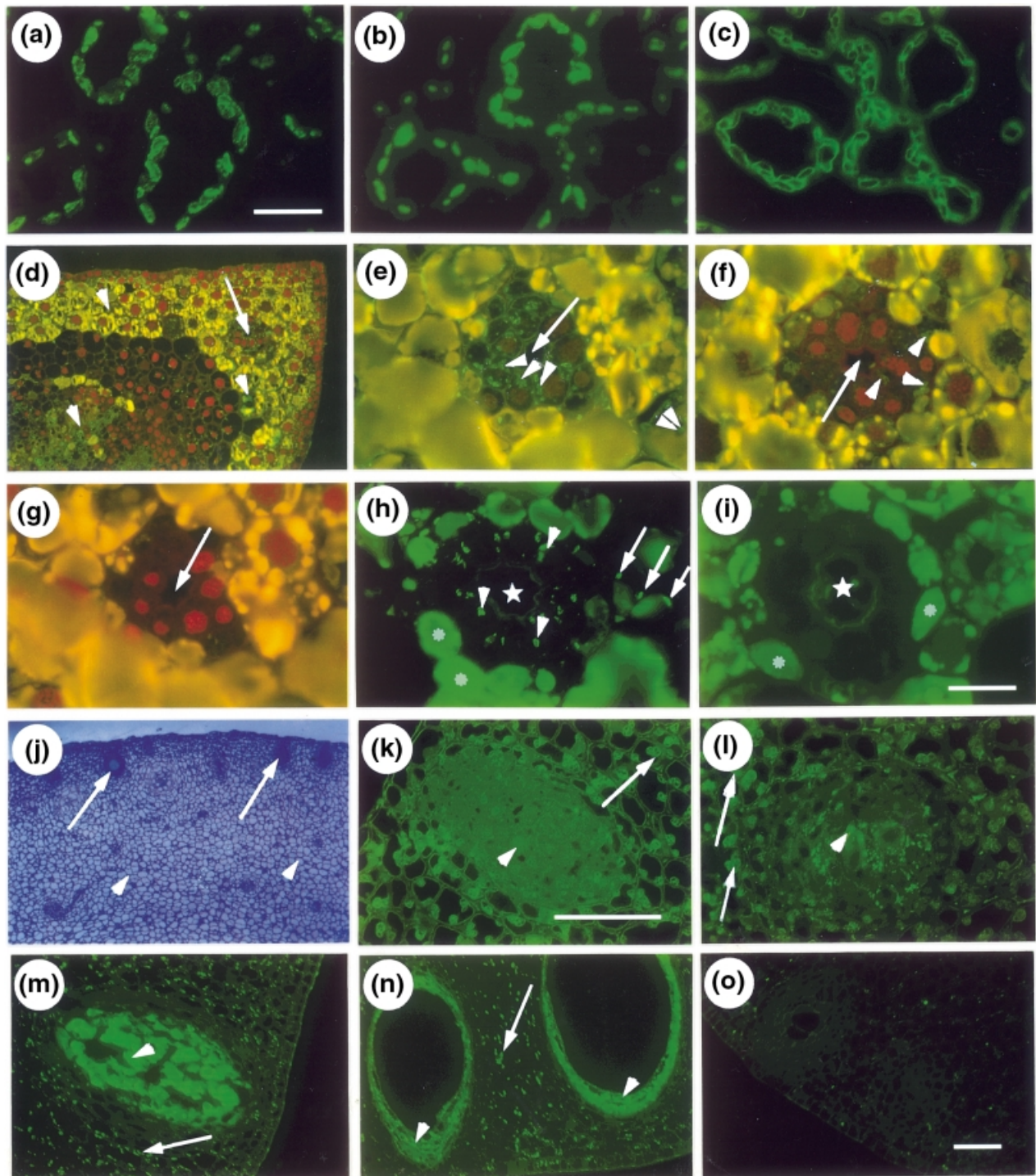
Figure 5. Immunohistochemistry of isoprenoid biosynthetic enzymes in non-secretory and secretory plant tissues.

Non-secretory tissues from *Arabidopsis* (a–c) and secretory tissues from pine needles containing resin ducts (d–i) and *Citrofortunella mitis* flavedo (exocarpium) (j–o) were fixed and double-labelled with the various specific antibodies and visualized by indirect immunofluorescence using FITC. Antibodies specifically recognizing GPPS (a), monoterpene synthase (b) and deoxy-xylulose-P synthase (c) were used. Note the intense punctate labelling of plastids. (d) Immunofluorescence of pine needle secretory tissue treated with GPPS antibodies. The cross-section shows photosynthetic parenchyma cells (arrowhead) and the central resin duct (arrow) bordered by epithelial secretory cells containing non-green plastids (arrow). Also visible are autofluorescent lipid bodies. (e) Higher magnification showing immunofluorescence of the resin duct area treated with GPPS antibodies. Note the positive labelling of non-green plastids (arrowhead) in the secretory cells bordering the resin duct (arrow) and parenchyma chloroplasts (double arrowheads). (f) The same structure and an identical labelling pattern as in (e) is observed after treatment with GGPPS antibodies. (g) Control sections similar to (e) and (f) treated with pre-immune sera, showing that plastids are not labelled. (h) Needle tissue treated with monoterpene synthase antibodies shows positive labelling of non-green plastids in the secretory cells (arrowhead) bordering the resin duct (star) and chloroplasts (arrow) inside photosynthetically active parenchyma cells. Note the artifactual labelling of lipid vesicles (asterisk). (i) Control fluorescence of needle tissue corresponding to (h). Note the absence of plastid labelling and the artifactual labelling of lipid bodies (asterisk) similar to that shown in (e). (j) Cross-section of the green flavedo (exocarpium) secretory tissue excised from *Citrofortunella mitis* showing photosynthetic parenchyma cells (arrowhead) and secretory oil cavities (arrow) containing non-green plastids. Flavedo tissue with a developing (k) and a mature (l) oil cavity were treated with GPPS antibodies. Note the labelling of non-green plastids (arrowhead) in the oil cavity and of chloroplasts (arrow) in the photosynthetic parenchyma. (m,n) Flavedo tissue as in (k) and (l) treated with monoterpene synthase antibodies shows an identical labelling pattern. (o) Representative control fluorescence of flavedo tissue treated with pre-immune sera. Fluorescence emission was monitored using a Leica DMRXA microscope without counterstaining (a–c, h, i, k–o) or a Zeiss Axiophot microscope following Evans blue counterstaining (d–g). Magnification for (a)–(c) is $\times 1100$; bar in (a) = 10 μm . Magnification for (d) is $\times 100$; (e)–(g) $\times 400$; (h), (i) $\times 1100$; bar in (i) = 20 μm . Magnification for (j) is $\times 50$; (k), (l), (o) $\times 400$; (m), (n) $\times 1800$. bar in (k) = 10 μm ; bar in (o) = 20 μm .

extracts derived from non-transformed *E. coli*, processed and incubated with the substrates according to the same procedure, did not reveal any products. Furthermore, anti-GPPS antibodies severely abolished the formation of GPPS (Figure 4d). Collectively, these data indicate that the cloned prenyltransferase encoded GPPS.

Immunocytochemistry of GPPS and its congeners

Intracellular localization of GPPS was compared in normal cells of *Arabidopsis* leaves and in specialized monoterpene secretory cells (Fahn, 1988) of *Pinus* needles and *Citrofortunella* fruit epicarp. *Arabidopsis* leaves were



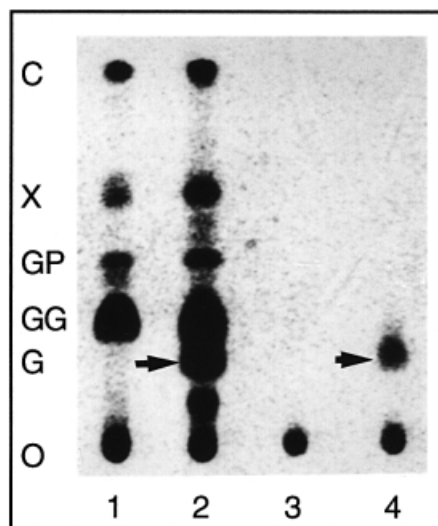


Figure 6. GPP synthesis by isolated chloroplasts and immunoaffinity-selected chloroplast GPPS.

Arabidopsis chloroplasts equivalent to 5 mg of protein were isolated and were incubated in a reaction mixture (500 μ l final volume) containing [14 C]IPP and DMAPP as specified for each lane. Lane 1: incubation in the presence of IPP (5 μ M) and in the absence of DMAPP. Lane 2: incubation in the presence of DMAPP (50 μ M). Lanes 3 and 4: following an osmotic shock, the stroma fraction equivalent to 10 mg of protein was loaded onto the GPPS immunoaffinity column and immunoselected GPPS was eluted as described in Experimental procedures before incubation in the presence of 2 μ M [14 C]IPP (lane 3) or in the presence of 2 μ M [14 C]IPP and 5 μ M DMAPP (lane 4) as prenyl diphosphate precursors. The dephosphorylated products were analysed and subjected to autoradiography as shown in Figure 4. O, origin; G, geraniol (arrowhead); GG, geranylgeraniol; GP, geranylgeranyl pyrophosphate and phytol zone; X, unidentified prenol; C, carotene zone.

examined by indirect fluorescence using anti-GPPS antibodies and goat anti-rabbit IgG coupled to fluorescein isothiocyanate (FITC) to reveal a punctate pattern that coincided with labelling of chloroplasts (Figure 5a). The presence of GPPS in the cytosol could not be unequivocally determined because at the level of photon microscopy, the cytoplasm, as usual in mature plant cells, was confined to a thin margin next to the cell wall where fluorescence was weak. Fluorescence labelling of the monoterpene synthase led to immunodecorated plastids (Figure 5b). This trend was further reinforced by the fact that deoxy-xylulose phosphate synthase, one of the first enzymes of the non-mevalonic pathway of IPP synthesis, was present in these plastids (Figure 5c). Under the same conditions, *Arabidopsis* tissues treated with the different pre-immune sera did not reveal any labelling as shown later for the secretory tissues (Figure 5o).

The secretory cells bordering the resin ducts of pine needles synthesize large quantities of monoterpenes (Wooding and Northcote, 1965) (Figure 5d). The non-green plastids of these secretory cells have been postulated as the primary source of these isoprenoids (Charon *et al.*, 1986; Wooding and Northcote, 1965). Antibodies to iso-

prenoid enzymes were used to evaluate the immunoreactivity of these plastids in pine needles. GPPS, monoterpene synthase and GGPPS were present in these plastids (Figure 5d–h). These same enzymes were also present in chloroplasts of neighbouring photosynthetic leaf parenchyma cells (Figure 5d–h). No labelling was observed using the different pre-immune sera (Figure 5i). In cells bordering the oil cavities of *Citrofortunella* fruit epicarps, where monoterpenes accumulate (Heinrich, 1966; Millet *et al.*, 1970) (Figure 5j), antibodies to GPPS and monoterpene synthase labelled both non-green plastids in secretory cells and chloroplasts in parenchyma cells (Figure 5k–n). Labelling was absent in control tissues treated with various pre-immune sera (Figure 5o), indicating that these antibodies to the isoprenoid enzymes recognized their corresponding endogenous enzymes *in vivo*.

Generation of GPP in isolated chloroplasts

Based on the above data, we attempted to characterize the production of GPP in isolated *Arabidopsis* chloroplasts. Incubation of isolated chloroplasts with labelled IPP revealed that only GGPP or geranylgeraniol accumulated as the major prenyl diphosphate or prenol (Figure 6, lane 1). However, when 25–50 μ M or higher concentrations of DMAPP were added to the incubation mixture, GPP or geraniol was formed (Figure 6, lane 2). This could be due to a premature abortion of the chain-lengthening activity of endogenous plastid GGPPS due to a displacement effect. Thus, we immunoselected the endogenous plastid GPP synthase as described above for the recombinant protein expressed in *E. coli*, using the GPPS immunoaffinity column. Under these conditions, the 36 kDa protein previously immunodecorated (Figure 3, lane 2) was isolated and used for *in vitro* incubation. In the absence of DMAPP, prenyl derivatives were not formed (Figure 6, lane 3) by the immunopurified plastidial GPPS. On the other hand, in the presence of DMAPP, the immunoselected protein catalysed exclusively the synthesis of GPP (Figure 6, lane 4). In conclusion, these data demonstrate the presence of a functional chloroplast GPPS whose product is readily channelled to the synthesis of higher-molecular-weight isoprenoids through the predominant GGPPS, thus impeding direct demonstration of GPPS activity in isolated chloroplasts or other non-green plastids except leucoplasts. A similar finding was also observed for isolated pepper chromoplasts (unpublished data).

Discussion

Roles of cytosolic and plastid GPPS

There are conflicting reports in the literature concerning the cellular and intracellular sites of GPPS in plant cells. The

data we report here indicate that a *bona fide* GPPS probably belongs to the homodimeric prenyltransferase family as shown by the characteristic domains DDXXD and FQXXDDXD (where X is any amino acid) involved in the binding of the substrates (Kellogg and Poulter, 1997). This differs from data published for the heterodimeric GPP synthase from *Mentha glandular* trichomes (Burke *et al.*, 1999). In the latter case, a small and a large subunit were assembled to yield an active enzyme. This type of prenyltransferase structure has not been previously described in plants. Except for some bacteria whose hexaprenyl or heptaprenyl diphosphate synthase genes are organized into operons (Koike-Takashita *et al.*, 1995), several lines of evidence suggest that homodimeric prenyltransferases could be engineered to produce prenyl diphosphates with variable chain lengths (for review, see Wang and Ohnuma, 1999), and thus reinforce the existence of homodimeric GPPS. In relation to GPPS, two cases are worth mentioning. First, a yeast FPPS synthase mutant in which Lys197 is mutated into Glu predominantly formed GPP in contrast to FPP, the natural product (Blanchard and Karst, 1993). Second, the conversion of a *Bacillus stearothermophilus* homodimeric FPPS to GPPS has been recently demonstrated by judicious site-directed mutagenesis of active amino acid residues (Narita and Ohnuma, 1999). The difference observed between *Mentha* and *Arabidopsis* GPP synthases could be due to the fact that the heterodimeric GPPS from *Mentha* is localized in leucoplast trichomes while the homodimeric form is compartmentalized in parenchyma tissues containing chloroplasts or other non-green plastids. Alternatively, this could reflect the plasticity observed with monoterpene synthases, as shown by the comparison of *S*- and *R*-linalool synthases of *Clarkia* (Dudareva *et al.*, 1996) and *Artemisia* (Jia *et al.*, 1999) which display only 22% identity. Our results suggest the existence of two forms of GPPS depending on the methionine used to initiate translation. Initiation from the upstream methionine produces a protein which possesses all of the structural determinants needed for the plastid-targeted GPPS isoform, while initiation from the second methionine produces a truncated GPPS isoform that could be targeted to the cytosol. The ChloroP program (Emanuelsson *et al.*, 1999) did not allow reliable cellular compartmentation of the cloned cDNA. This is illustrated by the differential localization predicted for our cloned cDNA and its EST homologue (Figure 1) derived from cotton, a monoterpene-producing plant (Loughrin *et al.*, 1994). In the isoprenoid series, a similar deviation with respect to the subcellular compartmentation could be observed for the plastidial enzyme kaurene synthase (Bensen *et al.*, 1995; Yamaguchi *et al.*, 1998).

The dual expression of two isoforms targeted to different cellular compartments from a single gene is not unique (for review, see Danpure, 1995). In the case of GPPS, the

presence or absence of the transit peptide is probably due to the use of alternative 5' exons as shown for the human dUTPase gene which encodes both nuclear and mitochondrial isoforms (Ladner and Caradonna, 1997). For the isoprenoid pathway, it has been shown that one gene for FPPS in *Arabidopsis* encodes both a cytosolic and mitochondrial form of the enzyme (Cunillera *et al.*, 1997). The question now is how do these GPPS isoenzymes operate during isoprenoid metabolism in plants?

GPPS has been partially characterized in undefined membrane fractions from *Pelargonium roseum* (Suga and Endo, 1991), and GPPS was indirectly localized in *Citrofortunella mitis* leucoplasts (Gleizes *et al.*, 1983) and in *Narcissus pseudonarcissus* chromoplasts (Mettal *et al.*, 1988). Additional evidence for GPPS localization in plastids came from studies of grape fruits (*Vitis vinifera* cv Muscat) (Clastre *et al.*, 1993). Moreover, all the monoterpene synthases functionally cloned to date from secretory tissues possess plastid targeting sequences (Bohlmann *et al.*, 1998). Thus, plastid-localized GPPS isoforms should provide the basic C₁₀ carbon skeletons required for these monoterpene synthases. Also, the compartmentation of GPPS in chloroplasts could explain, in relation to the availability of photosynthetic carbon substrates, how photo-regulation influences monoterpene emission in the environment by oak leaves (Loreto *et al.*, 1996; Staudt and Bertin, 1995).

A soluble form of GPPS used in the formation of shikonin, a monoterpene derivative, has been partially characterized from *Lithospermum erythrorhizon* cell cultures (Heide and Berger, 1989) and was localized to the cytosol (Sommer *et al.*, 1995). Biochemical data in support of this show that shikonin synthesis was blocked by mevinolin, an inhibitor specific to the cytosolic form of hydroxymethylglutaryl CoA reductase (Lange *et al.*, 1998). Additionally, *in vivo* incorporation and NMR analysis of ¹³C-labelled substrates provided strong evidence that shikonin biosynthesis operates through the cytosolic mevalonate pathway of isoprenoid biosynthesis (Li *et al.*, 1998). These data indicate that the cytosol is the site of shikonin synthesis, accumulation and secretion (Tsukada and Tabata, 1984).

The potential dual targeting of GPPS, coupled to the possible exchange of prenyl diphosphate substrates between plastid and cytosolic compartments, may reconcile the contradictory data of previous reports which described GPPS exclusively in the plastids or cytosol but not in both compartments. This is supported by examination of DNA sequences from *Arabidopsis thaliana* obtained through large sequencing programmes. The existence of open reading frames that code for putative monoterpene synthases located on chromosome II and IV was noted (accession numbers AAD03382 and CAB10448). The deduced peptide sequences of these genes display the

characteristic twin arginine motifs of other monoterpene synthases (Williams *et al.*, 1998). Furthermore, the sequence corresponding to the accession number AAD03382 displays an authentic plastid-targeting domain as shown by the ChloroP program (Emanuelsson *et al.*, 1999).

Exchanges between the plastid and the cytosol compartment could occur at the level of IPP or GPP. A shuttle involving IPP is easily conceivable due to its resistance to hydrolysis by endemic cellular phosphatases. The functional demonstration of a putative IPP transporter in plastids (Soler *et al.*, 1993) supports this view. This hypothesis is reinforced by the findings of studies in which *Catharanthus roseus* cells were fed 1-deoxy-D-xylulose *in vitro* to generate phytol and carotenoids that were differentially labelled in comparison to sterols (Arigoni *et al.*, 1997).

Compartmentation of monoterpenoid biogenesis in plant cells

The dual targeting of GPPS into green and non-green plastids and in the cytosol allows a reconsideration of the site of monoterpene formation and sequestration in plants. Until now, monoterpene synthesis was largely thought to occur exclusively in non-green plastids. The 'raison d'être' of this compartmentation was partly based on the toxicity that monoterpene concentrations as low as 100 ppm were supposed to have on biological structures (Weidenhamer *et al.*, 1993). This toxicity leads to growth inhibition, membrane disorganization, and mitochondrial and photosynthetic electron transfer perturbation in plants (Muller *et al.*, 1968). To circumvent these deleterious effects, monoterpenes are sequestered in specialized secretory structures which include glandular trichomes, secretory canals or cavities and idioblasts (Gershenzon and Croteau, 1990). For example, each glandular trichome of catmint (*Nepeta racemosa*) accumulates approximately 30 ng of nepetalactone monoterpenoid. This sequestration mechanism allows monoterpenes to accumulate up to 1.5–20% of the dry weight of lemongrass (*Cymbopogon* sp.) and *Eucalyptus* leaves (Lewinsohn *et al.*, 1998; Morrow and Fox, 1980). A characteristic feature of these secretory structures is the presence of non-green plastids that are frequently associated with the endoplasmic reticulum (Cheniclet and Carde, 1985; Wooding and Northcote, 1965). It was long held that these monoterpenes were synthesized by non-green plastids and that the endoplasmic reticulum facilitated their discharge and efflux to specialized structures. However, there are many instances where monoterpenes can be synthesized in cells lacking non-green plastids and can accumulate in non-specialized structures.

In lower plants, liverworts, the first land plants (Qiu *et al.*, 1998), are known to produce monoterpenes (Zinsmeister

et al., 1991) in the absence of non-green plastids. This has been recently ascertained by *in situ* immunolocalization of several isoprenoid biosynthetic enzymes in *Marchantia polymorpha* (Suire *et al.*, 2000). In the higher plant *Monarda fistulosa*, tissues lacking secretory cells are still competent for the production of monoterpene hydrocarbons (Pfaff *et al.*, 1980). The link between the synthesis and accumulation of essential oil isoprenoids and the presence of secretory structures also does not hold for the genus *Gossypium*. For instance, the *Gossypium* subgenus *Sturtia* possesses lysogenous cavities but does not produce gossypol (Brubaker *et al.*, 1996). In certain cotton plants (*Gossypium* sp.), the sesquiterpenoid aldehyde gossypol accumulates in lysogenous cavities (Stanford and Viehoveer, 1918). However, in *Gossypium hirsutum* mutants that are devoid of lysogenous cavities, gossypol is synthesized in response to fungal infection or xenobiotic treatments (Bell, 1967).

The lack of connection between monoterpene synthesis and secretory structures is also observed in plants not usually considered as monoterpene essential oil producers. Two examples are corn and *Phaseolus lunatus*, which transiently synthesize β -ocimene and linalool to attract predatory insects in response to feeding by phytophagous insects or parasitic fungus (Hopke *et al.*, 1994; Paré and Tumlinson, 1999). In *Phaseolus lunatus*, the monoterpene constituents derive either from the mevalonate or the deoxy-xylulose pathways (Piel *et al.*, 1998). Similarly, cabbage (*Brassica oleracea*), a relative of *Arabidopsis* belonging to the Cruciferae, emits several monoterpenoids including myrcene and limonene, when attacked by the caterpillar *Pieris brassicae* (Mattiacci *et al.*, 1995). This is even detected in field conditions in the absence of attack (Tollsten and Bergström, 1988) or with detached plantlets (Boland *et al.*, 1995). It should also be noted that limonene, sabinene, β -myrcene and linalool are the major compounds emitted by field-grown oilseed rape (Jakobsen *et al.*, 1994; McEwan and Macfarlane-Smith, 1998). Thus, the discovery of GPPS from *Arabidopsis* and the presence of two open reading frames in chromosomes II and IV, encoding two proteins (accession numbers AAD03382 and CAB10448), bearing all the characteristic of monoterpene synthases, should be expected in this species. These considerations could also be extended to other non-secretory organs such as tomato fruits (Buttery *et al.*, 1988; Stevens, 1970), pepper fruits (Buttery *et al.*, 1969; see also http://www.kmxq.com/hrbmoore/Constituents/Capsicum_annuum.txt) and sweet potatoes (Ohta *et al.*, 1991), whose aromas contain several monoterpenes including linalool, limonene, geranial and β -ocimene. Taking all these facts into consideration, we propose that monoterpene metabolism operates in several compartments (Figure 7) within photosynthetic and non-photosynthetic plant cells.

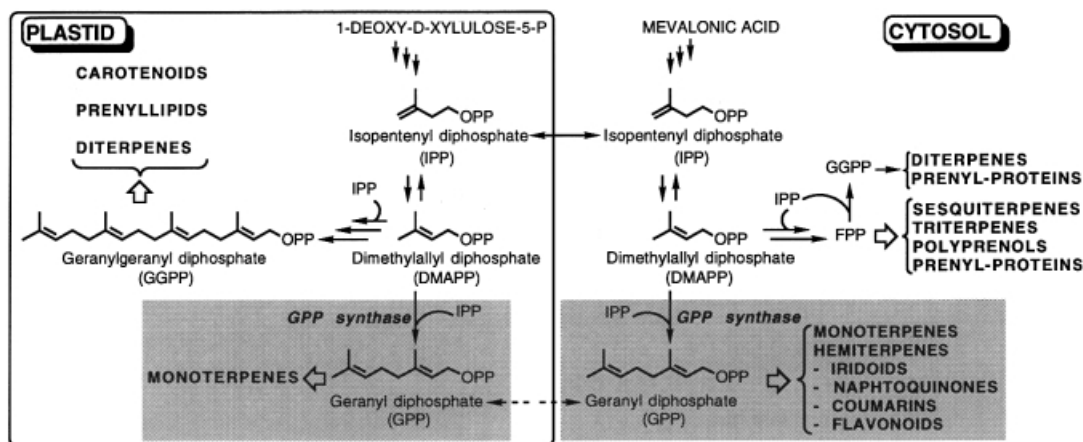


Figure 7. Compartmentation of GPP synthesis in plant cells.

GPP is synthesized in the plastids and the cytosol according to the deoxy-xylulose phosphate and the mevalonate pathways. In each compartment, IPP is partly channelled to the synthesis of specific monoterpenes and monoterpene derivatives by GPPS. Substrate exchanges between the plastid and the cytosolic pathways could occur at the level of IPP which is highly resistant to endemic phosphatases. Alternatively, the more labile GPP could be involved.

Experimental procedures

Plant material

Arabidopsis thaliana (ecotype Columbia) plants were grown under greenhouse conditions and treated with 1 mM jasmonic acid for 24 h before harvesting to enhance the defence responses. *Citrofortunella mitis* and *Citrus* sp. were obtained from local sources and *Pinus pinaster* L. needles were collected near Bordeaux (France).

CDNA isolation and sequencing

Unless otherwise stated, the molecular techniques were performed using standard protocols (Sambrook *et al.*, 1989). To clone GPPS, we used several oligonucleotide primers corresponding to two amino acid motifs VLAGDFLLS and FQLIDDDILD which are highly conserved between different prenyltransferases of several organisms and plants, including *A. thaliana*, and the two monoterpene-producing plants *Citrofortunella mitis* and *Citrus* sp. (F. Bouvier and B. Camara, unpublished data). The following sense primers 1S: GTA CTA GCA GGA GAC TTC CTG TTA TCC; 2S: GTC CTC GCT GGC GAT TTT CTA TTG TCA; 3S: GTG CTG GCG GGG GAC TTC CTT CTA TCG; 4S: GTA CTT GCA GGT GAT TTT TTA CTG AGC and 5S: GTG TTG GCT GGA GAC TTC TTG CTT AGT, and antisense primers 1R: ATC TAG TAT GTC ATC TAT AAG CTG AAA; 2R: ATC CAG GAT ATC ATC GAT GAG CTG AAA; 3R: GTC AAG AAT ATC GTC AAT CAG TTG GAA; 4R: GTC GAG TAT GTC GTC TAT TAG TTG GAA; 5R: GTC CAA GAT ATC GTC GAT TAA TTG GAA and 6R: ATC TAA AAT ATC ATC AAT CAA CTG AAA were used. Following RT-PCR of *Arabidopsis* mRNA (Bouvier *et al.*, 1998), the reaction products were cloned using the vector pCR^{2.1}-TOPO (Invitrogen) before sequencing and isolation of a full-length clone from *Arabidopsis* cDNA prepared from IZAP (Stratagene) according to the manufacturer's instructions.

Expression and enzymatic analysis of recombinant *E. coli* GPPS

Recombinant truncated GPPS starting from the peptide sequence LLSNKL was expressed in *E. coli* (TOP10) using the pBAD-

TOPO TA vector (Invitrogen). Following sequence verification, transformed *E. coli* were grown exponentially ($OD_{600} = 0.6$) in Luria-Bertani medium at 18°C before induction with arabinose. Bacterial cells were pelleted 3 h later, resuspended in the lysis buffer (Tris-HCl, 50 mM, pH 7.6 containing 0.1% Tween-80, and 0.1 M NaCl) before 5 sec sonication at 4°C (six times). The resulting lysate was centrifuged at 10 000 *g* for 30 min and the supernatant was used for enzyme assay. To avoid interference of endogenous *E. coli* prenyltransferases, the supernatant was loaded onto a protein A-Sepharose column (2 ml total volume) coupled to antibodies against GPPS (Harlow and Lane, 1988). Following extensive washing with Tris-HCl buffer (50 mM, pH 7.6) containing 0.5 M NaCl, the active fraction was eluted with 2.5 ml of 0.2 M glycine buffer (pH 2.3) containing 0.2 M NaCl and 0.25% of Tween-20 before immediate rebuffing to pH 7.6 with 1 M Tris followed by addition of 0.1% BSA, and *in vitro* assay in the incubation medium containing (in a final volume of 0.5 ml) 5 μ M [¹⁴C]IPP (40 mCi mmol⁻¹; NEN, France), 10 mM MgCl₂, 0.2 mM MnCl₂, 2 mM DTT, 10 μ M DMAPP, GPP, FPP or GGPP prepared as described previously (Camara, 1985) and 250 μ l of enzyme solution. Following incubation at 30°C for 1 h, the reaction products were dephosphorylated (Koyama *et al.*, 1985) and analysed by TLC (Dogbo *et al.*, 1987) and HPLC (Camara, 1985) using unlabelled authentic prenol standards.

Northern blot analysis, *in vitro* transcription-translation and plastid import

Northern blot analysis was performed using total RNA according to a previously described procedure (Bouvier *et al.*, 1998) and two DNA probes. The DNA probes were prepared by PCR using the sense (GTTCCAAAGCTTGCTCT) and the antisense (ATTCGC-ATGTTCCATGGC) oligonucleotides encoding the sequences VPKLAS and AMEHAN (probe 1) or the sense (ATGTTATTCACG-AGGAGT) and the antisense (AACCGGAGATTTCAATGA) oligonucleotides encoding, respectively, the sequences MLFTRS and SLKSPV (probe 2) of GPPS cDNA. *In vitro* transcription-translation was carried out using GPPS cDNA as a template and the TNT lysate procedure (Promega) and [³⁵S]methionine according to the manufacturer's instructions. Following 45 min incubation at 30°C, the reaction mixture was either diluted with SDS sample buffer,

boiled and subjected to SDS-PAGE and fluorography or used for *in vitro* import into purified intact pepper leaf chloroplasts (Camara, 1993). The import protocol and the thermolysin-protected GPPS analysis were conducted as described previously (Perry *et al.*, 1991) before SDS-PAGE and fluorography.

Antibodies and immunohistochemistry

Antibodies against GPPS were raised against the recombinant protein described above. Antibodies were raised against GGPPS purified from pepper chromoplasts (Dogbo and Camara, 1987) and FPPS from pepper fruit cytosol (Huguene and Camara, 1990). Anti-deoxy-xylulose-P-synthase was prepared from the recombinant pepper enzyme (Bouvier *et al.*, 1998). The anti-monoterpene synthase was prepared by coupling to the carrier protein KLH (keyhole limpet haemocyanin) the synthetic peptide (DDIYDVYGTLEELE) which is highly conserved between limonene synthase (Bohlmann *et al.*, 1998) and putative *Arabidopsis* limonene synthases (accession numbers AAD03382 and CAB10448). For immunohistochemistry, the plant tissues were fixed with 4% formaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2. The fixed tissues were probed with the different pre-immune sera and antibodies against the isoprenoid biosynthetic enzymes before indirect immunofluorescence analysis using goat anti-rabbit IgG coupled to fluorescein isothiocyanate (FITC) as described previously (Camara, 1993). The sections were mounted in Citifluor (Agar Aids R-1320). In some cases, the sections were analysed using an epifluorescence Leica DMRXA microscope fitted with an FITC set (excitation filter 450–490 nm; dichroic mirror at 510 nm; barrier filter 515–560 nm). Pictures were recorded with a cooled charge-coupled device Micromax camera (Princeton Instruments), processed by Metamorph II Universal Imaging, and printed on Sony UP-D8800 through Corel Xara. Other sections were counterstained with Evans blue (Merck 3169) and examined using an epifluorescence Zeiss Axiophot microscope fitted with the same FITC set, except a less selective filter barrier (from 520 nm) was used and colour slides were prepared using Agfachrome 100RS⁺ or Fujichrome Sensia 100 ISO.

Subcellular fractionation and GPP biosynthetic capacity of chloroplasts

Isolation of purified *Arabidopsis* chloroplasts and mitochondria and immunoblot analysis of protein were performed as described previously (Bouvier *et al.*, 1998). The stroma, i.e. the soluble plastid fraction, was obtained after subjecting the purified plastids to an osmotic shock in the presence of 50 mM Tris-HCl (pH 7.6), followed by centrifugation at 10 000 *g* for 30 min. Total protein was quantified as described previously (Smith *et al.*, 1985). The GPP biosynthetic capacity of intact chloroplasts was determined using a reaction mixture containing (in a final volume of 0.2 ml) 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 5 mM MnCl₂, 2 mM DTT, 1 mM ATP, 0.2% Tween-20, 5 mg of plastid protein, 5 μ M [¹⁴C]IPP and 50 μ M DMAPP as specified in the text. Following incubation at 30°C for 2 h, the reaction products were dephosphorylated and analysed as for the assay with recombinant GPPS described above. Alternatively, the stroma fraction equivalent to 10 mg of protein was loaded onto the GPPS immunoaffinity column, and GPPS was eluted as described above into 2.5 ml elution buffer and 250 μ l aliquots used for enzyme assay as shown above except that 2 μ M [¹⁴C] IPP and 5 μ M DMAPP were used.

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References

- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.
- Arigoni, D., Sagner, S., Latzel, C., Eisenreich, W., Bacher, A. and Zenk, M.H. (1997) Terpenoid biosynthesis from 1-deoxy-D-xylulose in higher plants by intramolecular skeletal rearrangement. *Proc. Natl Acad. Sci. USA*, **94**, 10600–10605.
- Bell, A.A. (1967) Formation of gossypol in infected or chemically irritated tissues of *Gossypium* species. *Phytopathology*, **57**, 759–764.
- Bensen, R.J., Johal, G.S., Crane, V.C., Tossberg, J.T., Schnable, P.S., Meely, R.B. and Briggs, S.P. (1995) Cloning and characterization of the maize *AN 1* gene. *Plant Cell*, **7**, 75–84.
- Blanchard, L. and Karst, F. (1993) Characterization of a lysine-to-glutamic acid mutation in a conservative sequence of farnesyl diphosphate synthase from *Saccharomyces cerevisiae*. *Gene*, **125**, 185–189.
- Bohlmann, J., Meyer-Gauen, G. and Croteau, R. (1998) Plant terpenoid synthases: molecular biology and phylogenetic analysis. *Proc. Natl Acad. Sci. USA*, **95**, 4126–4133.
- Boland, W., Hopke, J., Donath, J., Nüske, J. and Bublitz, F. (1995) Jasmonic acid and coronatin induce odor production in plants. *Angew. Chem. Int. ed. Engl.* **34**, 1600–1602.
- Bouvier, F., d'Harlingue, A., Suire, C., Backhaus, R.A. and Camara, B. (1998) Dedicated roles of plastid transketolases during the early onset of isoprenoid biogenesis in pepper fruits. *Plant Physiol.* **117**, 1423–1431.
- Brubaker, C.L., Benson, C.G., Miller, C. and Leach, D.N. (1996) Occurrence of terpenoid aldehydes and lysogenous cavities in the 'glandless' seeds of Australian *Gossypium* species. *Aust. J. Bot.* **44**, 601–612.
- Burke, C.C., Wildung, M.R. and Croteau, R. (1999) Geranyl diphosphate synthase: cloning, expression, and characterization of this prenyltransferase as a heterodimer. *Proc. Natl Acad. Sci. USA*, **96**, 13062–13067.
- Buttery, R.G., Seifert, R.M., Guadagni, D.G. and Ling, L.C. (1969) Characterization of some volatile constituents of bell peppers. *J. Agric. Food Chem.* **17**, 1322–1327.
- Buttery, R.G., Teranishi, R., Ling, L.C., Flath, R.A. and Stern, D.J. (1988) Quantitative studies on origins of fresh tomato aroma volatiles. *J. Agric. Food Chem.* **36**, 1247–1250.
- Camara, B. (1985) Carotene synthesis in *Capsicum* chromoplasts. *Methods Enzymol.* **110**, 244–253.
- Camara, B. (1993) Plant phytoene synthase complex: component enzymes, immunology, and biogenesis. *Methods Enzymol.* **214**, 352–365.
- Charon, J., Launay, J. and Vindt-Balguerie, E. (1986) Ontogenèse des canaux sécréteurs d'origine primaire dans le bourgeon de pin maritime. *Can. J. Bot.* **64**, 2955–2964.
- Cheniclet, C. and Carde, J.P. (1985) Presence of leucoplasts in secretory cells and monoterpenes in the essential oil: a correlative study. *Isr. J. Bot.* **34**, 219–238.

- Clastre, M., Bantignies, B., Feron, G., Soler, E. and Ambid, C.** (1993) Purification and characterization of geranyl diphosphate synthase from *Vitis vinifera* L. cv Muscat de Frontignan cell cultures. *Plant Physiol.* **102**, 205–211.
- Croteau, R. and Purkett, P.T.** (1989) Geranyl pyrophosphate synthase: characterization of the enzyme and evidence that this chain-length specific prenyltransferase is associated with monoterpene biosynthesis in sage (*Salvia officinalis*). *Arch. Biochem. Biophys.* **271**, 524–535.
- Cunillera, N., Boronat, A. and Ferrer, A.** (1997) The *Arabidopsis thaliana* FPS1 gene generates a novel mRNA that encodes a mitochondrial farnesyl diphosphate synthase isoform. *J. Biol. Chem.* **272**, 15381–15388.
- Danpure, C.J.** (1995) How can the products of a single gene be localized to more than one intracellular compartment? *Trends Cell Biol.* **5**, 230–238.
- Dogbo, O. and Camara, B.** (1987) Purification of isopentenyl pyrophosphate isomerase and geranylgeranyl pyrophosphate synthase from *Capsicum annuum* by affinity chromatography. *Biochim. Biophys. Acta*, **920**, 140–148.
- Dogbo, O., Bardat, F., Quennemet, J. and Camara, B.** (1987) Metabolism of plastid terpenoids: in vitro inhibition of phytoene synthesis by phenethyl pyrophosphate derivatives. *FEBS Lett.* **210**, 211–215.
- Dudareva, N., Cseke, L., Blanc, V.M. and Pichersky, E.** (1996) Evolution of floral scent in *Clarkia*: novel patterns of S-linalool synthase gene expression in the *C. breweri* flower. *Plant Cell*, **8**, 1137–1148.
- Emanuelsson, O., Nielsen, H. and Von Heijne, G.** (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci.* **8**, 978–984.
- Fahn, A.** (1988) Secretory tissues in vascular plants. *New Phytol.* **108**, 229–257.
- Gershenzon, J. and Croteau, R.** (1990) Regulation of monoterpene biosynthesis in higher plants. *Recent Adv. Phytochem.* **24**, 99–160.
- Gleizes, M., Pauly, G., Carde, J.P., Marpeau, A. and Bernard-Dagan, C.** (1983) Monoterpene hydrocarbon biosynthesis by isolated leucoplasts of *Citrofortunella mitis*. *Planta*, **159**, 373–381.
- Gould, M.N.** (1997) Cancer chemoprevention and therapy by monoterpenes. *Environ. Health Perspect.* **105**, 977–979.
- Harlow, E. and Lane, D.** (1988) Immunoaffinity purification. In *Antibodies: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, pp. 521–523.
- Heide, L. and Berger, U.** (1989) Partial purification and properties of geranyl pyrophosphate synthase from *Lithospermum erythrorhizon*. *Arch. Biochem. Biophys.* **273**, 331–338.
- Heinrich, G.** (1966) Licht- und elektronenmikroskopische Untersuchungen zur Genese der Exkrete in den lysigenen Exkretäumen von *Citrus medica*. *Flora Abt. A*, **156**, 541–546.
- Hopke, J., Donath, J., Bleichert, S. and Boland, W.** (1994) Herbivore-induced volatiles: the emission of acyclic homoterpenes from leaves of *Phaseolus lunatus* and *Zea mays* can be triggered by a β -glucosidase and jasmonic acid. *FEBS Lett.* **352**, 146–150.
- Hugueney, P. and Camara, B.** (1990) Purification and characterization of farnesyl pyrophosphate synthase from *Capsicum annuum*. *FEBS Lett.* **273**, 235–238.
- Hugueney, P., Bouvier, F., Badillo, A., Quennemet, J., d'Harlingue, A. and Camara, B.** (1996) Developmental and stress regulation of gene expression for plastid and cytosolic isoprenoid pathways in pepper fruits. *Plant Physiol.* **111**, 619–626.
- Jakobsen, H.B., Friis, P., Nielsen, J.K. and Olsen, C.E.** (1994) Emission of volatiles from flowers and leaves of *Brassica napus* in situ. *Phytochemistry*, **37**, 695–699.
- Jia, J.W., Crock, J., Lu, S., Croteau, R. and Chen, X.Y.** (1999) (3R)-Linalool synthase from *Artemisia annua* L. cDNA isolation, characterization, and wound induction. *Arch. Biochem. Biophys.* **372**, 143–149.
- Kellogg, B.A. and Poulter, C.D.** (1997) Chain elongation in the isoprenoid biosynthetic pathway. *Curr. Opin. Chem. Biol.* **1**, 570–578.
- Koike-Takashita, A., Koyama, T., Obata, S. and Ogura, K.** (1995) Molecular cloning and nucleotide sequences of the genes for two essential proteins constituting a novel enzyme system for heptaprenyl diphosphate synthesis. *J. Biol. Chem.* **270**, 18396–18400.
- Koyama, T., Fuji, H. and Ogura, K.** (1985) Enzymatic hydrolysis of polyprenyl pyrophosphates. *Methods Enzymol.* **110**, 153–155.
- Kuntz, M., Römer, S., Suire, C., Hugueney, P., Weil, J.H., Schantz, R. and Camara, B.** (1992) Identification of a cDNA for the plastid located geranylgeranyl pyrophosphate synthase from *Capsicum annuum*: correlative increase in enzyme activity and transcript level during fruit ripening. *Plant J.* **2**, 25–34.
- Ladner, R.D. and Caradonna, S.J.** (1997) The human dUTP gene encodes both nuclear and mitochondrial isoforms. *J. Biol. Chem.* **272**, 19072–19080.
- Laferrière, A. and Beyer, P.** (1991) Purification of geranylgeranyl pyrophosphate synthase from *Sinapis alba* etioplasts. *Biochim. Biophys. Acta*, **1077**, 167–172.
- Lange, B.M., Severin, K., Bechthold, A. and Heide, L.** (1998) Regulatory role of microsomal 3-hydroxy-3-methylglutaryl-coenzyme A reductase for shikoniol biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures. *Planta*, **204**, 234–241.
- Langenheim, J.H.** (1994) Higher plant terpenoids: a phytocentric overview of their ecological roles. *J. Chem. Ecol.* **20**, 1223–1280.
- Lewinsohn, E., Dudai, N., Tadmor, Y., Katzir, I., Ravid, U., Putievsky, E. and Joel, D.M.** (1998) Histochemical localization of citral accumulation in lemongrass leaves (*Cymbopogon citratus* D.C. Stapf. Poaceae). *Ann. Bot.* **81**, 35–39.
- Li, S.M., Hennig, S. and Heide, L.** (1998) Shikoniol: a geranyl diphosphate-derived plant hemiterpenoid formed via the mevalonate pathway. *Tetrahedron Lett.* **39**, 2721–2724.
- Loreto, F., Ciccioli, P., Cecinato, A., Brancaleoni, E., Frattoni, M., Fazio, C. and Tricoli, D.** (1996) Evidence of the photosynthetic origin of monoterpenes emitted by *Quercus ilex* L. leaves by ^{13}C labelling. *Plant Physiol.* **110**, 1317–1322.
- Loughrin, J.H., Manukian, A., Heath, R.R., Turlings, T.J.C. and Tumlinson, J.H.** (1994) Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plants. *Proc. Natl Acad. Sci. USA*, **91**, 11836–11840.
- McEwan, M. and Macfarlane-Smith, W.H.** (1998) Identification of volatile organic compounds emitted in the field by oilseed rape (*Brassica napus* ssp. *oleifera*) over the growing season. *Clin. Exp. Allergy*, **28**, 332–338.
- Mattiacci, L., Dicke, M. and Posthumus, M.A.** (1995) β -glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proc. Natl Acad. Sci. USA*, **92**, 2036–2040.
- Mettal, U., Boland, W., Beyer, P. and Kleinig, H.** (1988) Biosynthesis of monoterpene hydrocarbons by isolated chromoplasts from daffodil flowers. *Eur. J. Biochem.* **170**, 613–616.
- Millet, C.R., Monghal, M.A., Rollet, M. and Dorche, J.** (1970) L'essence d'Orange obtenue par ponction directe dans les glandes sécrétrices. *Ann. Pharm. Fr.* **28**, 63–68.

- Morrow, P.A. and Fox, L.R. (1980) Effects of variation in *Eucalyptus* essential oil yield on insect growth and grazing damage. *Oecologia*, **45**, 209–219.
- Mukumoto, F., Hirose, S., Imaseki, H. and Yamazaki, K. (1993) DNA sequence requirement of a TATA element-binding protein from *Arabidopsis* for transcription in vitro. *Plant Mol. Biol.* **23**, 995–1003.
- Mulder-Krieger, T.H., Verpoorte, R., Baerheim, S.A. and Scheffer, J.J.C. (1988) Production of essential oils and flavours in plant cell and tissue cultures. *Plant Cell Tissue Organ Cult.* **13**, 85–154.
- Muller, P., Lorber, P. and Haley, B. (1968) Volatile growth inhibitors produced by *Salvia leucophylla*: effect on seedling growth and respiration. *Bull. Torrey Bot. Club*, **95**, 415–422.
- Narita, K. and Ohnuma, S.I. (1999) Protein design of geranyl diphosphate synthase. Structural features that define the product specificities of prenyltransferases. *J. Biochem.* **126**, 566–571.
- Ohta, T., Omori, T., Shimojo, H., Hashimoto, K., Samuta, T. and Ohba, T. (1991) Identification of monoterpene alcohol β -glucosides in sweet potatoes and purification of a *Shiro-koji* β -glucosidase. *Agric. Biol. Chem.* **55**, 1811–1816.
- Okada, K., Suzuki, K., Kamiya, Y., Zhu, X., Fujisaki, S., Nishimura, Y., Nishino, T., Nakagawa, T., Kawamukai, M. and Matsuda, H. (1992) Polyprenyl diphosphate synthase essentially defines the length of the side chain of ubiquinone. *Biochim. Biophys. Acta*, **1302**, 217–223.
- Okada, K., Kamiya, Y., Zhu, X., Suzuki, K., Tanaka, K., Nakagawa, T., Matsuda, H. and Kawamukai, M. (1997) Cloning of the *sdsA* gene encoding solanesyl diphosphate synthase from *Rhodobacter capsulatus* and its functional expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *J. Bacteriol.* **179**, 5992–5998.
- Paré, P.W. and Tumlinson, J.H. (1999) Plant volatiles as a defense against insect herbivores. *Plant Physiol.* **121**, 325–331.
- Perry, S.E., McClure, M.R. and Keegstra, K. (1991) *In vitro* reconstitution of protein transport into chloroplasts. *Methods Cell Biol.* **34**, 327–344.
- Pfab, I., Heinrich, G. and Schultze, W. (1980) Über das ätherische Öl in den Öldrüsen und im außendrüsenartigen Bereich von *Monarda fistulosa*. *Biochem. Physiol. Pflanzen*, **175**, 29–44.
- Piel, J., Donath, J., Bandemer, K. and Boland, W. (1998) Mevalonate-independent biosynthesis of terpenoid volatiles in plants: induced and constitutive emission of volatiles. *Angew. Chem. Int. ed. Engl.* **37**, 2478–2481.
- Qiu, Y.L., Cho, Y., Cox, J.C. and Palmer, J.D. (1998) The gain of three mitochondrial introns identifies liverworts as the earliest land plants. *Nature*, **671**, 671–674.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76–85.
- Soler, E., Feron, G., Clastre, M., Dargent, R., Gleizes, M. and Ambid, C. (1992) Evidence for a geranyl diphosphate synthase located within the plastids of *Vitis vinifera* L. cultivated *in vitro*. *Planta*, **187**, 171–175.
- Soler, E., Clastre, M., Bantignies, B., Marigo, G. and Ambid, C. (1993) Uptake of isopentenyl diphosphate into plastids isolated from *Vitis vinifera* L. suspension. *Planta*, **324**, 324–329.
- Sommer, S., Severin, K., Camara, B. and Heide, L. (1995) Intracellular localization of geranyl pyrophosphate synthase from cell cultures of *Lithospermum erythrorhizon*. *Phytochemistry*, **38**, 623–627.
- Stanford, E.E. and Viehoveer, A. (1918) Chemistry and histology of the glands of cotton plant with special notes on the occurrence of similar glands in related plants. *J. Agric. Res.* **51**, 419–435.
- Staudt, M. and Bertin, N. (1995) Light dependent emissions of monoterpenes by Holm oak (*Quercus ilex* L.). *Naturwissenschaften*, **82**, 89–92.
- Stevens, A.A. (1970) Relationship between polyene-carotene content and volatile compound composition of tomatoes. *J. Am. Soc. Hort. Sci.* **95**, 461–464.
- Suga, T. and Endo, T. (1991) Geranyl diphosphate synthase in leaves of *Pelargonium roseum*. *Phytochemistry*, **30**, 1757–1761.
- Suire, C., Bouvier, F., Backhaus, R.A., Bégu, D., Bonneau, M. and Camara, B. (2000) Cellular localization of isoprenoid biosynthetic enzymes in *Marchantia polymorpha*. Uncovering a new role of oil bodies. *Plant Physiol.* **124**, in press.
- Tollsten, L. and Bergström, G. (1988) Headspace volatiles of whole plants and macerated plants parts of *Brassica* and *Sinapis*. *Phytochemistry*, **27**, 2073–2077.
- Tsukada, M. and Tabata, M. (1984) Intracellular localization and secretion of naphthoquinone pigments in cell cultures of *Lithospermum erythrorhizon*. *Planta Med.* **50**, 338–341.
- Turner, G., Gershenzon, J., Nielson, E.E., Froehlich, J.E. and Croteau, R. (1999) Limonene synthase, the enzyme responsible for monoterpene biosynthesis in peppermint, is localized to leucoplasts of oil gland secretory cells. *Plant Physiol.* **120**, 879–888.
- Von Heijne, G., Hirai, T., Klösken, R.B., Steppuhn, J., Bruce, B., Keegstra, K. and Herrmann, R.G. (1991) CHLPEP-A database of chloroplast transit peptides. *Plant Mol. Biol.* **9**, 104–126.
- Wang, K. and Ohnuma, S.I. (1999) Chain-length determination mechanism of isoprenyl diphosphate synthase and implication for molecular evolution. *Trends Biochem. Sci.* **24**, 445–451.
- Weidenhamer, J.D., Macias, F.A., Fischer, N.H. and Williamson, G.B. (1993) Just how insoluble are monoterpenes? *J. Chem. Ecol.* **19**, 1799–1807.
- Williams, D.C., McGarvey, D.J., Katahira, E.J. and Croteau, R. (1998) Truncation of limonene synthase preprotein provides a fully active 'pseudomature' form of this monoterpene cyclase and reveals the function of the amino-terminal arginine pair. *Biochemistry*, **37**, 12213–12220.
- Wooding, F.B.P. and Northcote, D.H. (1965) The fine structure of the mature resin canal cells of *Pinus pinea*. *J. Ultrastruct. Res.* **13**, 233–244.
- Yamaguchi, S., Sun, T., Kawaide, H. and Kamiya, Y. (1998) The GA2 locus of *Arabidopsis thaliana* encodes ent-kaurene synthase of gibberellin biosynthesis. *Plant Physiol.* **116**, 1271–1278.
- Zinsmeister, H.D., Becker, H. and Eicher, T. (1991) Bryophytes, a source of biologically active, naturally occurring material? *Angew. Chem. Int. ed. Engl.* **30**, 130–147.

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