

Peroxisomal localisation of the final steps of the mevalonic acid pathway in *planta*

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Abstract In plants, the mevalonic acid (MVA) pathway provides precursors for the formation of triterpenes, sesquiterpenes, phytosterols and primary metabolites important for cell integrity. Here, we have cloned the cDNA encoding enzymes catalysing the final three steps of the MVA pathway from Madagascar periwinkle (*Catharanthus roseus*), mevalonate kinase (MVK), 5-phosphomevalonate kinase (PMK) and mevalonate 5-diphosphate decarboxylase (MVD). These cDNA were shown to functionally complement MVA pathway deletion mutants in the yeast *Saccharomyces cerevisiae*. Transient transformations of *C. roseus* cells with yellow fluorescent protein (YFP)-fused constructs reveal that PMK and MVD are localised to the peroxisomes, while MVK was cytosolic. These compartmentalisation results were confirmed using the *Arabidopsis thaliana* MVK, PMK and MVD sequences fused to YFP. Based on these observations and the arguments raised here we conclude that the final steps of the plant MVA pathway are localised to the peroxisome.

Keywords *Arabidopsis* · *Catharanthus* · Isoprenoid · Mevalonic acid pathway · Peroxisome

Abbreviations

AACT	Acetoacetyl-CoA thiolase
FOA	5-fluoroorotic acid
HMGR	3-hydroxy-3-methylglutaryl-CoA reductase
HMGS	3-hydroxy-3-methylglutaryl-CoA synthase
IDI	Isopentenyl diphosphate isomerase
MVA	Mevalonic acid
MVD	Mevalonate 5-diphosphate decarboxylase
MVK	Mevalonate kinase
PMK	5-phosphomevalonate kinase
PTS	Peroxisomal targeting signal
YFP	Yellow fluorescent protein

Introduction

Isoprenoids constitute the largest classes of natural products, with over 22,000 different compounds identified. In higher plants, isoprenoids carry out numerous essential roles in plant developmental processes including respiration, photosynthesis, growth, reproduction and adaptation to environmental challenges as well as being involved in plant defence (Bouvier et al. 2005; Gershenzon and Dudareva 2007).

Isoprenoids are derived from a common C₅ precursor, isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). Two separate IPP-forming pathways are known to co-exist in plants. The 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway that provides the precursors for the synthesis of monoterpenes, carotenoids, apocarotenoids and the side chain of chlorophylls, tocopherols and prenylquinones and the

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mevalonic acid (MVA) pathway that provides precursors for the formation of triterpenes, sesquiterpenes, phytosterols, ubiquinone, vitamin D and primary metabolites important for cell integrity (Rodríguez-Concepción and Boronat 2002; Bouvier et al. 2005). However, the relative contributions of each pathway to the biosynthesis of these various compounds have yet to be determined since cross-talk between these two IPP-forming pathways has been documented (Hemmerlin et al. 2003; Rodríguez-Concepción 2006). Such exchanges involve tightly regulated transports across intracellular membranes due to the subcellular compartmentalisation of both biosynthetic pathways. The MEP pathway enzymes have been entirely localised within plastids (Hsieh et al. 2008); however, the current state of knowledge concerning the localisation of the enzymes of the MVA pathway remains unclear, even though numerous studies have attempted to address the issue, especially in mammalian systems.

The MVA pathway produces IPP through a series of six enzymatic reactions (Bouvier et al. 2005) beginning with the condensation of two molecules of acetyl-CoA to produce acetoacetyl-CoA, a reaction catalysed by acetoacetyl-CoA thiolase (AACT; EC 2.3.1.9). An additional condensation of acetyl-CoA catalysed by the hydroxy-3-methylglutaryl (HMG)-CoA synthase (HMGS; EC 2.3.3.10) allows the formation of HMG-CoA that is subsequently reduced by the HMG-CoA reductase (HMGR; EC 1.1.1.34) to generate mevalonate. Mevalonate then undergoes two successive phosphorylation steps catalysed by mevalonate kinase (MVK; EC 2.7.1.36) and 5-phosphomevalonate kinase (PMK; EC 2.7.4.2) to form mevalonate diphosphate. The final step in the MVA pathway is the decarboxylation of mevalonate diphosphate by mevalonate 5-diphosphate decarboxylase (MVD; EC 4.1.1.33) which yields IPP. IPP undergoes a reversible isomerisation step catalysed by the IPP isomerase (IDI; EC 5.3.3.2) which leads to the generation of DMAPP, thus allowing the biogenesis of terpenoid compounds via IPP/DMAPP condensations (Dewick 2002). Figure 1 shows an overview of the MVA pathway and the related farnesyl diphosphate synthase (FPS) leading to the formation of C₁₅ farnesyl diphosphate, which is the precursor of sterols and sesquiterpenes.

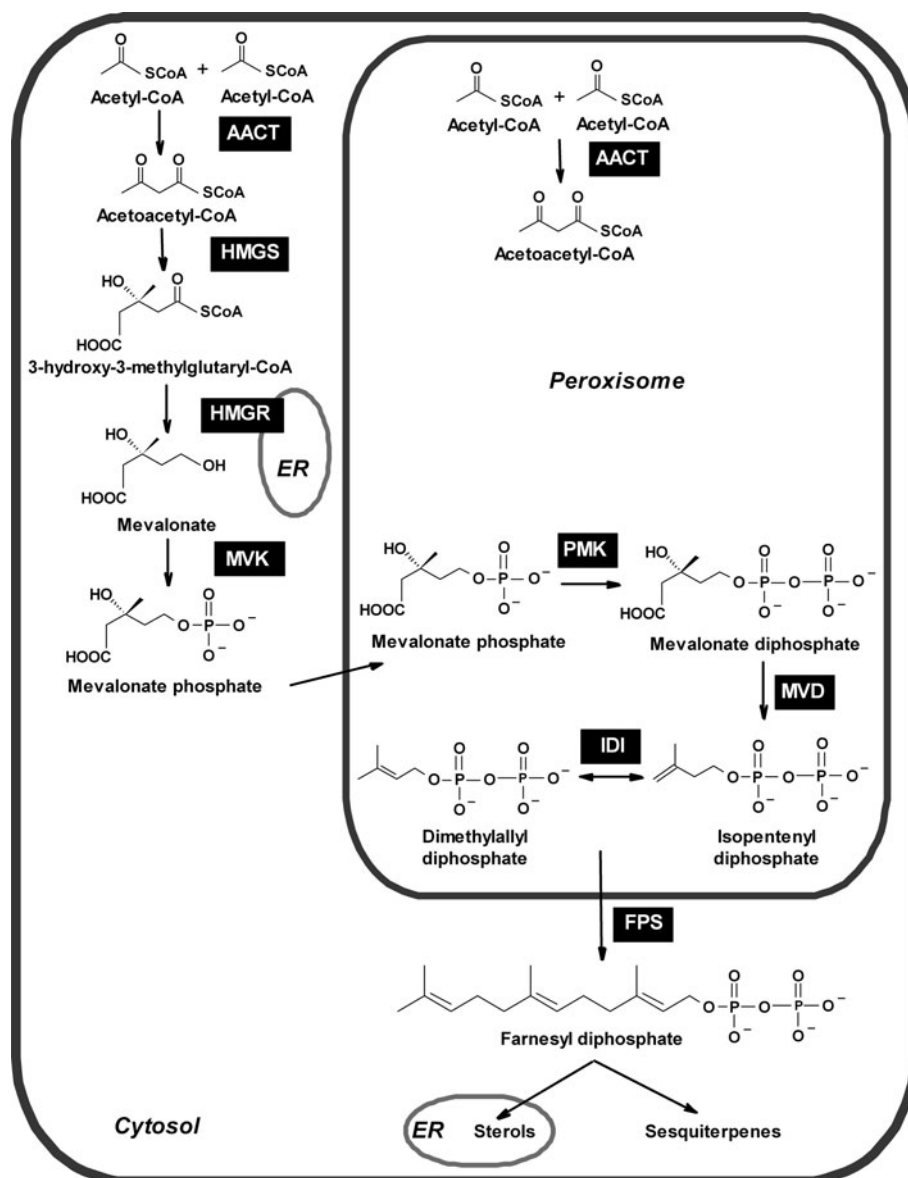
For a long time, the MVA pathway has been considered to be a cytosolic pathway due to the localisation of HMGS in the cytosol (Nagegowda et al. 2005) and the anchoring of HMGR to the endoplasmic reticulum (ER), exposing the protein towards the cytosol (Campos and Boronat 1995). However, recent studies opened new perspectives on the subcellular distribution of the MVA pathway (Reumann et al. 2007; Sapir-Mir et al. 2008).

Proteome analysis of *Arabidopsis thaliana* leaf peroxisomes identified AACT as a peroxisomal protein (Reumann et al. 2007). The involvement of peroxisomes in IPP biosynthesis was also reinforced by the work on the localisation of the two *Arabidopsis* IDI enzymes. The two IDI genes are transcribed each as long and short isoforms. The long versions of protein products are targeted to chloroplasts/mitochondria whereas the short versions, lacking the N-targeting signal, are localised to peroxisomes in agreement with the presence of a peroxisomal targeting signal (PTS) type 1 at their terminal end (Sapir-Mir et al. 2008). Two types of PTS (PTS1 and PTS2) mediate the peroxisome import of proteins by the PTS1 receptor PEX5 and the PTS2 receptor PEX7 (Kaur et al. 2009). PTS1 is a C-terminal tripeptide with the consensus sequence (S/C/A) (K/R/H)(L/M) and PTS2 is a nonapeptide located internally or near the N-terminus and having the usual consensus sequence (R/K)(L/V/I)X₅(H/Q)(L/A/F) (Petřiv et al. 2004).

In addition to the characterisation of the IDI enzyme localisation, Sapir-Mir et al. (2008) identified PTS2-related nonapeptides in *Arabidopsis* HMGS, MVK and MVD. Based on these observations, the authors proposed a new model for the compartmentalisation of the plant MVA pathway in which all the enzymes, except for HMGR bound to the endoplasmic reticulum membrane, are localised to the peroxisomes. But until now, to the best of our knowledge, there are no experimental data directly showing the localisation of the final steps of the MVA pathway in plants. Moreover, several studies have shown that the early steps in the isoprenoid/cholesterol pathway occur in peroxisomes of mammalian cells although conflicting results have been obtained for the enzymes MVK, PMK and MVD including cytosolic localisation (Hogenboom et al. 2004a, b, c) as well as peroxisomal targeting (Olivier et al. 1999, 2000). In 2007, the group of Krisans issued a clarification of their results which confirms previous data showing peroxisomal localisation of several mammalian enzymes of the MVA pathway (Kovacs et al. 2007).

To clarify the situation in plant cells, we have studied the subcellular distribution of three enzymes of the MVA pathway in the medicinal plant Madagascar periwinkle (*Catharanthus roseus*) widely used to study the architecture of the isoprenoid biosynthetic pathways leading to the formation of monoterpenoid indole alkaloids (Hedhili et al. 2007; Oudin et al. 2007), some of which are well known for their pharmacological potential (van der Heijden et al. 2004). This paper reports on the cloning, functional characterisation in yeast and subcellular localisation of the *C. roseus* MVK, PMK and MVD in periwinkle cells. Furthermore, subcellular localisations of the three enzymes were confirmed by studies using the corresponding orthologs from *A. thaliana*.

Fig. 1 Overview of the MVA pathway leading to sesquiterpenes and sterols. The scheme proposes new view of the sub-cellular localisation of the MVA pathway according to all the data that have been experimentally validated for the peroxisomal localisation of the enzymes. *AACT* acetoacetyl-CoA thiolase, *FPS* farnesyl diphosphate synthase, *HMGS* hydroxymethylglutaryl-CoA synthase, *HMGR* hydroxymethylglutaryl-CoA reductase, *MVK* mevalonate kinase, *PMK* 5-phosphomevalonate kinase, *MVD* mevalonate 5-diphosphate decarboxylase, *IDI* isopentenyl diphosphate isomerase



Materials and methods

Reverse transcription and cloning of cDNA sequences from the MVA pathway

Total RNA was extracted from *Catharanthus roseus* [L.] G. Don, Pacifica pink (botanical garden of Tours) and *Arabidopsis thaliana* L. Col-0 (seeds from Nottingham Arabidopsis Stock Centre, UK) leaf samples using the NucleoSpin® RNA Plant Kit (Macherey–Nagel). The cDNAs were synthesised from total RNA using the oligo-dT primer AP according to the protocol in the Superscript II Reverse Transcriptase kit (Invitrogen). A detailed description of the MVA pathway cDNA sequences isolation is provided online as Supplementary methods and the

primers used in the protocol are described in Supplementary Table S1. Database searches for similar protein sequences were performed by using NCBI's BLAST network service. Protein sequence alignment was performed using ClustalW from the Mac Vector program (Oxford Molecular Ltd, UK). The full-length coding sequences of the *C. roseus* *MVK*, *PMK* and *MVD* cDNAs have been deposited at NCBI under Genbank accession numbers: HM462019 (*CrMVK*), HM462020 (*CrPMK*) and HM462021 (*CrMVD*).

Generation of constructs for subcellular localisation studies

MVK, *PMK* and *MVD* were cloned from either *C. roseus* or *A. thaliana* RT-generated cDNAs using primers described

in Supplementary Table S2. Pfu PCR products were digested with *NheI* and cloned into the *SpeI* site of vector pSCA-cassette YFPi to make YFP-cDNA fusions or cloned into the *NheI* site of the same vector to make cDNA-YFP fusions (Guirimand et al. 2009).

Biolistic transformation of *C. roseus* suspension cells and YFP imaging

Transient transformation of *C. roseus* cells by particle bombardment and YFP imaging were performed following the procedures described by Guirimand et al. (2009, 2010). Briefly, *C. roseus* cells plated onto solid culture medium were bombarded with DNA-coated gold particles (1 µm) and 1,100 psi rupture disc at a stopping-screen-to-target distance of 6 cm, using the Bio-Rad PDS1000/He system. Cells were cultivated during 15 to 48 h, and the protein subcellular localisation was determined using an Olympus BX-51 epifluorescence microscope equipped with an Olympus DP-71 digital camera. The pattern of localisation presented in this work is representative of ca. 100 observed cells. Two organelle markers were used for co-transformation studies: the “peroxisome”-CFP (CD3-977) marker obtained from the ABRC (<http://arabidopsis.org>) and the cytosolic CFP-GUS marker described in Guirimand et al. (2010). The “peroxisome”-CFP marker has been obtained by addition of a PTS1 (SKL) at the C-termini of CFP, allowing in turn an efficient peroxisomal targeting (Nelson et al. 2007).

Generation of constructs for yeast complementation assays

The full-length coding sequences of the periwinkle *MVK*, *PMK* and *MVD* cDNAs were recovered by RT-PCR. The cDNAs were amplified with PfuTurbo DNA polymerase (Stratagene) and the primers shown in Supplementary Table S2.

A cDNA of 1,164 bp was recovered by PCR using the pair of primers MVKf and MVKr. The resulting amplicon was digested with *HindIII* and *XbaI* and cloned into the corresponding restriction sites of plasmid pYES2 (Invitrogen) to make the plasmid pYES2-*CrMVK*.

PCR with primers PMKf and PMKr generated the 1,580-bp amplicon of periwinkle *PMK* which was digested with *SacI* and *BamHI* and cloned into the corresponding restriction sites of plasmid pYES2 to make the plasmid pYES2-*CrPMK*.

The 1,260-bp fragment of periwinkle *MVD* was recovered by PCR using primers MVDf and MVDr. The amplicon was digested with *HindIII* and *XbaI* and cloned into the corresponding restriction sites of plasmid pYES2 to make the plasmid pYES2-*CrMVD*.

Yeast complementation assays

Yeast Magic Marker strains were obtained from Thermo Scientific ABgene (Epsom, UK). All strains used in this study are listed in Supplementary Table S3. Yeast transformation was performed as follows: The equivalent of ten yeast colonies from YMR208W, YMR220W and YNR043W heterozygous diploid strains were recovered with a pipette tips from a 24-h-grown YPD (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) plate and suspended in 100 µL PALD (45% polyethylene glycol 4,000, 100 mM lithium acetate, 50 mM DTT). Plasmid DNA (1–10 µg in 1–10 µL of sterile water) was added and mixed vigorously. After heat-shock (30 min, 42°C), cells were directly plated on YCS selective medium (0.67% Yeast Nitrogen Base with ammonium sulphate and without amino acids; 0.08% SC-Ura; 2% dextrose; 2% agar) and incubated at 30°C for 3 days. Plasmid-transformed heterozygous diploid strains [Sc (*erg12Δ/ERG12*) + pYES2-*CrMVK*; Sc (*erg8Δ/ERG8*) + pYES2-*CrPMK* and Sc (*mvd1Δ/MVD1*) + pYES2-*CrMVD*] were allowed to sporulate by plating 10⁹ cells onto ACK medium (1% potassium acetate, 0.25% yeast extract, 2% agar) at 28°C for 7 days. Haploid transformed cells harbouring both the disrupted allele and the plasmid-borne *C. roseus* MVA cDNA [Sc (*erg12Δ*) + pYES2-*CrMVK*; Sc (*erg8Δ*) + pYES2-*CrPMK* and Sc (*mvd1Δ*) + pYES2-*CrMVD*] were selected by plating the sporulated culture of heterozygous diploid pools on a modified galactose-containing magic medium (2% galactose, 0.67% Yeast Nitrogen Base with ammonium sulphate and without amino acids, 0.06% SC-Leu-His-Arg-Ura drop out mix, 200 µg/mL G418, 60 µg/mL canavanine, 2% agar) (Pan et al. 2004) and incubated at 30°C for 3 days. Haploid transformant cells harbouring both wild-type allele and the plasmid-borne *C. roseus* MVA cDNA [Sc (*ERG12*) + pYES2-*CrMVK*; Sc (*ERG8*) + pYES2-*CrPMK* and Sc (*MVD1*) + pYES2-*CrMVD*] were selected by plating the sporulated culture of heterozygous diploid pools on a modified dextrose-containing magic medium (2% dextrose, 0.67% Yeast Nitrogen Base with ammonium sulphate and without amino acids, 0.06% SC-Leu-His-Arg-Ura drop out mix, 60 µg/mL canavanine, 2% agar) and incubated at 30°C for 3 days. The functional complementation was triggered by spotting 5 µL drops (10⁴ cells) of each haploid transformant on YPD or YPG (1% yeast extract, 2% peptone, 2% galactose, 2% agar) and incubated at 30°C for 2 days. Plasmid loss ability was analysed by spotting 5-µL drops (10⁴ cells) of each haploid transformant on 5-fluoroorotic acid (FOA)—containing plates (0.67% Yeast Nitrogen Base with ammonium sulphate and without amino acids; 0.08% SC-Ura; 2% galactose; 1 mg/mL FOA, 50 µg/mL uracil, 2% agar) and incubated at 30°C for 2 days.

Results

Isolation and identification of MVA pathway cDNAs from *C. roseus*

Until now, the *C. roseus* cDNA sequences encoding MVK, PMK and MVD have not been isolated and characterised. Thus, partial cDNA encoding MVA pathway candidate genes were isolated from *C. roseus* cDNA using degenerate primers as described in Supplementary methods. The sequences of the 5' and 3' extremities were determined by 3'RACE-PCR and GenomeWalker. The full-length cDNA sequences were named *CrMVK* (*Catharanthus roseus* mevalonate kinase), *CrPMK* (*Catharanthus roseus* 5-phosphomevalonate kinase) and *CrMVD* (*Catharanthus roseus* mevalonate 5-diphosphate decarboxylase).

The *CrMVK* cDNA encoded a protein of 387 amino acids in length with a calculated mass of 41.0 kDa. *CrMVK* possesses strong similarity with MVKs from dicotyledons such as *Hevea brasiliensis* (77% identity) and *A. thaliana* (67% identity). The similarity with MVKs from monocotyledons such as *Oryza sativa* and *Zea mays* is lower, 59 and 56% identity, respectively. Furthermore, *CrMVK* possesses 47% identity with the ortholog present in the moss *Physcomitrella patens subsp. patens* and 27% identity with the yeast (*S. cerevisiae*) MVK and 37% identity with the human (*Homo sapiens*) and rat (*Rattus norvegicus*) MVKs.

The *CrPMK* cDNA encoded a protein of 498 amino acids in length with a calculated mass of 53.9 kDa. As with *CrMVK*, *CrPMK* possesses strong similarity with orthologs from *H. brasiliensis* (74% identity) and *A. thaliana* (72% identity). The protein also shows lower similarity with orthologs from maize (63% identity), rice (65% identity), *P. patens* (51% identity) and from *S. cerevisiae* (28% identity). However, plant PMKs are very different from mammalian PMKs and cannot be considered orthologs.

The *CrMVD* cDNA encoded a protein of 421 amino acids in length with a calculated mass of 46.6 kDa. In plants several isoforms of MVD exists, including two in *A. thaliana*. *CrMVD* has 80% identity with *AtMVD2* (At3g54250) and 77% identity with *AtMVD1* (At2g38700). *CrMVD* has strong similarity with orthologs from *H. brasiliensis* (83% identity), maize (74% identity), rice (73% identity) and *P. patens* (63% identity). Finally, lower similarities are observed with yeast MVD (46% identity with the ortholog from *S. cerevisiae*) and the mammalian MVDs (47% identity with the ortholog from human and rat).

The similarity of the deduced amino acid sequence of the three identified cDNAs with the amino acid sequences from the plant orthologs of the MVA pathway can be seen in Supplementary Fig. S1, S2 and S3.

Functional complementation of MVA pathway cDNAs from *C. roseus* in yeast

Yeast complementation method was previously used to characterise several cDNAs from the mevalonate pathway. First, Riou et al. (1994) functionally characterised *MVK* from *A. thaliana* and Cordier et al. (1999) characterised the *A. thaliana* *AtMVD1*. More recently, the same technique was used to functionally characterise *AACT*, *HMGS* and *PMK* from *H. brasiliensis* (Sando et al. 2008).

It is now well characterised that, in the yeast *S. cerevisiae*, the MVA pathway remains the unique source of the IPP precursor for the biosynthesis of ergosterol, an essential fungal terpenoid. Gene disruption of enzymes involved in MVA branch is thus lethal in yeast. As a consequence, a complementation assay was performed using a haploid strain lacking a single allele of each target gene to validate the function of the cloned *C. roseus* cDNAs. In this study, we used the full-length coding sequences of *CrMVK*, *CrPMK* and *CrMVD*. It remains unknown at this time if the enzymes of the MVA pathway in yeast are peroxisomal. However, it should be noted that the MVK, PMK and MVD of *S. cerevisiae* contain PTS2-related nonapeptides (Table 1).

Diploid heterozygous *S. cerevisiae* colonies carrying MVA-plasmids or empty-vector-pYES2 were plated onto sporulation medium and haploid colonies were recovered. No haploid cells harbouring both the disrupted allele and the empty control plasmid pYES2 were obtained suggesting that the empty pYES2 is unable to complement the *Saccharomyces erg12Δ* (*mvk*), *erg8Δ* (*pmk*) or *mvd1Δ* (*mvd*) disrupted alleles.

A yeast strain carrying both *erg12Δ* (*mvk*) disrupted allele and the plasmid pYES2-*CrMVK* [Sc (*erg12Δ*) + pYES2-*CrMVK*] could grow on YPG (galactose-containing medium) expression medium, but not on the non-expression medium YPD (dextrose-containing medium) (Fig. 2). This indicated the *CrMVK* had mevalonate kinase activity and could complement the *erg12Δ* (*mvk*) disrupted allele.

The activities of *CrPMK* and *CrMVD* were determined in the same way. A yeast strain carrying both *erg8Δ* (*pmk*) disrupted allele and plasmid pYES2-*CrPMK* [Sc (*erg8Δ*) + pYES2-*CrPMK*] and a yeast strain carrying both *mvd1Δ* (*mvd*) disrupted allele and plasmid pYES2-*CrMVD* [Sc (*mvd1Δ*) + pYES2-*CrMVD*] could grow on YPG medium, but not on YPD medium (Fig. 2), showing that these two *C. roseus* cDNAs were able to complement their respective *S. cerevisiae* alleles. Moreover, all haploid transformed strains harbouring both a wild-type allele and the plasmid-borne *C. roseus* cDNA [Sc (*ERG12*) + pYES2-*CrMVK*; Sc (*ERG8*) + pYES2-*CrPMK* and Sc (*MVD1*) +

Table 1 Sequences of the putative PTS2 motifs within MVK, PMK and MVD

Protein	Organism	Accession number ^a	PTS2-related nonapeptide	Positions ^b	Localisation	References
MVK	<i>C. roseus</i>	HM462019 (Gb)	KIILAGEHA	10–18	Cytosol	This study
	<i>A. thaliana</i>	At5g27450 (AGI)	KIILAGEHA	10–18	Cytosol	This study
	<i>S. cerevisiae</i>	NP_013935 (GP)	KVIIFGEHS	12–20	nd	–
	<i>R. norvegicus</i>	NP_112325 (GP)	KVILHGEHA	13–21	Peroxisome	Kovacs et al. 2007
PMK	<i>C. roseus</i>	HM462020 (Gb)	DVKLTSPQM	57–65	Peroxisome	This study
	<i>A. thaliana</i>	At1g31910 (AGI)	DVKLTSPQL	57–65	Peroxisome	This study
	<i>S. cerevisiae</i>	NP_013947 (GP)	KDGEWLYHI	63–71	nd	–
	<i>H. sapiens</i>	AAH07694 (GP)	no PTS2 ^c	–	Peroxisome	Kovacs et al. 2007
MVD	<i>C. roseus</i>	HM462021 (Gb)	SVTLDP AHL	42–50	Peroxisome	This study
	<i>A. thaliana</i>	At2g38700 (AGI)	SVTLDPDHL	40–48	Peroxisome	This study
		At3g54250 (AGI)	SVTLDPDHL	40–48	nd	–
	<i>S. cerevisiae</i>	NP_014441 (GP)	SISVTLSQD	34–42	nd	–
	<i>R. norvegicus</i>	AAH81784 (GP)	SVTLHQDQL	41–49	Peroxisome	Olivier et al. 2000
Consensus PTS2			(R/K) (L/V/I) X ₅ (H/Q) (L/A)			Petriv et al. 2004

nd not determined

^a Accession number from Genbank (Gb), GenPept (GP) or *Arabidopsis* Genome Initiative (AGI)

^b Indicate the position of the putative PTS2 motif in the amino acid sequence of the protein

^c The human PMK contains a PTS1 motif and is nonorthologous to plant PMK enzymes

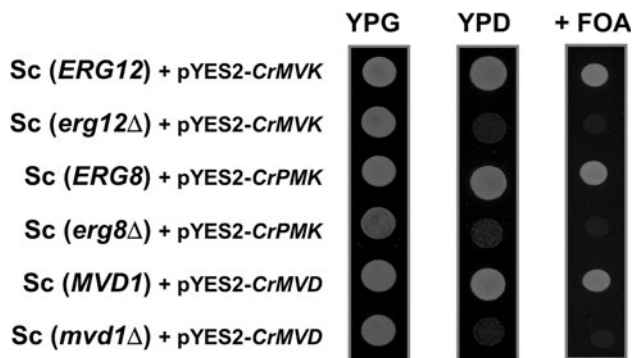


Fig. 2 Yeast complementation assay. Drops containing 10^4 cells from haploid transformant strains harbouring both wild-type allele and the plasmid-borne *C. roseus* cDNA [Sc (*ERG12*) + pYES2-CrMVK; Sc (*ERG8*) + pYES2-CrPMK and Sc (*MVD1*) + pYES2-CrMVD] and from haploid transformed strains harbouring both the disrupted allele and the plasmid-borne *C. roseus* cDNA [Sc (*erg12Δ*) + pYES2-CrMVK; Sc (*erg8Δ*) + pYES2-CrPMK and Sc (*mvd1Δ*) + pYES2-CrMVD] were spotted onto YPG, YPD or FOA-containing (+FOA) plates. YPG contains galactose necessary for the induction of the *C. roseus* enzymes. Each plate was incubated for 1 day at 30°C and photographed

pYES2-CrMVD] were able to grow on both YPG and YPD medium (Fig. 2).

Finally, haploid transformed cells were plated onto FOA-containing medium to test the ability of each strain to lose their plasmid. None of the haploid disrupted strains harbouring the plasmid-borne *C. roseus* cDNA [Sc (*erg12Δ*) + pYES2-CrMVK; Sc (*erg8Δ*) + pYES2-CrPMK and Sc (*mvd1Δ*) + pYES2-CrMVD] could grow on a FOA-contain-

ing medium, confirming that these strains were not able to lose their plasmid (Fig. 2). In contrast, all haploid transformed strains harbouring both a wild-type allele and the plasmid-borne *C. roseus* cDNA [Sc (*ERG12*) + pYES2-CrMVK; Sc (*ERG8*) + pYES2-CrPMK and Sc (*MVD1*) + pYES2-CrMVD] were able to grow on FOA containing medium (presence of a wild-type *Saccharomyces* allele) confirming that they were able to lose their plasmid. All these results clearly indicate that CrMVK, CrPMK and CrMVD display MVK, PMK and MVD activity, respectively.

Mevalonate kinase is a cytosolic enzyme

The deduced amino acid sequences of CrMVK and AtMVK (*A. thaliana* MVK) were analysed for the existence of PTS1 and PTS2 motifs. The N-terminus of CrMVK and of AtMVK was shown to contain the sequence KIILAGEHA which conforms to the PTS2 consensus sequence (R/K)(L/V/I)X₅(H/Q)(L/A) (Table 1). CrMVK and AtMVK were cloned into pSCA-cassette YFPi vector to create MVK-YFP and YFP-MVK constructs. In vivo localisation of CrMVK and AtMVK was determined through a biolistic-mediated transient expression of YFP-fused protein in *C. roseus* cells according to Guirimand et al. (2009). In transiently transformed cells, CrMVK-YFP and YFP-CrMVK showed a diffuse pattern of fluorescence coinciding perfectly with the fluorescence signal of the cytosolic CFP-GUS marker (Fig. 3a–c, e–g, respectively) arguing thus for a cytosolic localisation. Similarly, AtMVK-YFP

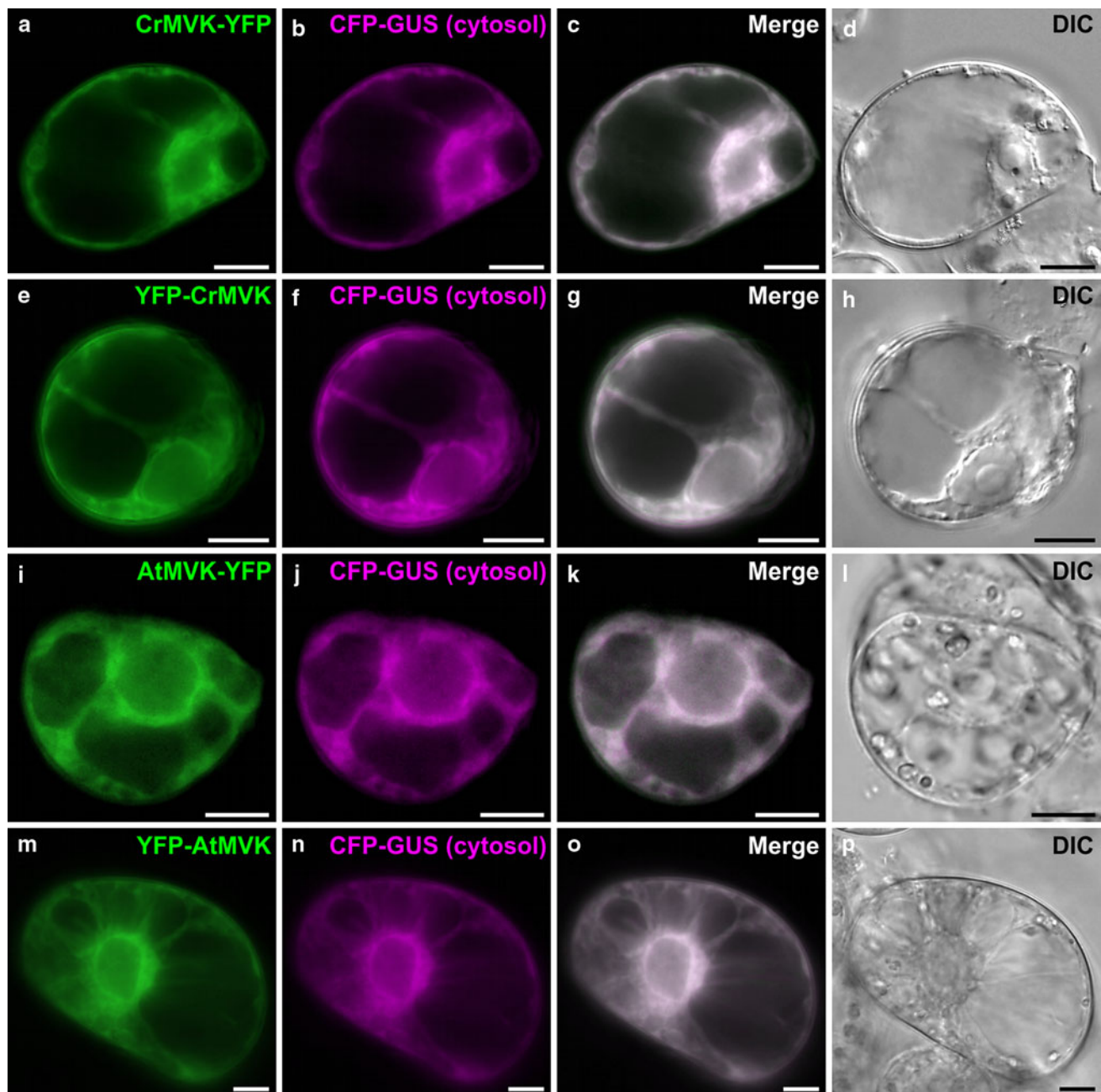


Fig. 3 Subcellular localisation of mevalonate kinase. *C. roseus* cells were transiently co-transformed with plasmids expressing either CrMVK-YFP (**a**), YFP-CrMVK (**e**), AtMVK-YFP (**i**) or YFP-AtMVK (**m**) and the cytosolic CFP-GUS marker (**b**, **f**, **j**, **n**). Co-localization of

two fluorescence signals appeared in *white* (**c**, **g**, **k**, **o**) when merging two individual (*green/magenta*) false-colour images. The morphology (**d**, **h**, **l**, **p**) is observed with differential interference contrast (DIC). Bars 10 μ m

and YFP-AtMVK were also exclusively localised in the cytosol (Fig. 3i–k, m–o, respectively).

5-phosphomevalonate kinase and mevalonate 5-diphosphate decarboxylase are targeted to the peroxisome

It has previously been postulated that the human PMK is predominantly localised in the peroxisome (Olivier et al.

1999). This peroxisomal targeting is dependent on the presence of a PTS1 located at the C-terminal end of mammalian PMKs. On the contrary, no such PTS1 could be found within the amino acid sequences of CrPMK or AtPMK (*A. thaliana* PMK) as well as no PTS2 consensus sequences. However, the PTS2-related nonapeptide DVK (L/V)TSPQ(L/M) was identified in several plant PMKs including CrPMK and AtPMK (Table 1; Supplementary Fig. S2), although its role in peroxisomal targeting has

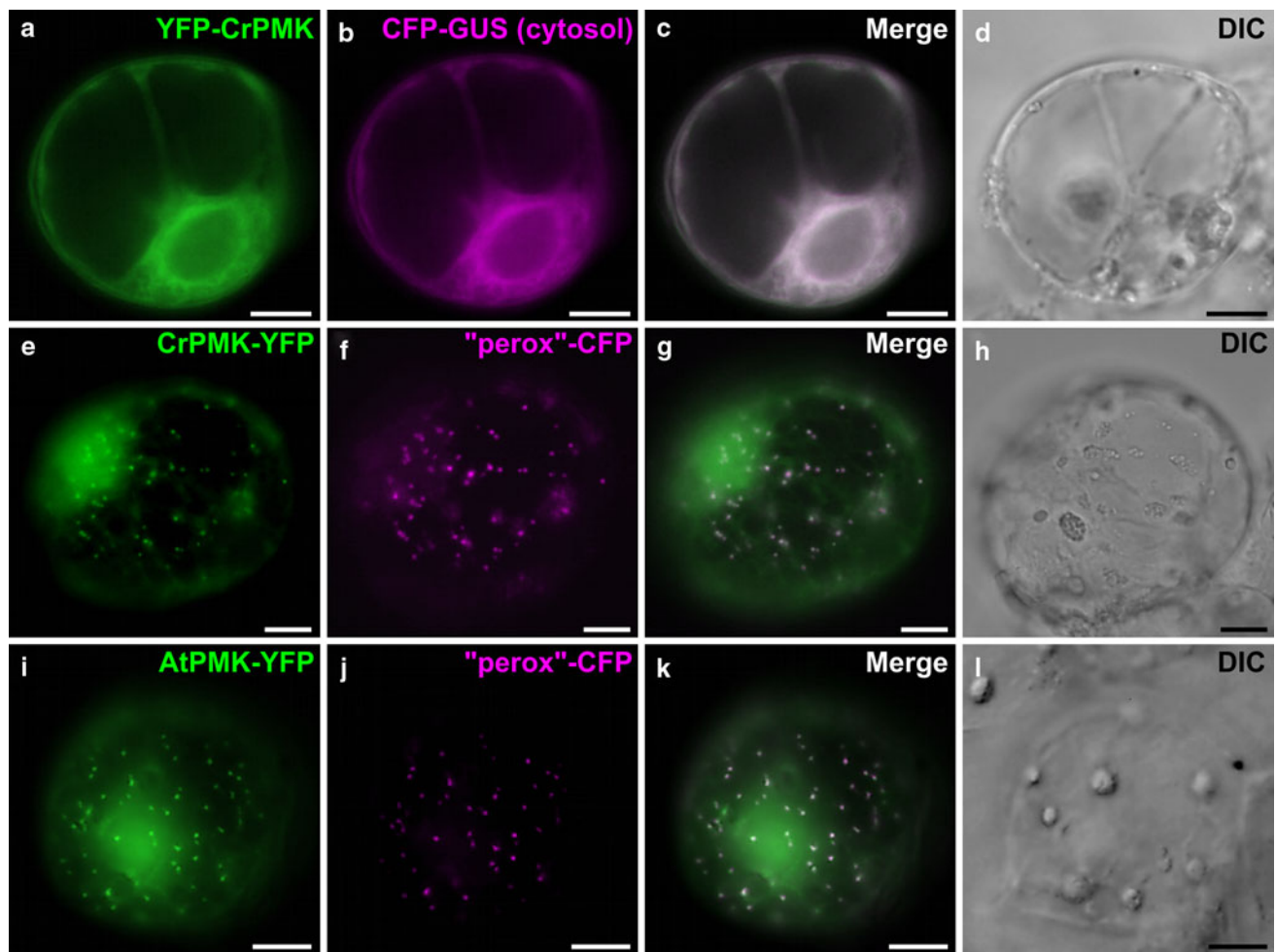


Fig. 4 Subcellular localisation of 5-phosphomevalonate kinase. *C. roseus* cells were transiently co-transformed with plasmid expressing either YFP-CrPMK (a), CrPMK-YFP (e) or AtPMK-YFP (i) and the cytosolic CFP-GUS marker (b) or the peroxisomal marker CD3-

977 (f, j). Co-localization of two fluorescence signals appeared in *white* (c, g, k) when merging two individual (green/magenta) false-colour images. The morphology (d, h, l) is observed with differential interference contrast (DIC). Bars 10 μ m

yet to be determined. This prompted us to engage the study of the subcellular localisation of both CrPMK and AtPMK. In *C. roseus* cells, the YFP-CrPMK fusion protein showed a diffuse pattern of fluorescence characteristic of the cytosol as revealed by the merging with the fluorescence signal of the cytosolic CFP-GUS marker (Fig. 4a–c).

However, in the opposite orientation, leaving the CrPMK N-terminal end accessible, CrPMK-YFP displayed a punctuated fluorescence pattern, which perfectly co-localised with the fluorescence pattern of the peroxisomal marker (Fig. 4e–g). Peroxisomes appear as round or elongated particles distributed randomly in the cytoplasm (Schrader and Fahimi 2008). The peroxisomal localisation of CrPMK was confirmed using AtPMK-YFP which was also shown to co-localise with the peroxisomal marker (Fig. 4i–k). These results indicate that the CrPMK and AtPMK proteins

contain a putative PTS2 that could mediate their targeting to peroxisomes.

The N-terminal end of numerous plant MVDs also contains a PTS2-related nonapeptide (SVTLDPXHL), which can be found in CrMVD and in the two *A. thaliana* MVD isoforms AtMVD1 (At2g38700) and AtMVD2 (At3g54250) (Table 1; Supplementary Fig. S3). This nine amino-acid sequence is similar to the amino-acid motif SVTLHQDQL previously identified in the mammalian MVD and required to target the protein to peroxisomes (Olivier et al. 2000). As a consequence, we have also investigated the subcellular localisation of CrMVD and AtMVD1 in *C. roseus* cells. The fusion protein engaging the N-terminal end of MVD displayed a cytosolic fluorescence signal (Fig. 5a–c) whilst the fusion proteins CrMVD-YFP and AtMVD-YFP which leave the N-terminal extremity accessible showed a punctuate fluorescence distribution

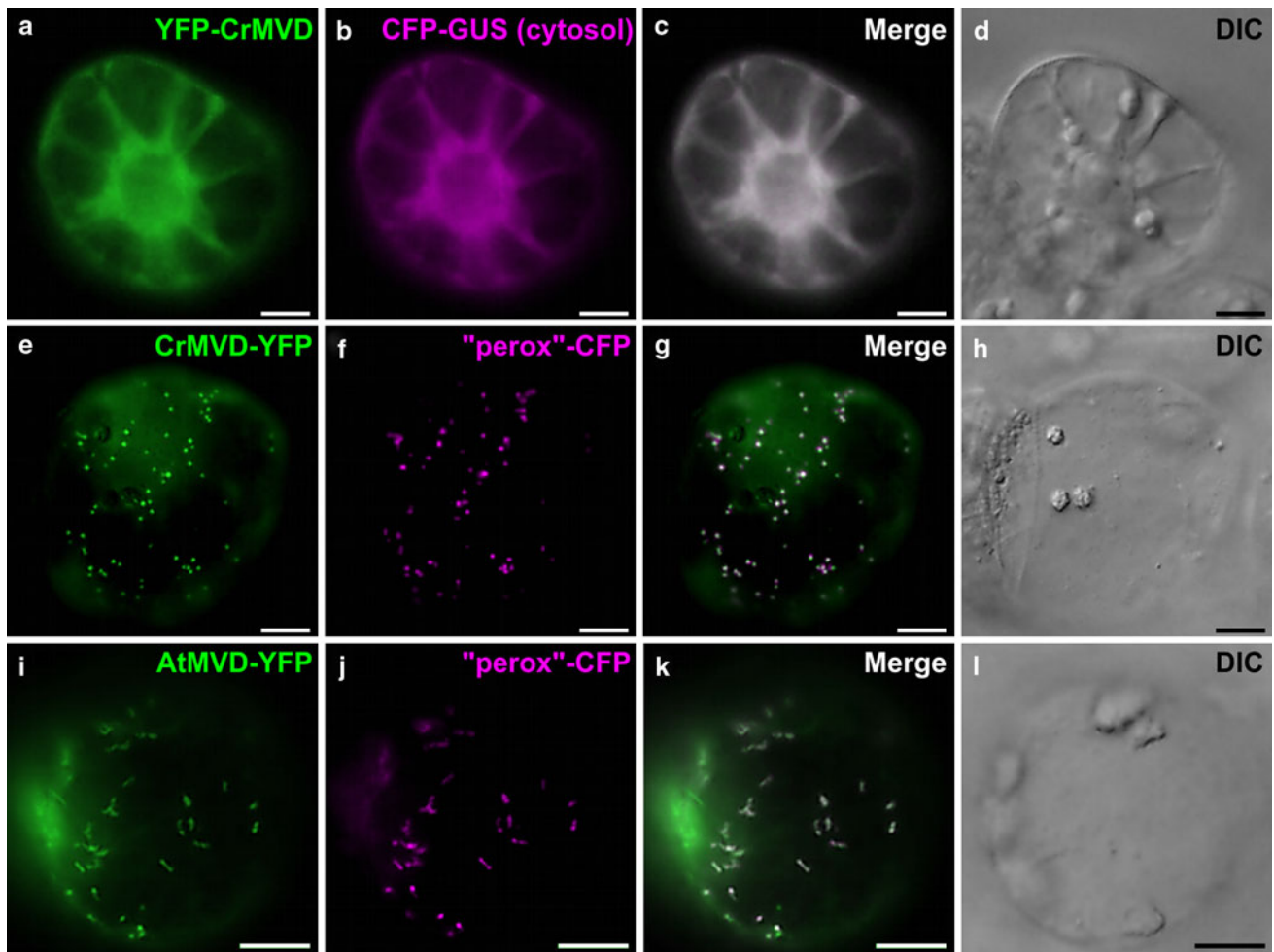


Fig. 5 Subcellular localisation of mevalonate 5-diphosphate decarboxylase. *C. roseus* cells were transiently co-transformed with plasmid expressing either YFP-CrMVD (**a**), CrMVD-YFP (**e**) or AtMVD-YFP (**i**) and the cytosolic CFP-GUS marker (**b**) or the peroxisomal marker

CD3-977 (**f, j**). Co-localization of two fluorescence signals appeared in *white* (**c, g, k**) when merging two individual (*green/magenta*) false-colour images. The morphology (**d, h, l**) is observed with differential interference contrast (DIC). Bars 10 μ m

superimposable over that of the peroxisomal marker (Fig. 5e–g, i–k, respectively). These results reveal that CrMVD and AtMVD1 are also targeted to peroxisomes.

It should be noted that a diffuse pattern of fluorescence is also apparent in Figs. 4e, i, 5e, i, suggesting that a portion of the PMK-YFP and MVD-YFP fusion proteins remained in the cytosol. Similar observations could be made for the localisation of the short version of the *Arabidopsis* IDI2 enzyme since transfected tobacco protoplasts display a strong punctuate peroxisomal pattern and a weakly diffuse pattern that could be associated with the cytosol (Sapir-Mir et al. 2008).

Discussion

In this work, we have isolated and functionally characterised three *C. roseus* cDNAs encoding enzymes of the

final steps of the MVA pathway prior to engage the analysis of their compartmentalisation within the cell. Such studies constitute the first report of the subcellular localisation of the plant MVA pathway enzymes MVK, PMK and MVD. Our results clearly show that MVK is a cytosolic enzyme whereas PMK and MVD are located in the peroxisome.

For these last two enzymes, these data are in agreement with previous reports showing the localisation of the mammalian orthologous enzymes in the peroxisomes (Olivier et al. 2000; Kovacs et al. 2007). They are also in agreement with part of the plant MVA pathway compartmentalisation model proposed by Sapir-Mir et al. (2008) and based on bioinformatic analyses suggesting the existence of PTS in MVA pathway enzymes. Proteins can be addressed to the peroxisomes by several mechanisms (Wolf et al. 2010). A majority of them possess a PTS motif. Proteins containing a PTS1 motif at the C-terminal are imported via the PEX5

receptor. Those possessing a PTS2 motif in the N-terminal region are imported via the receptor PEX7.

Based on subcellular experiments, PTS2 consensus sequences are regularly redefined, rendering their identification more difficult (Petriv et al. 2004; Reumann 2004; Reumann et al. 2009). In the present study, the first invariant residue (R or K) of the PTS2 motif defined by Petriv et al. (2004) is altered in the PTS2-related nonapeptides identified in the peroxisomal PMK (first residue: D) and MVD (first residue: S) from *C. roseus* and *A. thaliana* (Table 1). However, it has been shown that the first invariant residue can be S since the nonapeptide SVX₅QL was clearly identified as a PTS2 motif which utilises the PTS2 import pathway (Olivier et al. 2000). In the majority of cases the PTS2 motif is close to the N-terminal and is cleaved by a protease at a site situated 15–25 residues after the PTS2 motif generating the mature peroxisomal protein (Hayashi et al. 2002). However, in *C. roseus* and *A. thaliana*, the PTS2 motifs of PMK and MVD are situated relatively far from the N-terminal extremity (Table 1), and we cannot confirm that a pre-sequence is cleaved following import in order to generate the mature protein. The cleavage of a PTS2 pre-sequence may not be an exclusive mechanism given that some proteins possess internal PTS2 motifs (Lamberto et al. 2010). There exists also a group of proteins that do not contain classic PTS motifs and are addressed to the peroxisome via a mechanism known as ‘piggy-backing’ in which they form a complex with a protein containing a PTS motif. Finally, the fact that only one construction type (PMK-YFP and MVD-YFP) results in peroxisomal targeting can be explained by a masking of the PTS2 by the fluorescent protein when it is situated in position N-terminal: in this orientation the protein remains confined to the cytosol (Tian et al. 2004).

The cytosolic localisation of MVK is different from that of the mammalian MVK, which is targeted to the peroxisome by the PTS2 KVILHGEHA (Kovacs et al. 2007). While CrMVK and AtMVK also contain the sequence KIILAGEHA that conforms to the PTS2 consensus sequence (R/K)(L/V/I)X₅(H/Q)(L/A) (Table 1), no peroxisomal targeting has been observed for either of the YFP fusion orientations. This shows that the presence of a canonical PTS2 motif is not necessary sufficient to target the protein to peroxisome. Indeed, Mizuno et al. (2008) showed that of five mouse proteins containing conserved PTS2 motifs identified by bioinformatic approaches, none located to peroxisome. The authors pointed out the poor performance of computer-aided predictions of PTS2-containing proteins.

Furthermore, some proteins showing dual compartmentalisation are located in the cytosol or peroxisomes depending on the surface accessibility of their PTS motifs. Thus, the PTS1-mediated peroxisomal import of the human solu-

ble epoxide hydrolase is dependent on the protein expression level and on the protein quaternary structure, which affects the accessibility of PTS1 to the PEX5 receptor (Luo et al. 2008). Furthermore, the over-expression of MVK under these experimental conditions could affect the protein quaternary structure and in consequence mask the PTS2. Another explanation is that the fusion of the YFP at the N- or C-terminal could physically obstruct the PTS2 (Tian et al. 2004).

In regard to plants, there are no data reporting the compartmentalisation of all the MVA pathway enzymes in the same species although some individual enzyme localisations in different model systems have been published (Fig. 1). Thus, the *A. thaliana* AACT seems to have dual localisation in both the peroxisome and the cytosol (Carrie et al. 2007; Ahumada et al. 2008). However, AACT is not restricted to the MVA pathway, and it has been shown that *A. thaliana* contains two genes *AACT1* and *AACT2* encoding three and two proteins, respectively. GFP experiments showed that the AACT1.3 isoform is targeted to peroxisomes, while the others remain cytosolic. On the basis of a microarray analysis which revealed that the gene *AACT2*, encoding the cytosolic isoforms AACT2.1 and AACT2.2, co-expresses highly with *HMGS* and *MVD*, it has been suggested that the AACT isoform involved in the MVA pathway is encoded by *AACT2* and is therefore a cytosolic enzyme (Carrie et al. 2007). When transiently expressed in onion epidermal cells and tobacco BY-2 cells, the *Brassica juncea* HMGS was shown to be localised in the cytosol (Nagegowda et al. 2005). HMGR has been shown to be anchored to the ER by a trans-membrane domain, exposing the catalytic domain of the enzyme to the cytosol (Campos and Boronat 1995). Furthermore, HMGR has been localised within ER and within spherical vesicular structures in *Arabidopsis* plants (Leivar et al. 2005) and tobacco BY-2 cells (Merret et al. 2007). As determined in this work, in *C. roseus* cells, MVK occurs in the cytosol while PMK and MVD reside in peroxisomes.

Although these data provide evidence for complex compartmentalisation of the MVA pathway suggesting subcellular trafficking of pathway intermediates, we conclude, according to this study and that of Sapir-Mir et al. (2008) that the last three steps catalysed by PMK, MVD and IDI are located to peroxisomes (Fig. 1). Therefore, it will be necessary to revisit the classical sequestration of isoprenoid biosynthesis between plastids and cytoplasm by including the peroxisome as an additional isoprenoid biosynthetic compartment within plant cells.

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