Critical Reviews in Biochemistry and Molecular Biology, 34(2):107–122 (1999)

Mevalonate Biosynthesis in Plants

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CONTENTS

I. SYNTHESIS OF MEVALONIC ACID

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGR, *R*-mevalonate: $NADP^+$ oxidoreductase, CoA-acylating, EC 1.1.1.34) catalyzes the reductive conversion of HMG-CoA to mevalonic acid (MVA). This reaction is generally considered as a key controlling step in plant isoprenoid biosynthesis.[1–4] However, the role of HMGR in the overall control of plant isoprenoid biosynthesis has not yet been unequivocally established. In spite of the interest in plant HMGR, the molecular characterization of this enzyme was hampered by the fact that it is membrane bound and difficult to purify (see Bach et al.[3,5] for literature). That is why many of the molecular properties of plant HMGR did not emerge until the genes encoding the enzyme had been cloned.

After characterization of a considerable number of HMGR genes (for literature see Stermer et al.[4]), it now seems clear that plant HMGR is encoded by multigene families. The number of genes comprising each multigene family varies, depending on the species, ranging from the two genes found in *Arabidopsis thaliana*[6,7] to the at least seven genes reported to occur in potato.[4] This is in sharp contrast with animal systems in which the enzyme is encoded by a single gene. The presence of multiple plant HMGR isozymes is consistent with the proposed key role of this enzyme in plant isoprenoid biosynthesis.

Up to now, *Arabidopsis* is the only plant species from which all the HMGR genes have been cloned and characterized.[6,7] The simplicity of the *Arabidopsis* HMGR gene family, together with the well-recognized advantages of this plant for molecular and genetic studies, make *Arabidopsis* an attractive model system to elucidate the regulatory role of HMGR in plant isoprenoid biosynthesis.

II. *ARABIDOPSIS* CONTAINS TWO DIFFERENTIALLY EXPRESSED GENES ENCODING HMGR

The two HMGR genes (*HMG1* and *HMG2*) have recently been cloned and their structure and expression analyzed. *HMG1* and *HMG2* consist of four exons and three small introns that interrupt the coding

sequence at equivalent positions.[7] A similar pattern of organization was found for the HMGR genes isolated from *Hevea brasiliensis*,[8] tomato,[9] and *Camptotheca acuminata*.[10]

By using gene-specific probes, we have shown that *Arabidopsis HMG1* and *HMG2* are differentially expressed. *HMG1* mRNA accumulates at relatively high levels in all parts of the plant, whereas *HMG2* mRNA is detected at a much lower level and its expression is restricted to seedlings, roots, and inflorescences.[7] A more detailed analysis using transgenic plants has confirmed the generalized expression of *HMG1*. As to *HMG2*, expression of this gene is restricted to meristematic (root tip and shoot apex) and floral tissues (secretory zone of the stigma, mature pollen grains, gynoecium, vascular tissue, and fertilized ovules).[11] While the broad expression of *HMG1* suggests that it may encode a housekeeping form of HMGR, the enzyme encoded by *HMG2* might play a role in the synthesis of specific isoprenoids in specific cell types or during specific developmental stages. The expression of *HMG2* in meristematic tissues could be primarily related with the high rate of sterol biosynthesis that is required to sustain the active synthesis of membranes in actively dividing cells. In addition, the expression of *HMG2* could also be related to protein prenylation, a process known to be essential for cell growth and division[12,13] (see also elsewhere in this volume). In contrast to this, the expression of *HMG2* in the floral tissues does not appear to be related to cell division. The high level of expression in pollen grains may be required for the synthesis of specific isoprenoid compounds (mainly sterols) during the rapid growth of the pollen tube. The expression in fecundated ovules might be involved in the synthesis and accumulation of storage isoprenoids (or isoprenoid precursors) in the seeds. This possibility is supported by the observation that both germination and the early stages of seedling development are not affected by mevinolin, a specific inhibitor of HMGR (A. Boronat and T. J. Bach, unpublished results). The presence of a pool of isoprenoid compounds or intermediates in maize seeds has also been proposed by Moore and Oishi.[14]

As a first approach towards the identification of the control mechanisms underlying the differential expression of the *HMG1* and *HMG2* genes, we have analyzed the expression of truncated versions of their promoters fused to a reporter gene using both transgenic plants and transfected protoplasts. The results obtained indicate that the expression of these genes is controlled by complex mechanisms which involve the use of alternative promoters and *cis*-regulatory elements located both in the 5′-flanking region and in the leader sequence (V. Lumbreras, C. Marín and A. Boronat, unpublished results).

A. CHARACTERIZATION OF THE *ARABIDOPSIS* HMGR ISOFORMS

The use of alternative promoters in the *HMG1* gene results in the synthesis of two HMGR isoforms (HMGR1S and HMGR1L), which differ in their N-terminal region. The presence of an in-phase upstream AUG initiator-codon in the HMGRL mRNA results in the synthesis of a second HMGR1 isoform that has 50 additional amino acid residues at its N-terminal end (Lumbreras et al., submitted) (Figure 1). Therefore, the two *Arabidopsis* HMGR genes encode at least three different HMGR isoforms.

A structural model for plant HMGR has been proposed on the basis of primary sequence comparisons.[3,15] Four regions have been defined in the protein: the N-terminal region (highly divergent), the conserved membrane domain (containing two hydrophobic sequences), the linker region (also highly divergent), and the highly conserved catalytic domain. The analysis of the primary structure of the three *Arabidopsis* HMGR isoforms reveals that they have the general structure previously defined for plant HMGR. However, while the isoforms HMGR1S and HMGR1L are almost identical (differing only in their N-terminal end), HMGR2 represents a highly diverged form of the enzyme that has no counterpart among the plant HMGR isozymes characterized so far.[7] These data, together with the qualitative and quantitative differences observed in the expression pattern of the *Arabidopsis* HMGR genes[7] (Lumbreras et al., submitted), raises the question about the specific role of each HMGR isoform in the isoprenoid biosynthetic pathway. The differences observed in the primary sequence of the three *Arabidopsis* HMGR isoforms might result in different kinetic and/or regulatory properties, intracellular compartmentation or membrane topology.

The catalytic domains of isoforms HMGR1S/L and HMGR2 have been expressed in *E. coli* in a catalytically active form. After purification it was revealed that the kinetic parameters for both enzyme preparations were very similar, thus indicating that the three *Arabidopsis* HMGR isoforms do not significantly differ in their catalytic properties (M. Arró, A. Boronat and A. Ferrer, unpublished results). Similar observations were made when recombinant, truncated forms of radish HMGR1 and HMGR2 were overexpressed in *E. coli* and kinetically analyzed (A. Ferrer, A. Boronat, S. Zeiler and T. J. Bach, unpublished). However, posttranslational modification(s) in response to various environmental stimuli (reviewed by Stermer et al.[4]) could specifically affect each individual isoform, inducing changes in their kinetic parameters. Recent results have shown that the catalytic domain of both *Arabidopsis* HMGR1S/L

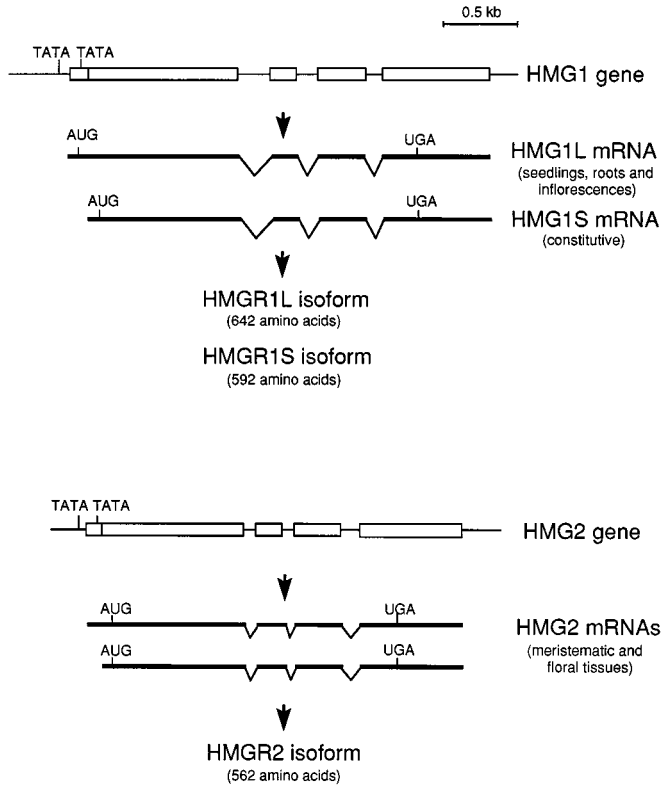

Figure 1 Schematic representation of the *Arabidopsis thaliana HMG1* and *HMG2* genes and their corresponding transcripts and encoded proteins. White boxes indicate exons.

and HMGR2 can be phosphorylated *in vitro* by cauliflower HMGR kinase A. Furthermore, phosphorylation of the catalytic domain of HMGR1S/L results in the concomitant loss of enzyme activity (Dale et al., submitted). The mechanism of this deactivation might be similar to that recently described for the phosphorylation-dependent inactivation of hamster HMGR.[16,17] However, the importance of this regulatory mechanism in the control of plant HMGR activity *in vivo* yet remains to be established, although *in vitro* assays provide evidence for the presence of a protein kinase cascade in higher plants.[18]

B. INTRACELLULAR LOCALIZATION OF *ARABIDOPSIS* HMGR ISOZYMES

One of the most challenging and controversial aspects of plant isoprenoid biosynthesis concerns the intracellular location of the enzymes involved in the synthesis of mevalonic acid (see Kleinig;[19] Bach et al.;[3,5] Stermer et al.;[4] Bach[20] for literature and discussion). Although there is general agreement upon plant HMGR being associated with microsomal membranes, there are reports that claim that the enzymatic activity is also present in plastids and mitochondria.[21,22] We have analyzed the targeting of the *Arabidopsis* HMGR isoforms to endoplasmic reticulum (ER)-derived microsomal membranes, using a wheat germ *in vitro* translation system. Our results show that the three HMGR isoforms are cotranslationally inserted into the ER membrane, where they behave as integral membrane proteins[7] (Lumbreras et al., submitted). The observations are consistent with the model that the insertion of HMGR into ER membranes is specifically mediated by the interaction of the two hydrophobic sequences with the signal recognition particle (SRP) (Campos and Boronat, submitted). Because of the differences in the molecular mechanisms which are involved in the targeting of proteins to the ER and to plastids or mitochondria, it seems very unlikely that any of the *Arabidopsis* HMGR isoforms could also be directed to these organelles.

In order to define the topology of *Arabidopsis* HMGR in the ER membranes, we have performed posttranslation analysis involving digestion with proteinase K, in combination with fractionation of the microsomal vesicles. Our observations clearly demonstrate that the proteins contain two membrane-spanning sequences, as was proposed previously.[6] The catalytic domain and the N-terminal region are

located at the cytosolic side of the ER membrane, while only a short hydrophilic region, rich in charged amino acids, is exposed to the luminal side (Figure 2). Considering the fact that the membrane domain is highly conserved among plant HMGR isozymes and that the two hydrophobic sequences are responsible for the targeting to the ER membrane (Campos and Boronat, manuscript in preparation), it is likely that all plant HMGR reported so far are targeted to the ER and exhibit the same membrane topology. Our results support the hypothesis that the formation of mevalonate in higher plants occurs solely in the cytosol.[19,23] Further support is given by the fact that only one gene for MVA kinase seems to be present in *Arabidopsis*.[24] However, as will be discussed below, this does not necessarily mean that there is only one intracellular location of isopentenyl pyrophosphate (IPP) formation.

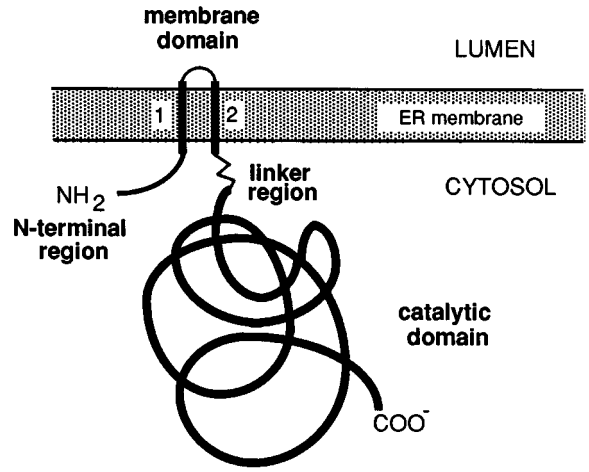

Figure 2 Topological model of plant HMGR in the endoplasmic reticulum (ER) membrane. The two hydrophobic sequences of the membrane domain are indicated (1 and 2). For simplicity, the different regions of the enzyme are not drawn to scale.

III. SYNTHESIS AND METABOLISM OF HMG-CoA

For yeast and mammalian tissue it has been well documented (*cf.* Bach et al.[2,5] and literature cited therein) that the conversion of three units of acetyl-CoA to HMG-CoA is catalyzed by two distinct enzymes: a) acetoacetyl-CoA thiolase (AACT, acetyl-CoA acetyl transferase, E.C. 2.1.3.9) catalyzing a Claisen-type condensation, and b) 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS, *(S)*-3-hydroxy-3-methylglutaryl-coenzyme A: acetoacetyl-coenzyme A lyase (CoA acylating), EC 4.1.3.5), catalyzing an aldol condensation. Besides being the substrate for HMGR, HMG-CoA serves as a putative intermediate in the degradation of leucine *via* HMG-CoA lyase (HMGL, *(S)*-3-hydroxy-3-methylglutaryl coenzyme A lyase, EC 4.1.3.4).

A. FUNCTION OF THIOLASES

Two different types of thiolase are found both in eukaryotes and in prokaryotes: AACT (EC 2.3.1.9) and 3-ketoacyl-CoA thiolase (= 3-oxoacyl-CoA thiolase, OACT, EC 2.3.1.16). OACT (also called thiolase I) has a broad chain-length specificity for its substrates and is involved in degradative pathways such as fatty acid β-oxidation. AACT (also called thiolase II) is specific for the thiolysis of acetoacetyl-CoA and involved in biosynthetic pathways such as poly-β-hydroxybutyrate synthesis or isoprenoid biogenesis. In eukaryotes, there are two forms of OACT: one located in mitochondria and the other in peroxisomes (for literature see Peoples and Sinskey,[25] Igual et al.[26]). In yeast, the formation of acetoacetyl-CoA appears to be an important step in the regulation of ergosterol biosynthesis.[27,28]

B. ENZYME PURIFICATION AND CHARACTERIZATION FROM PLANTS

For plants only little was known until recently as to enzymology and genetics of the aforementioned reactions.[1–3,29–32] For instance, we have purified and characterized a membrane-associated enzyme system from 4-day-old etiolated radish seedlings (*Raphanus sativus* L.), capable of converting acetyl-CoA into HMG-CoA.[30] This enzyme system, apparently catalyzing both partial reactions, is further referred to as

AACT/HMGS. The quite unusual *in vitro* properties of this enzyme system led us to believe that plants might have developed an additional mechanism of HMG-CoA synthesis different from other eukaryotes. The most puzzling feature of radish AACT/HMGS is its apparent *in vitro* activation by redox cycling as mediated by the artificial redox couple Fe(II)/pyrroloquinoline quinone (PQQ), or, alternatively, by Fe(II) and higher concentrations of H_2O_2, suggesting the involvement of hydroxyl radicals formed by a Fenton-Haber-Weiss reaction [$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$]. This effect can be seen with crude solubilized radish AACT/HMGS, but it is more pronounced with purified enzyme, and it can be further enhanced by addition of PQQ.[33] It is evident that during the purification we remove a heat-stable cofactor system (that remains yet to be identified), which can not only partially replace the PQQ, but also to a certain extent the Fe^{2+}. However, at present we cannot exclude that this new system represents a multifunctional enzyme or even an intrinsic side activity of a plant-specific enzyme, which might be less specific than we thought before. Although on sodium dodecyl sulfate (SDS) gels we detected only a prominent band of about 56 kDa,[30] in good agreement with values obtained by gel filtration on various materials,[2,5] an underlying impurity cannot be excluded. This requires further studies. A final proof of the existence of the so-called AACT/HMGS enzyme system will need to await the isolation of the corresponding genes.

C. CLONING OF A cDNA FROM RADISH ENCODING CYTOSOLIC (BIOSYNTHETIC) AACT

1. Generation and Use of Suitable Yeast *erg* Mutants

For the cloning of genes involved in the conversion of acetyl-CoA to HMG-CoA we have chosen the technique of complementation of suitable ergosterol-deficient (*erg*) and thermo-labile yeast mutants.[34] *erg* mutants that are impaired in AACT range into two linked complementation units, *erg10A* and *erg 10B,* while mutants deficient in HMGS belong to two unlinked complementation groups, *erg11* and *erg13*.[35] Whereas to the best of our knowledge no HMGS gene could be isolated by complementation analysis, although gene disruption of yeast HMGS has recently been reported,[36] the *erg10* mutant has been successfully used to clone an AACT gene from the industrial yeast *Saccharomyces uvarum.*[37] The original ergosterol-auxotroph yeast F2SP5 (*erg10A*)[35] is a derivative of the strain FL100, which bears a mutation in the *GAL2* gene, encoding galactose transferase. Thus, for heterologous expression of plant cDNAs, placed under the *GAL1* promoter, it was mandatory to generate an *erg10A* recipient strain with the wild-type allele *GAL2* being reconstituted, which was achieved by crossing of the two haploid strains F2SP5 (Mat a) and W303-1B (Mat α, see Thomas and Rothstein[38]) permitting galactose uptake. This allowed for the use of a newly established radish cDNA expression library in which the genes are placed under the control of the *GAL1* promoter. We succeeded in the isolation of a radish cDNA, complementing the *erg10* mutation of yeast and apparently coding for a cytosolic (biosynthetic) AACT.[39] Some technical details of this latter work have been presented.[40] A full description has appeared elsewhere.

2. Sequence Analysis

Sequence analysis of the radish cDNA insert of plasmid pYRS10, conferring ergosterol-autotrophy and thermoresistance to the yeast strain KV5 (*erg10*), revealed the existence of an open reading frame containing the entire coding unit for a protein of about 42 kDa, in agreement to thiolase proteins from other organisms. There is no evidence for the presence of a leader peptide characteristic of peroxisomal OACT from cucumber,[41,42] where the precursor form is approximately 4 kDa larger than the 45-kDa subunit of the mature enzyme. A search on the GenBank/EMBL databanks by means of the BLAST network service[43] revealed the existence of significant sequence homologies with domains of various eukaryotic and prokaryotic thiolases (see for instance Peoples et al.[44] Peoples and Sinskey[25]). Conserved domains in the carboxy-terminal end of thiolases are shown in Figure 3. The consensus sequence for the putative active center of thiolases has been determined by Yang et al.[45] and is also present in radish AACT (identical amino acids are underlined):

$$\underline{\text{Asn}}\text{-}X_1\text{-}X_2\text{-}\underline{\text{Cys}}\text{-}X_3\text{-}\underline{\text{Ser}}\text{-}\underline{\text{Gly}}(\text{Ala})\text{-}X_4\text{-}X_5$$

In most cases, X_1 represents a basic residue (Arg, Lys). X_2 is a hydrophobic amino acid (Leu, Val, Phe), with the exception of most peroxisomal thiolases, where this place is occupied by a Gln. X_3 is a Gly, Ala, or a Ser. In position X_4 we find hydrophobic residues such as Leu, Phe, Met. The amino acid residue at position X_5 seems to play an important role in the substrate specificity: in OACT it is occupied by Gln or Met, while in acetoacetyl-CoA-specific thiolases a basic residue is present (Arg, Lys). This

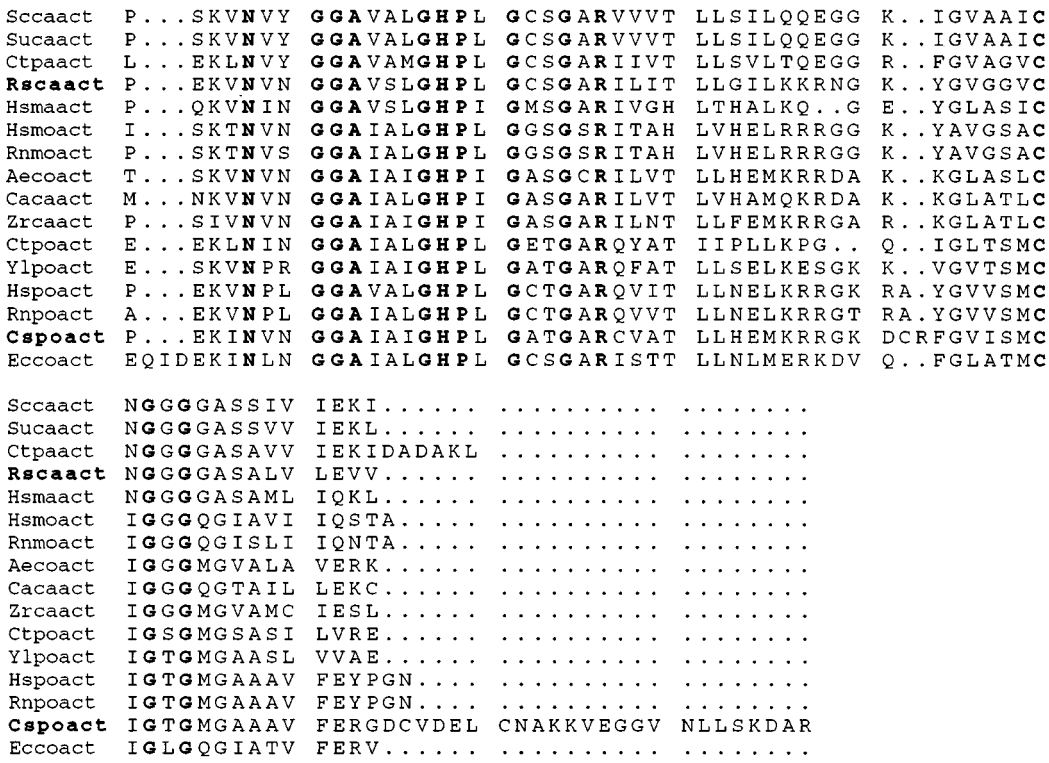

Figure 3 Conserved domains at the carboxy-terminal end of eukaryotic and prokaryotic thiolases. Amino acid residues that are common to all sequences are in bold letters. Only full-length sequences have been considered. The nucleic acid sequence of cRS10 and the deduced amino acid sequence were used to search database libraries for homologies with the DNASIS/PROSIS® software from Hitachi. Further sequence analyses were performed with the BLAST network service.[43] Sequence alignments and further analyses were performed by using the Genetics Computer Group sequence analysis software package.[72] aact, acetoacetyl-CoA thiolase, oact, 3-oxoacyl-CoA thiolase. Putative subcellular localization: c, cytosolic; m, mitochondrial; p, peroxisomal. Databank accession numbers are given in parentheses: Ae, *Alcaligenes eutrophus* (J04987); Ca,*Clostridium acetobutylicum* (U08465); Cs, *Cucumis sativus* (X67696); Ct, *Candida tropicalis* (aact: D13471; oact: D17321); Ec, *Escherichia coli* (fadA gene, J05498); Hs, *Homo sapiens* (aact, mitochondrial: D90228 M61117; oact, peroxisomal: X12966); Rn, *Rattus norvegicus* (aact, mitochondrial: D00512; oact, mitochondrial: X05341; peroxisomal: D90063 J05269); Rs, *Raphanus sativus* (radish; X78116); Sc, *Saccharomyces cerevisiae* (L20428); Su, *Saccharomyces uvarum* (X07976); Yl, *Yarrowia lipolytica* (X69988; Zr, *Zoogloea ramigera* (J02631).

latter observation is further indicative of having isolated a radish gene coding for AACT rather than for OACT.

Fukao et al.[46] argued against the existence of hydrophobic domains which could act as potential binding sites of carbohydrate chains being present in acyl-CoA substrates. However, homology research with the radish AACT cDNA revealed the existence of a conserved hexapeptide Leu-Gly-His-Pro-Leu-Gly-Cys (five identical residues, two conservative exchanges) with a 6-amino acid peptide of human phosphatidyl sterol acyltransferase (AA 202 to 207),[47] which was described to function as an interfacial lipid binding site. This sequence is embedded into a highly conserved domain in the carboxy-terminal region with the following consensus sequence (AA residues in radish AACT underlined):

Gly-Gly-Ala-Ile/Val-Ser/Val-Leu/Ile-Gly-His-Pro-
Ile/Leu-Gly-X(Cys)-Ser/Thr-Gly-X(Ala)-Arg

This sequence seems to be essential for the stability of thiolase proteins as was revealed by site-directed mutagenesis, using the human OACT gene.[48,49] A further, highly conserved cysteine residue close to the carboxy-terminal end (see Figure 3) appears to participate in the catalysis during the second

partial reaction of the Claisen reaction, which involves proton abstraction from the acyl-CoA residue that is not bound to the enzyme and thus generates a nucleophile enolate ion or the corresponding enol.[50]

3. Southern and Northern Blot Analyses

Southern blot analysis with the radish cDNA (cRS10) coding for AACT under stringent hybridization conditions suggests the presence of a single gene, but most possibly having several introns[39] (Vollack and Bach, submitted). Preliminary studies as to the expression of the AACT gene in radish showed the presence of a 1.5 kb transcript.[39] Apparently, the transcription rate did not change significantly during the development of seedlings (day 2 to 8), similarly to radish *HMG2* (functionally identical to *HMG1* in *Arabidopsis*), which was determined in parallel. There is some apparent induction of AACT mRNA in light-treated radish cotyledons, while expression in hypocotyls and roots was hardly affected.[39] The signal having the higher intensity was found with RNA isolated from roots, stems, and cotyledons of radish seedlings, suggesting a constitutive expression of the corresponding AACT gene in all major parts. This implies that the AACT gene has more a housekeeping function. A detailed study will require a careful promoter analysis as outlined above for the examination of HMGR regulation in *Arabidopsis*.

When radish seedlings were grown in the presence of the HMGR inhibitor mevinolin, Northern blot analysis revealed the appearance of a further strongly hybridizing band of ~800 bp.[39] Under conditions where the *de novo* phytosterol biosynthesis is blocked (see Bach et al.[5] and literature cited therein) this might indicate some sort of stress response. Though this 800-bp band was already weakly visible with RNA isolated from control seedlings, the sharp increase following mevinolin treatment might be interpreted to mean that either a gene is activated that bears considerable sequence homology to the AACT gene we have identified, or that, for instance by alternative splicing, we have a further transcript that might encode a small protein having a rescue function. TATA box elements in the noncoding strand could also give rise to an antisense transcript (see below).

The synthesis of (radish) AACT mRNA in the yeast can be estimated to be several 1000-fold higher than in 4-day-old radish seedlings. We noted the appearance of an additional, smaller hybridizing transcript (about 1.2 kb) that appeared under glucose-repressed conditions. Since no signal was obtained with control cells, the conclusion can only be that it arises from a transcript of the radish AACT cDNA, which is not controlled by glucose repression of the *GAL1* promoter. Analysis of the nucleotide sequence of the AACT clone cRS10 in the 3′-flanking, nontranslated region showed the presence of TA-rich nucleotide stretches. On the antisense strand several TATA box sequences could be identified[39] (Vollack and Bach, in preparation), which could give rise to antisense mRNA species in the transformed yeast and in radish.

4. AACT Activity in Transformed Yeast

For the assay of AACT activity in cell-free yeast extracts we adopted the method described for the coupled test system AACT/HMGS described in detail.[30] The assay is actually a modification of the method used for the assay of avian HMGS introduced by Clinkenbeard et al.[51] Here we use ^{14}C-labeled acetyl-CoA as the only substrate, which is then converted to acetoacetyl-CoA by action of AACT. Endogenous yeast HMGS will use this intermediate for the condensation with a further acetyl-CoA unit to yield *(S)*-HMG-CoA. After the reaction is stopped by addition of HCl, followed by heating of samples to 110°C, the thioesters are cleaved and unreacted substrate (in the form of acetate or acetoacetate that is converted to acetone) will evaporate while HMG acid remains. Hence, incorporation of radioactivity into heat- and acid-stable product indicates the combined activity of AACT and HMGS. When the yeast strain KV10 that carries the plasmid bearing the gene encoding radish AACT was grown on glucose minimal medium (supplemented with 4 mg/l of ergosterol in liquid broth) at the permissive temperature of 26°C, there was only little AACT activity measurable in total cellular protein extracts prepared in the presence of detergent (Basson et al.[52]). As expected, wild-type yeast (strain W303B) showed similar AACT/HMGS activity when grown either on glucose or on galactose medium. In strain KV10, under inducing conditions, *viz.*, in the presence of galactose, expression of radish AACT led to a 10-fold increase of apparent AACT/HMGS activity over that of the wild-type. In the presence of 1233A (= F 244 = L-659,699), a potent inhibitor of HMGS,[53,54] this activity was completely blocked.[39] The ergosterol-auxotrophic strain used for transformation was apparently slightly leaky. (A complete knockout of the yeast *ERG10* gene is lethal.[28] Only thermolabile *erg10* mutants could be isolated, which will grow at the permissive temperature of 26°C, but not at 36°C.[28] The lethality of a complete lack of AACT might be indicative of a further role of this enzyme, independent of its inclusion in the sterol pathway, for instance in the biosynthesis of pre-squalene products that are essential for cell-cycle regulation.)

When cell homogenates of wild-type and transformed yeast were subfractionated and assayed for AACT/HMGS, it was clearly revealed that enzyme activity was associated with the cytosolic fraction, but not with resuspended membrane pellets (Figure 4). This is in clear contrast to the intracellular localization of two intact radish HMGR isozymes expressed in a mevalonate-auxotroph yeast mutant, where enzyme activity was exclusively associated with membranes.[55] The absence of potential transit-peptide signals for import into mitochondria or chloroplasts[56] or for peroxisomes[57] obviously leads to the formation of largely soluble AACT. These observations provide further evidence that we have successfully cloned a cDNA containing the entire coding unit for a cytosolic radish AACT. Tenfold overexpression of AACT as compared to the wild-type values, nicely fits the observations made by Dequin et al.,[58] who overexpressed AACT from *S. uvarum* in a yeast *erg10* strain. These authors used an assay system that exclusively measures thiolase activity, in the cleavage direction which is thermodynamically favored. In our coupled AACT/HMGS assay such an apparent increase in enzyme activity can be interpreted to mean that, at least in *S. cerevisiae*, for the two-step conversion of acetyl-CoA to HMG-CoA, AACT appears to catalyze the rate-limiting step.

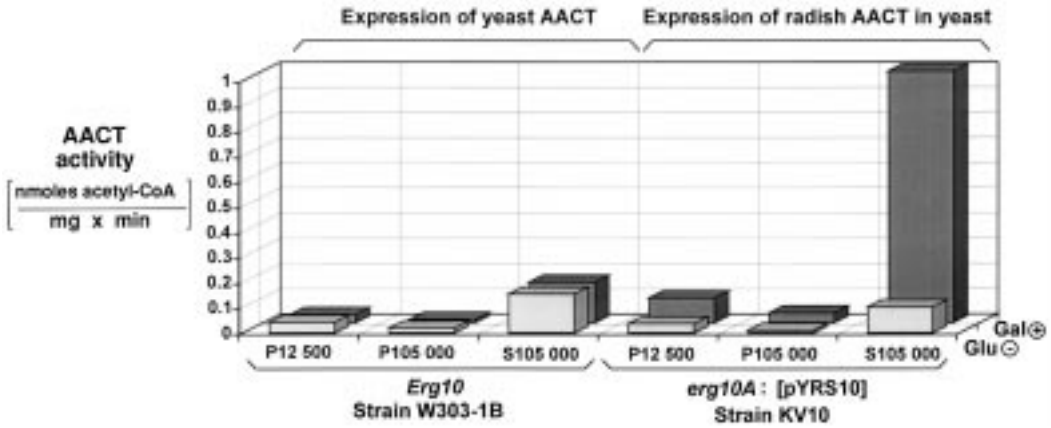

Figure 4 Heterologous expression of radish AACT in yeast. Homogenates and membrane fractions were prepared from a) wild-type strain W303-1B (*ERG10*) and from strain KV10 (*erg10A*: [PYRS10]), carrying a full-length radish cDNA expressing AACT, placed under the control of the *GAL1* promoter. Strains were grown at 28°C on minimal medium YNB, supplemented with 50 mg/l each of histidine, adenine and leucine. Glu, 2% glucose (plus 80 mg/l ergosterol; non-inducing conditions: –); Gal, in the presence of 2% galactose (inducing conditions: +). P12500, heavy membrane fraction; P105000, microsomal fraction; S105000, soluble proteins. Activity was measured as incorporation of [1-^{14}C]acetyl-CoA into heat-stable HMG-CoA (Weber and Bach, 1994). Note that in this coupled assay in both cases endogenous yeast HMGS activity will participate in the overall reaction. While AACT activity in strain W303-1B is not affected when glucose is replaced by galactose, an about tenfold increase of apparent enzyme activity can be seen in soluble extracts from transformed strain KV10.

Overexpression of cytosolic radish AACT in the yeast has no apparent negative effect on the growth behavior of yeast cells. This has already been shown with yeast overexpressing the AACT gene isolated from *S. uvarum*,[58] or for yeast overexpressing phosphomevalonate kinase.[59] Although we have not yet made an analysis of sterols in yeast transformed with the radish AACT gene, from the results obtained by Dequin et al.[58] it appears as if overexpression of AACT does not significantly affect the sterol pattern, nor its total content. Thus, other enzymatic steps downstream the pathway must be responsible for substrate flow regulation from acetyl-CoA to ergosterol as the predominant sterol in yeast.[60,61] As a candidate for such a regulatory enzyme farnesyl-pyrophosphate synthase has been proposed.[26]

When we determined [2-^{14}C]acetyl-CoA incorporation into HMG-CoA in the presence of Fe(II) and PQQ, with cell-free extracts of transformed yeast, we could not observe any significant stimulation of enzyme activity (Figure 5). From these observations we conclude that the product of the cloned gene is not identical to the membrane-associated AACT/HMGS system described before.[30] Cloning of this AACT gene would rather support the presence of separate AACT and HMGS enzymes in plant cells, although they seem to behave similarly during various purification steps.[31,32] This latter purification scheme, using cell-free preparations of *Catharanthus roseus* cell cultures as an enzyme source, did apparently not (yet) result in a separation of AACT and HMGS activities. In our test system for AACT/HMGS we could not

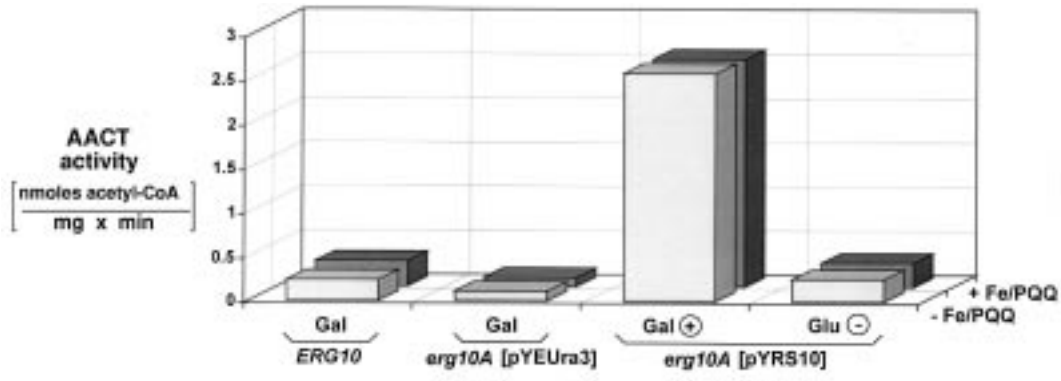

Figure 5 Galactose-inducible AACT activity in total protein extracts of strain KV10 (*erg10A* [pYRS10], strain KV5 (*erg10A* [pYEUra3], recipient ergosterol-deficient strain, transformed with the plasmid only), and from wild-type strain W303-1B (*ERG10*). Activity was determined in the absence (-) or in the presence (+) of Fe(II)/PQQ (50/25 μM, see Weber and Bach.[30] No *in-vitro* stimulation is visible as was observed for the coupled enzyme system AACT/HMGS isolated from radish membranes. Note that the mutant *erg10A*-strain (KV5) still exhibits some measurable AACT activity (see text for explanations).

trap free acetoacetyl-CoA as a putative intermediate.[5] Thus, the situation is still far from being clear. Furthermore, the exact intracellular localization of HMG-CoA synthesis (i.e., cytoplasm versus plastids and/or mitochondria) remains obscure unless corresponding genes have been cloned.

5. Further Possible Features of Radish AACT

Recently, an AU-rich RNA-binding protein has been isolated from the flesh fly *Sarcophaga peregrina*, which was subsequently identified as a thiolase.[62] The partial amino acid sequences of two peptides obtained from the 39-kDa protein showed high similarities to rat and yeast OACT, and exhibited in fact thiolase activity. Since rat mitochondrial OACT showed affinity to the AU-rich RNA, this RNA-binding activity might be an intrinsic character of thiolase.[62] AU-rich sequences in 3′-untranslated regions of short-lived mRNA species appear to be needed for their rapid degradation through the rapid removal of the poly(A) moiety, resulting in destabilization of the mRNAs (see Nanbu et al.[62] for literature). It is tempting to assume that in this way thiolase protein could somehow downregulate its own synthesis once sufficient amounts have been formed. The sequence of the 3′-untranslated region of our cDNA clone could possibly match such a requirement. A similar regulatory function through binding to sequence motifs of mRNA was described for the iron-responsive element binding-protein (IRE-BP) having aconitase activity.[63,64]

The putative antisense transcript of the AACT gene in radish (0.8 kb) is much shorter than in transformed yeast (1.2 kb). This discrepancy might be due to the existence of different signals for the processing and the polyadenylation of mRNA in plants[65] and in yeast.[66] Moreover, while in transformed yeast a cDNA sequence serves as a matrix, in radish seedlings it is genomic DNA; the putative intron sequences might thus be responsible for an early termination of transcription. Synthesis of an AACT antisense mRNA would provide the means for a downregulation of AACT activity, which could be rapidly reversed due to the small life time of RNA-RNA duplex hybrids (see Inouye[67]). Such a mechanism, including the synthesis of antisense mRNA for *in vivo* expression, was described for α-amylase from barley[68] (see also Mol et al.[69]). Future work, including isolation and sequencing of corresponding genomic clones also from other plants such as *Arabidopsis*, will be needed to clarify these questions.

Seedorf et al.[70] sequenced mammalian sterol-carrier protein (SCP2)-encoding cDNAs and found inframe 5′-extensions of up to 1,250 nucleotides upstream of the initiator ATG of the cDNA encoding pre-SCP2. Computerized alignment showed that the catalytically active cysteine in *E. coli* OACT (Cys91) aligns with Cys93 and Cys94 on human and rat 58-kDa precursor (SCPx) of SCP2 (nonspecific lipid-transfer protein), respectively.[71] Purified recombinant SCPx was capable of cleaving 3-oxoacyl$_{(n)}$-CoA to yield acetyl-CoA and acyl$_{(n-2)}$-CoA. Thus, SCPx, localized in peroxisomes and having an intrinsic *in vitro* sterol-carrier and phosphatidylcholine-transfer activity, represents an example of a multifunctional enzyme.[70] Thus, in view of the various functions of thiolases discussed above, such a further role even in plants would be no surprise.

D. REACTIONS COMPETING FOR HMG-CoA

The membrane fraction used to isolate the AACT/HMGS enzyme system from radish is also a major source for HMG-CoA lyase (HMGL EC 4.1.3.4) catalyzing the conversion of HMG-CoA into acetyl-CoA and acetoacetate.[73] To our knowledge there is no clear information as to the metabolic fate in plants of acetoacetate generated by the HMGL reaction. Likewise, there are no clear data available that would indicate the presence of a so-called HMG-CoA cycle and ketone body formation as described for vertebrate systems.[51] However, besides being involved in leucine catabolism, HMGL might also participate in the process of shunting carbon units away from their inclusion into the isoprenoid pathway, *via* the *trans*-methylglutaconyl-CoA or mevalonate shunt mechanism as originally envisaged by Popják.[74] The existence of this shunt, which combines the synthetic pathways leading to fatty acids and to isoprenoids with the catabolism of leucine, has been demonstrated in vertebrates (see Landau and Brunengraber[75] for review of literature), in insects[76] and in wheat seedlings, as demonstrated by incorporation of [2-^{3}H]mevalonate, but not of [5-^{3}H]mevalonate into long-chain fatty alcohols.[77] Tritium from [2-^{3}H]mevalonate, if routed through the mevalonate shunt, must appear in the acetoacetate fragment of the HMGL-catalyzed cleavage of HMG-CoA. Because the label appeared in the long-chain fatty alcohols, the acetoacetate had to be used as a precursor of fatty-acid biosynthesis, most likely through previous conversion to acetate.[77]

HMGL was solubilized from radish membranes and purified by 154-fold.[73] The apparent molecular weight under nondenaturating conditions was determined to be about 70 kDa. The enzyme has a broad pH optimum around 8.0 and its activation energy as determined from the linear part of an Arrhenius plot is 137.1 kJ/mol. The K_m with respect to *(S)*-HMG-CoA is 40 µM. The behavior during free-flow isoelectric focusing[5] suggested a pI value around pH 7.0 both for radish and maize HMGL. The enzyme was found to be extremely unstable and rapidly loses activity even when kept on ice, but retains some activity over several weeks when stored at –80°C. Hitherto, this instability has hampered further purification. A partial purification of HMGL from *Catharanthus roseus*[32] and also from spinach leaves[29] has been achieved, but details were not given.

The activity of another key enzyme of leucine degradation and of the MVA shunt, 3-methylcrotonyl-CoA carboxylase (2-methylcrotonyl-CoA:carbon-dioxide ligase(ADP-forming), EC 6.4.1.4) has been demonstrated in plants,[78–82] and corresponding genes have been cloned.[83,84] The presence of *trans*-3-methylglutaconyl-CoA hydratase (3-hydroxy-3-methylglutaryl-CoA hydrolyase, EC 4.2.1.18) in extracts from *Catharanthus roseus* cells was shown by van der Heijden et al.[31,32] However, further evidence of an alternative peroxisomal pathway of leucine degradation has been presented by Gerbling,[85] including free acids rather than CoA esters. At present the interdependence of those intervening and/or scavenging pathways is not yet known, nor is their regulation. Thus, the exact physiological role of HMGL in the plant cell remains obscure at present.

A further enzyme utilizing HMG-CoA has been described and partially purified by van der Heijden et al.,[31,32] a nonspecific 3′-nucleotidase, which cleaves off a phosphate unit from the CoA moiety of *(R,S)*-HMG-CoA. It appears as if the modified substrate *(S)*-3′-dephospho-HMG-CoA is still recognized and converted to MVA in the presence of truncated recombinant radish HMGR purified from *E. coli* (van der Heijden, personal information).

IV. ALTERNATE PATHWAYS OF MVA/IPP FORMATION?

It must be noted that plants might very well have developed further strategies for the biosynthesis of HMG-CoA, or even mechanisms that bypass MVA formation for the synthesis of IPP. Recently, Rohmer et al.[86] have provided convincing evidence for the existence of an alternate pathway in certain bacteria, among others in *E. coli*, starting from a thiamine diphosphate (TPP)-activated acetaldehyde generated by pyruvate decarboxylation, which would be added to the C-2 carbonyl group of a dihydroxyacetone derivative, followed by a transposition reaction. The following reactions (reduction, isomerization, elimination of water) remain speculative, but the authors argue that they could be similar to those found in L-valine biosynthesis (see Rohmer et al.[86] for details). In a recent study, Cartayrade et al.[87] compared the biosynthesis of β-sitosterol and of diterpenoid ginkgolides in embryos of *Ginkgo biloba*. In *Ginkgo biloba* the IPP precursor of β-sitosterol is assembled from MVA formed by the classical pathway from acetyl-CoA *via* acetoacetyl-CoA and HMG-CoA. In contrast, the IPP units used for formation of ginkgolides are apparently assembled through decarboxylative condensation of pyruvate with a triose phosphate, similar or identical to the Rohmer[86] pathway.[87,88] Hano et al.[89] have used callus and cell suspension cultures of *Morus alba* L. to study the biosynthesis of optically active prenylchalcones such

as chalcomoracin. [2-^{13}C]MVA was not incorporated into the prenyl moiety of chalcomoracin, while it was incorporated into β-sitosterol. The labeling pattern of chalcomoracin from [2-^{13}C]-L-leucine was the same as from [1-^{13}C]acetate. Feeding D-[U-$^{13}C_6$]glucose, [1,3-$^{13}C_2$]- and [2-^{13}C]glycerol (which formed [1,3,4,6-$^{13}C_4$]- and [2,5-$^{13}C_2$]glucose, respectively) demonstrated that of the three acetate units composing the hemiterpene moiety the starter acetate unit might be of glycolytic origin, whereas the second and third acetate units might originate from the pentose phosphate cycle.[89] Triose phosphates such as dihydroxyacetone phosphate and glyceraldehyde-3-phosphate are postulated to occupy the junction of this chimeric hemiterpene pathway with glycolysis and the pentose phosphate cycle. The major difference in the isotopic enrichments between the Rohmer pathway[86] and the mechanism described by Hano et al.[89] is that ^{13}C-label coming from [1-^{13}C]acetate appears in C-4 of IPP, while the hemiterpene moieties of prenylchalcones were not enriched, and second in *M. alba* cell cultures, label from [1,3-$^{13}C_2$]- and [2-^{13}C]glycerol labeled the C-1 and C-5 positions, or the C-2, C-3, and C-4 positions of IPP, respectively. Although the nuclear magnetic resonance (NMR) data are quite conclusive, it is still difficult to rationalize in this model of how acetyl-CoA units can be distinguished in the formation of HMG-CoA as an intermediate[89] if not assuming intermediates that are recognizable through tight binding to proteins/enzymes, beyond differential subcellular localization. But in any case, this model[89] would also require a mechanism of HMG-CoA formation that is clearly distinct from the classical pathway. If the model of Hano et al.[89] is true, then differential formation of MVA used either for sterol formation or for prenylchalcone synthesis must remain susceptible to inhibition by inhibitors such as mevinolin, which affects HMGR,[90] or of F-244, blocking HMGS.[53,54]

From such inhibition studies, using intact radish seedlings or cell cultures of *Silybum marianum* treated with mevinolin (for literature and discussion see Bach et al.[5]), it was concluded that within the plant cell there must exist a compartment in which the formation of IPP units takes place, independent of its cytosolic formation, there providing sterol formation with its substrate. The accumulation of plastid prenyl lipids such as carotenoids, chlorophylls, plastoquinone, phylloquinone, and α-tocopherol was not inhibited by mevinolin, which was interpreted to mean that the compound cannot penetrate the plastid envelope and thus cannot inhibit the putative plastid HMGR isozyme.[5] On the other hand, as outlined above, since there is no evidence for a gene encoding a chloroplast-specific HMGR form, and in view of these recent findings on the Rohmer pathway,[86] this effect can quite easily be explained by operation of one or more alternative pathways (see Bach[20] for a detailed discussion).

Is there a cooperation of alternative pathways? Stress conditions under which the classical pathway is blocked might lead to the activation of rescue mechanisms. It should also be considered that "overloading" of a system with a precursor, as is required for NMR analyses of products having incorporated stable isotopes,[86–89] might lead to the activation of salvage pathways that are not operational under normal conditions. Future work might reveal that, depending on the plant species and on their developmental stage, different strategies might be used to ensure a sufficient MVA/IPP synthesis in the cytoplasm and in the organelles. If such anaplerotic and presumably compartmentalized pathways coexist, then we have to look for some molecular switch or sensor responsible for activating genes for different biosynthetic routes.

ACKNOWLEDGMENTS

Studies carried out at the University of Karlsruhe were supported by the Deutsche Forschungsgemeinschaft (Ba 871/3), those at the IBMP, Strasbourg by the Centre National de la Recherche Scientifique, and those at the University of Barcelona by the Dirección General de Investigación Científica y Técnica (Grants No. PB90-0492 and PB93-0753). Collaboration between our laboratories has been made possible by a NATO Collaborative Research Grant (No. 0538/88). We are grateful to Prof. Francis Karst for providing us with an original *erg10* yeast strain, for stimulating discussions and for introducing one of us (K.-U. V.) into some techniques of yeast genetics during a short research stay in his laboratory at the University of Poitiers.

NOTES ADDED IN PROOF

The citation "Lumbreras et al., submitted" has been published as:

Lumbreras, V., Campos, N., and Boronat, A., The use of an alternative promoter in the *Arabidopsis thaliana HMG1* gene generates a mRNA that encodes a novel 3-hydroxy-3-methylglutaryl coenzyme A reductase isoform with an extended N-terminal region, *Plant J.*, 8, 541, 1995.

The citation "Dale et al., submitted" has been published as:

Dale, S., Arró, M., Becerra, B., Morrice, N. G., Boronat, A., Hardie, D. G., and Ferrer, A., Bacterial expression of the catalytic domain of 3-hydroxy-3-methylglutaryl-CoA reductase (isoform HMGR1) from *Arabidopsis thaliana*, and its inactivation by phosphorylation at Ser577 by *Brassica oleracea* 3-hydroxy-3-methylglutaryl-CoA reductase kinase, *Eur. J. Biochem*., 233, 506, 1995.

The citations "Campos and Boronat, submitted" and "Campos and Boronat, manuscript in preparation" have been published as:

Campos, N., and Boronat, A., Targeting and topology in the membrane of plant 3-hydroxy-3-methylglutaryl coenzyme A reductase, *Plant Cell*, 7, 2163, 1995.

The citations "Vollack and Bach, submitted" and "Vollack and Bach, in preparation" have been published as:

Vollack, K.-U., and Bach, T. J., Cloning of a cDNA encoding cytosolic acetoacetyl-coenzyme A thiolase from radish by functional complementation in *Saccharomyces cerevisiae*, *Plant Physiol.*, 111, 1097, 1996.

Since the submission of this chapter several reviews have been published, covering different aspects of mevalonate synthesis and function in plants:

Weissenborn, D. L., Denbow, C. J., Laine, M., Lång, S. S., Yang, Z., Yu, X., and Cramer, C. L., HMG-CoA reductase and terpenoid phytoalexins: molecular specialization within a complex pathway, *Physiol. Plant.*, 93, 393, 1995.

Chappell, J., The biochemistry and molecular biology of isoprenoid metabolism, *Plant Physiol.*, 107, 1, 1995.

Chappell, J., Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants, *Annu. Rev. Plant Physiol. Mol. Biol.*, 46, 521, 1995.

A cDNA from *A. thaliana* coding for HMGS was serendipidously cloned through complementation of a yeast mutation that causes a deficiency in amino acid uptake. However, this cDNA was then used to transform a HMGS-deficient yeast strain, which led to full complementation of this latter mutation and thereby provided a functional proof:

Montamat, F., Guilloton, M., Karst, F., and Delrot, S., Isolation and characterization of a cDNA encoding *Arabidopsis thaliana* 3-hydroxy-3-methylglutaryl coenzyme A synthase, *Gene*, 167, 197, 1995.

Cloning of this cDNA and of the radish cDNA encoding AACT described in this chapter provides convincing evidence for the presence of separate AACT and HMGS enzymes in plant cells, although they seem to behave similarly during various purification steps. Through refinement of chromatographic methods our preliminary observations suggest that the combined AACT/HMGS assay, which works without any problems when for instance enzyme extracts from yeast are used, might lead to the formation of (a) further as yet unidentified product(s) with plant protein preparations. This side reaction appears to interfere with the assay of HMGS and even of HMGR (A. Galichet and T.J. Bach, unpublished).

In the meantime, by the use of triose-phosphate isomerase-deficient *E. coli* mutants, the first reaction within the alternative bacterial pathway for IPP formation has been further characterized by assigning to glyceraldehyde-3-phosphate the exclusive role as substrate for the condensation reaction with pyruvate:

Rohmer, M., Seemann, M., Horbach, S., Bringer-Meyer, S., and Sahm, H., Glyceraldehyde-3-phosphate and pyruvate as precursors of isoprenic units in an alternative pathway for terpenoid biosynthesis, *J. Am. Chem. Soc*., 118, 2564, 1996.

Interestingly, in the green alga *Scenedesmus obliquus* all isoprenoids analyzed so far, even including sterols, are exclusively synthesized via the pyruvate/glyceraldehyde-3-phosphate (Rohmer) pathway:

Schwender, J., Seemann, M., Lichtenthaler, H. K., and Rohmer, M., Biosynthesis of isoprenoids (carotenoids, sterols, prenyl side-chains from chlorophylls and plastoquinone) via a novel pyruvate/glyceraldehyde-3-phosphate non-mevalonate pathway in the green alga *Scenedesmus obliquus*. *Biochem. J.*, 316, 73, 1996.

More recently, by the use of a cell culture of the gymnosperm *Taxus chinensis*, evidence has been presented that the taxane carbon skeleton of the diterpenoid taxol is apparently synthesized via a pathway that is similar or identical to the Rohmer pathway:

Eisenreich, W., Menhard, B., Hylands, P. J., Zenk, M. H., and Bacher, A., Studies on the biosynthesis of taxol: The taxane carbon skeleton is not of mevalonoid origin, *Proc. Natl. Acad. Sci.*, 93, 6431, 1996.

The observations made with *S. obliquus* can now be extended to the synthesis of major isoprenoids occurring in chloroplasts of higher plants:

Lichtenthaler, H. K., Schwender, J., Disch, A., and Rohmer, M., Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a so far unexpected novel pathway, submitted for publication.

In order to demonstrate the importance of an intact MVA biosynthesis we have used highly synchronizable tobacco BY-2 cells. By an array of different methods and approaches we have shown that mevinolin (= lovastatin), an inhibitor of MVA formation, when applied in (gap) phase G1, S (DNA synthesis) and G2 (preparation for mitosis) has no effect, but that cells are sensitive in late phase M (mitosis; check point to initiate a new round of cell division), leading to an arrest in G1. Those TBY-2 cells that pass the block at G1/S appear to be arrested in G2 and partially die later on. This might be indicative of an interaction with a further control point, and some evidence exists that apoptosis (programmed cell death) is induced. Thus, MVA synthesis is essential for the initiation of DNA synthesis, cell division and viability. This also indicates, at least in higher plant cells, that the presence of an alternate, presumably compartmentalized pathway for IPP formation does apparently not lead to complementation of MVA deficiency in the cytosol:

Hemmerlin, A, and Bach, T. J., Blockage of cell cycle progression in tobacco BY-2 cells by the HMG-CoA reductase inhibitor mevinolin, (Abstract 729), *Plant Physiol.*, 111 (supplement), 158, 1996.

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