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Is the Reaction Catalyzed by 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase a Rate-Limiting Step for Isoprenoid Biosynthesis in Plants?[1]

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3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyzes the irreversible conversion of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate and is considered a key regulatory step controlling isoprenoid metabolism in mammals and fungi. The rate-limiting nature of this enzyme for isoprenoid biosynthesis in plants remains controversial. To investigate whether HMGR activity could be limiting in plants, we introduced a constitutively expressing hamster HMGR gene into tobacco (*Nicotiana tabacum* L.) plants to obtain unregulated HMGR activity. The impact of the resulting enzyme activity on the biosynthesis and accumulation of particular isoprenoids was evaluated. Expression of the hamster HMGR gene led to a 3- to 6-fold increase in the total HMGR enzyme activity. Total sterol accumulation was consequently increased 3- to 10-fold, whereas end-product sterols such as sitosterol, campesterol, and stigmasterol were increased only 2-fold. The level of cycloartenol, a sterol biosynthetic intermediate, was increased more than 100-fold. Although the synthesis of total sterols appears to be limited normally by HMGR activity, these results indicate that the activity of one or more later enzyme(s) in the pathway must also be involved in determining the relative accumulation of end-product sterols. The levels of other isoprenoids such as carotenoids, phytol chain of chlorophyll, and sesquiterpene phytoalexins were relatively unaltered in the transgenic plants. It appears from these results that compartmentation, channeling, or other rate-determining enzymes operate to control the accumulation of these other isoprenoid end products.

HMGR catalyzes the irreversible conversion of HMG-CoA to mevalonate and is considered a key regulatory step controlling isoprenoid metabolism in mammals (Goldstein and Brown, 1990), fungi (Basson et al., 1986), and perhaps insects (Morgen et al., 1982; Feyereisen and Farnsworth, 1987) and plants (Bach, 1987). The pivotal nature of HMGR in mammalian systems was initially suspected on the basis of theoretical considerations of pulse-labeling experiments that measured [^{14}C]acetate and [^{3}H]mevalonate incorporation into cholesterol by sterol-starved and sterol-supplemented mammalian cells (Siperstein and Guest, 1960). Those pulse-labeling studies demonstrated that [^{14}C]acetate incorporation into cholesterol was inversely proportional to the availability of exogenous sterols, whereas [^{3}H]mevalonate incorporation was much less responsive. Subsequent comparisons between the activities of several isoprenoid biosynthetic enzyme activities and the in vivo accumulation rates of cholesterol established the reaction catalyzed by HMGR as the rate-limiting step for sterol biosynthesis and the isoprenoid biosynthetic enzyme exhibiting the greatest degree of feedback regulation (Goldstein and Brown, 1990).

Evidence for the importance of a regulated HMGR activity in plant isoprenoid metabolism is as yet controversial. For example, a number of investigators (Suzuki et al., 1975; Stermer and Bostock, 1987; Narita and Gruissem, 1989), including us (Chappell and Nable, 1987), have reported a correlation between the induction of isoprenoid biosynthesis, particularly of sesquiterpenoids, and HMGR enzyme activity. However, pulse-labeling studies with [^{14}C]acetate and [^{3}H]mevalonate and subsequent assays of squalene synthase and sesquiterpene cyclase have demonstrated that these enzymes are also stringently regulated and might be key regulatory (control) points controlling carbon flow into these branch pathways (Vögeli and Chappell, 1988; Chappell et al., 1989).

Also important and useful in elucidating the contribution of HMGR activity to the production of particular isoprenoids was the discovery of two very potent competitive inhibitors, mevinolin (Alberts et al., 1980) and compactin (Endo, 1980). These two structural analogs of HMG-CoA have K_i values of less than that of the K_m for HMG-CoA. In numerous studies, mevinolin and compactin have proven to be selective inhibitors of sterol metabolism in mammalian cells with minimal side effects and have been found to have clinical application in the control of cholesterol me-

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Abbreviations: CaMV, cauliflower mosaic virus; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase.

tabolism. Mevinolin and compactin also inhibit plant HMGR activity, and their exogenous application stunts plant growth and development, presumably by limiting the availability of mevalonate for isoprenoid metabolism (Bach and Lichtenthaler, 1983; Hashizume et al., 1983; Hata et al., 1987; Narita and Gruissem, 1989). In addition, we previously used mevinolin to inhibit inducible HMGR activity in elicitor-treated tobacco cell-suspension cultures and demonstrated a dose-dependent inhibition of sesquiterpenoid accumulation (Chappell and Nable, 1987). Unexpectedly, mevinolin also inhibited the incorporation of radioactive mevalonate into sesquiterpenoids by elicitor-treated tobacco cell cultures and in vitro measurements of sesquiterpene cyclase activity (Vögeli and Chappell, 1991). These results indicated possible secondary effects of these inhibitors and, hence, a limited usefulness of these compounds for defining the importance of HMGR activity to isoprenoid biosynthesis in general.

The current experiments were undertaken to evaluate more directly the role of HMGR in regulating carbon flow into plant isoprenoids. Our results demonstrate that an enhanced HMGR enzyme activity is indeed sufficient to direct more carbon into sterols. However, the transgenic plants accumulate cycloartenol, an intermediate in the sterol biosynthetic pathway, and the enhanced HMGR activity does not seem to affect the level of several other classes of isoprenoids. These results have important implications for identifying the rate-limiting step(s) for sterol biosynthesis in plants and suggest possible metabolic channels for isoprenoid metabolism in plants.

MATERIALS AND METHODS

Isotopes and Materials

DL-3-Hydroxy-3-methyl-[3-^{14}C]glutaryl CoA (43.2 Ci/mol) was purchased from New England Nuclear.

Gene Construction and Transformation

The hamster HMGR gene used in all these experiments was originally isolated in the laboratories of Goldstein and Brown (Chin et al., 1984) and further modified in the same laboratories by removing nucleotides 28 to 1023 to generate a truncated form of the gene in a plasmid referred to as pRED-Δ227 (Gil et al., 1985). The pRED-Δ227 gene was kindly provided by Dr. K.L. Luskey (University of Texas Health Science Center at Dallas). A *Bam*HI/*Sst*I fragment of the Δ227 construct comprising −22 to 3388 bp of the truncated HMGR cDNA was inserted into an intermediate vector, pKYLX 61, and an *Eco*RI/*Cla*I fragment of that construct was subsequently inserted into the corresponding site of pKYLX71 (Schardl et al., 1987), a Ti-based plasmid, to generate pKYLX71-Δ227 (Fig. 1). Expression of the truncated HMGR gene in this construct is controlled by a CaMV 35S promoter.

The pKYLX71-Δ227 plasmid was mobilized into *Agrobacterium tumefaciens* harboring GV3850, a disarmed Ti plasmid, using standard triparental mating procedures (Schardl et al., 1987). Leaf discs of *Nicotiana tabacum* L. cv Xanthi were inoculated with the *Agrobacterium* carrying the pKYLX71-Δ227 construct, and transgenic plants were regenerated using the protocol of Schardl et al. (1987).

HMGR Enzyme Assay

Frozen tissue was homogenized in ice-cold 100 mM K-phosphate (pH 7.0), 4 mM $MgCl_2$, and 5 mM DTT, and the homogenate was centrifuged at 10,000*g* for 15 min at 4°C. The 10,000*g* supernatant was subsequently centrifuged at 100,000*g* for 45 to 60 min. The HMGR activity in the 100,000*g* supernatant and pellet (microsomal fraction) resuspended with 100 mM K-phosphate (pH 7.0) and 50 mM DTT was determined using a modified assay. Aliquots of supernatant or microsomes (equivalent to 1–10 μg of protein) were incubated in 100 mM K-phosphate (pH 7.0), 3 mM NADPH, and 20 μM [^{14}C]HMG-CoA (58 μCi/μM) in a final assay volume of 26 μL for 15 to 60 min at 30°C. The assay was terminated by the addition of 5 μL each of 1 mg/mL mevalonate lactone and 6 N HCl, and the mixture was incubated an additional 15 min at room temperature to allow any radiolabeled mevalonate formed to lactonize.

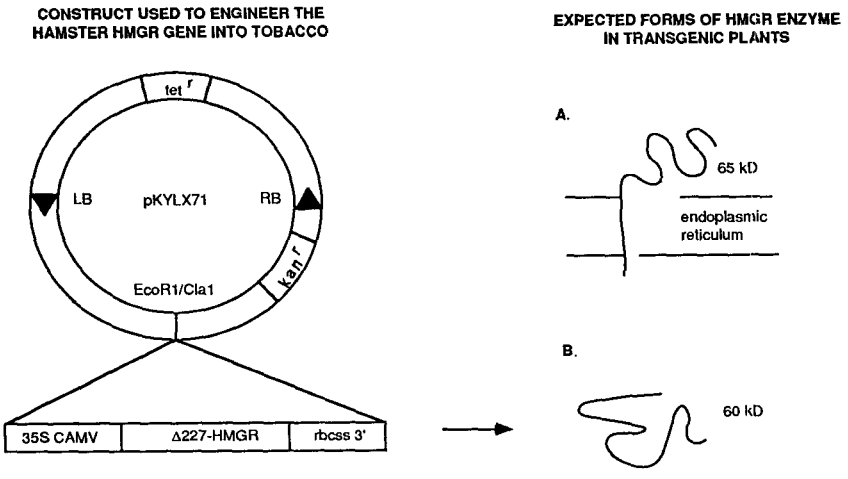

Figure 1. Expected forms of HMGR enzyme in transgenic plants. The HMGR enzyme encoded by the endogenous tobacco HMGR gene (A) would be anchored to the ER by a hydrophobic domain of the protein (Genschik et al., 1992). The HMGR enzyme encoded by the hamster Δ227 construct (Gil et al., 1985) would not have any such targeting signals and, thus, would behave as a soluble, cytosolic form (B).

The [^{14}C]mevalonate formed was separated from the HMG-CoA by an organic partitioning method originally suggested by Dr. R. Feyereisen (University of Arizona, Tucson). After 125 μL of saturated K-phosphate (pH 6.0) and 300 μL of ethyl acetate were added, the samples were briefly vortexed and centrifuged, and an aliquot of the upper organic phase was used to determine radioactivity by scintillation spectrophotometry. Assay controls included the omission of NADPH as well as an acid stop prior to the addition of substrate. Assays were performed in duplicate with less than 10% variation between replicates.

Chemical Analyses

The total levels of sterols and phytol were determined by a GC analysis. Lyophilized plant material (0.1–1 g) was pulverized and extracted/saponified in 5 mL of a 20% KOH ethanol:methanol:water (6:1:4) solution for 30 min at 70°C in a shaking water bath. Extracts were cooled and then extracted three times with 15 mL *n*-heptane. The combined heptane extracts were reduced to dryness under a stream of nitrogen and resuspended in heptane containing cholestane, used as an internal standard. GC analyses were performed on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector. Individual sterols and tocopherol were resolved from one another at 270°C on an Alltech (Deerfield, IL) 30-m × 0.25-mm i.d. RSL-200 capillary column. Phytol was resolved from other extracted constituents using the same GC column but operated at 240°C. Identification of individual components was determined by GC-MS and co-injection with commercially available standards. Total Chl and carotenoid levels in acetone extracts were estimated spectrophotometrically (Scott et al., 1994). Sesquiterpenes were quantified according to the method of Chappell and Nable (1987).

RESULTS

HMGR Gene Construct and Expression in Transgenic Plants

The gene construct used to engineer elevated HMGR enzyme activity into tobacco cells is shown in Figure 1. The enzyme activity expected from expression of the hamster HMGR transgene would be soluble, since the N-terminal, membrane-spanning domains of the hamster gene had been deleted (Gil et al., 1985). The resulting truncated form of the hamster enzyme was previously shown to contain catalytic activity in Chinese hamster ovary cells (Gil et al., 1985). Expression of the hamster transgene would also be expected to generate enzyme activity in addition to that already encoded for by the endogenous tobacco gene(s) (Genschik et al., 1992). However, transcriptional control of the truncated HMGR transgene was directed by the CaMV 35S promoter (Schardl et al., 1987), a constitutive promoter, whereas the endogenous tobacco gene(s) would be subject to endogenous transcriptional and translational regulatory mechanisms. Attempts to express a full-length hamster HMGR gene were unsuccessful as measured by enhanced microsomal enzyme activity.

Expression of the truncated HMGR transgene was initially monitored as the HMGR enzyme activity in the 100,000*g* supernatant of young leaves taken from plants grown in a greenhouse (Table I). In comparison to control tissue, several independent transgenic plant lines contained 3 to 12 times more soluble reductase activity. However, this is a minimal estimate, since the soluble reductase activity in control tissue was very low and near the detection limits of the assay. The soluble HMGR activity in control tissue extracts ranged from undetectable to the maximum level shown in Table I and may represent microsomal contamination of the 100,000*g* supernatant or a false-positive activity in the enzyme assay.

Table I. *HMGR enzyme activity in control and transgenic tobacco plants*

Seeds from each of the initial regenerated plants designated below were germinated on media containing kanamycin and scored for segregation of the antibiotic resistance marker, and 10 of the resistant progeny plants for each line were grown in the greenhouse. Lines 14, 15, 18, 23, and the control segregated 3:1, and line 5 segregated 15:1 for kanamycin resistance. Leaves 1 to 2 cm in length from 10 siblings were combined and used to determine the HMGR activity. Soluble and membrane-associated HMGR activity is operationally defined as that activity in a 100,000*g* supernatant and pellet, respectively. Act., Activity; Sp. Act., specific activity.

Plant	Soluble		Membrane Associated		Sum Total[a]	Fold[b] Increase	Soluble Activity[c]
	Total Act.[a]	Sp. Act.	Total Act.	Sp. Act.			
	nmol h^{-1}	*nmol h^{-1} mg^{-1} protein*	*nmol h^{-1}*	*nmol h^{-1} mg^{-1} protein*	*nmol h^{-1}*		*%*
Δ227–5	0.98	1.32	0.58	2.35	1.56	2.1	62
Δ227–14	3.45	3.16	1.00	2.58	4.45	6.0	77
Δ227–15	1.44	0.95	0.86	1.74	2.30	3.1	62
Δ227–18	1.13	0.96	0.58	1.45	1.71	2.3	66
Δ227–23	1.67	1.35	0.94	2.17	2.61	3.5	63
Control[d]	0.27	0.135	0.46	0.70	0.73	1.0	37

[a] Calculated as the total activity in each fraction of an extract. The sum total was calculated as the sum of the total soluble plus membrane-associated activities. [b] Calculation based on the sum total activities. [c] Calculated as the total soluble activity per sum total activity. [d] Control plants were derived similarly to the Δ227 transformants except that they were transformed with a pKYLX71 plasmid lacking the hamster HMGR gene sequence.

The level of microsomal HMGR activity was also significantly elevated in the transgenic plants, showing up to 2 times more microsomal activity than in control plants (Table I). This was unexpected, because the truncated form of the hamster HMGR gene lacked a putative ER-targeting domain. However, the enhanced microsomal activity could reflect trapping of soluble activity within the microsomal pellet. Tissue samples were initially homogenized with as little homogenization buffer as possible to generate a very protein-rich 100,000*g* supernatant. Trapping of soluble, cellular macromolecules in the high-speed pellet is likely under these conditions. Further evidence for expression of the foreign HMGR gene was also obtained by RNA blot analysis, which demonstrated the presence of the hamster HMGR mRNA in only transgenic plants (data not shown).

Stable integration and expression of the HMGR transgene were confirmed by segregation analysis of the T_1 generation for the kanamycin-resistance trait, total HMGR enzyme activity, total sterol content, and gene copy number as determined by Southern blot hybridization. All of the data were consistent with one to two independent insertions per transgenic plant. The results of a segregation analysis of the progeny from a control transformant (vector alone) and one transgenic line (Δ227-KYLX71–14) for HMGR activity and sterol content are presented in Table II. The HMGR activity determinations were measured using a low-speed supernatant (10,000*g*), which included the soluble and microsomal activities. Of the 12 progeny plants derived from the initial transformant, 4 had sterol levels and total HMGR activity comparable to those of control plants. One of these 4 plants (designated 14–9) contained approximately 2 times higher levels of reductase activity than controls. The remaining 8 progeny plants had consistently higher levels of sterols and HMGR enzyme activity. Although plants having very high levels of reductase and sterols and those having intermediate levels of both were observed, correlation of these traits with the homozygosity/heterozygosity of the transgene was not verified in subsequent generations. Such observations most likely reflected the heterogeneity of the tissues sampled.

Table II. *Segregation of HMGR enzyme activity and sterol levels in a T_1 population of control and Δ227 transgenic plants*

Seeds were germinated and grown under routine greenhouse conditions. One small apical leaf (2–4 cm in length, 50–150 mg fresh weight) was used for each determination of HMGR activity in a 10,000*g* supernatant. The remaining leaves from each plant were combined and lyopholized, and a portion was used for sterol determinations.

Plant	HMGR Activity	Sterols
	pmol h^{-1} mg^{-1} protein	*% dry wt*
Control 1	184	0.20
Control 2	146	0.25
Control 3	154	0.29
Control 4	180	0.31
Control 5	176	0.36
Control 6	183	0.22
14–1	115	0.21
14–2	156	0.17
14–3	1168	1.10
14–4	520	0.74
14–5	394	1.59
14–6	143	0.25
14–7	2426	2.05
14–8	402	0.97
14–9	366	0.19
14–10	1444	1.02
14–11	334	1.10
14–12	416	1.29

Sterol Composition and Content

Comparing the sterol content in callus, leaf, and root tissue of control and transgenic plant lines extended the initial findings (Table III). Transgenic tissues accumulated 3 to 8 times more sterol than the respective control tissues. In general, the increased sterol levels corresponded to an equal increase in the level of esterified sterols (data not shown). However, important tissue-specific differences were noted with regard to the abundance of particular molecular species of sterol. For example, campesterol, sitosterol, and stigmasterol accounted for 80% or more of the total sterol in control callus and leaf tissue, whereas campesterol and other unidentified sterols constituted the bulk of sterol in control root tissue. In contrast, approximately 50% or more of the total sterol content in all three transgenic tissues was cycloartenol, an early intermediate in the sterol biosynthetic pathway (Goodwin, 1981). These cycloartenol levels represent 300-, 60-, and 20-fold higher levels in the transgenic callus, leaf, and root tissues, respectively, than in the respective control tissues.

The impact of the HMGR transgene on the level of other sterols was less consistent. For example, whereas campesterol, sitosterol, and stigmasterol levels were all increased approximately 3-fold in the transgenic callus culture cells, the campesterol levels were unchanged in leaf tissue and suppressed in root tissue. Likewise, stigmasterol levels were suppressed in leaf tissue but significantly elevated in the root tissue. Sitosterol levels were more consistently increased 2- to 6-fold in all of the transgenic tissues examined.

Sterol accumulation was also dependent on the developmental age of the tissue analyzed (Table IV). The accumulation of sterol in apical buds, stems, and newly formed, rapidly expanding, and fully expanded leaves was determined for control and transgenic plants. In control plants, total sterol levels of 0.1 to 0.2% of the dry weight varied little between the various tissues tested, and cycloartenol never accumulated to more than 12% of the total sterol. In contrast, the sterol content was significantly higher in all tissues of the transgenic plants tested, with the highest levels of accumulation occurring in the oldest and most fully expanded leaves. Cycloartenol accounted for 20 to 25% of the total sterols in the young transgenic tissues and upward of 60% in the mature leaf tissue.

Effects on Other Isoprenoids

The levels of other isoprenoids were determined to assess the contribution of HMGR activity to the accumulation

Table III. *Levels of sterols and sterol biosynthetic intermediates in control and Δ227 transgenic plant tissues*

Tissues at comparable stages of development were used for analysis, and the amount of each constituent is expressed as a percentage of the tissue's dry weight. Values represent the averages of triplicate determinations (variation of 5–10%) of a single transgenic line and a companion control line. tr, Trace amounts detected; nd, not determined.

Compound Analyzed	Callus		Leaf		Root	
	Control	Transgenic	Control	Transgenic	Control	Transgenic
			% dry wt			
Total sterol	0.05	0.41	0.31	1.10	0.35	1.36
Campesterol	0.009	0.020	0.057	0.056	0.058	0.021
Cholesterol	0.004	nd	tr	nd	nd	nd
Cycloartenol	0.003	0.258	0.010	0.677	0.038	0.641
Sitosterol	0.026	0.076	0.083	0.187	0.029	0.194
Stigmasterol	0.003	0.011	0.131	0.078	tr	0.238
Unknowns	0.003	0.046	0.025	0.141	0.220	0.179
Sterol classes						
Cycloartenol	0.003	0.258	0.010	0.677	0.038	0.640
Δ5 sterols	0.046	0.156	0.299	0.422	0.311	0.718

of these products (Table IV). Chl levels were measured as an additional means for evaluating the tissues sampled, and these were similar between the control and transgenic plants. Phytol, the long-chain isoprenoid found covalently linked to the Chl chromophore, was increased approximately 3-fold in the leaf tissues in this particular transgenic line. The phytol levels in leaf samples of other transgenic lines were, however, more similar to those observed in control plant tissues. Because a consistent increase in the phytol levels in all of the independent transgenic lines was not observed, as seen for the sterol levels, the enhanced phytol levels in this one transgenic line could be a manifestation of some other change introduced during the tissue culture/regeneration manipulations. Carotenoid and tocopherol levels were unchanged. In other experiments, leaf sections were treated with 1 to 10 μg/mL cellulase, an elicitor of sesquiterpene accumulation in tobacco (Chappell et al., 1991), and the level of capsidiol was determined 12 h later. Both control and transgenic tissues accumulated similar levels of this sesquiterpene phytoalexin, 1.2 ng capsidiol mg^{-1} dry weight.

DISCUSSION

These experiments show that up-regulation of HMGR enzyme activity in tobacco does lead to a marked increase in the accumulation of total sterols, which is consistent with the idea that HMGR activity is rate-determining for sterol synthesis under normal circumstances. However, the accumulation of normal end-product sterols, such as stigmasterol, sitosterol, and campesterol, is not influenced nearly so much by this up-regulation of HMGR activity as is the intermediate cycloartenol. In tobacco, at least one enzymatic step subsequent to cycloartenol, perhaps the methyltransferase catalyzing the conversion of cycloartenol to 24-methylene cycloartenol, appears to be limiting under these conditions of HMGR overexpression. Fang and Baisted (1975) came to a similar conclusion based on their

Table IV. *Comparison of sterol and other isoprenoid levels in control and Δ227 transgenic plant tissues as a function of tissue development*

Sibling plants representing the T_2 generation and true breeding for the absence (14–2) or presence (14–8) of the Δ227 HMGR gene were used for analysis. Tissues used for this comparison included the apical bud at the onset of flowering; the top three expanding leaves (>5 cm); three middle, fully expanded leaves; three bottom, fully mature and partially senescing leaves; and stem tissue. Tissues from 10 individual plants for each genotype were pooled, and assays were performed in triplicate. Values shown are averages with a variance of 5 to 10% for sterol, cycloartenol, Chl, phytol, and carotenoid determinations and a 10 to 20% variance for tocopherol determinations. n.d., Not determined.

Sample	Sterols	Cycloartenol	Chl	Phytol	Carotenoid	Tocopherol
	% dry wt	*% dry wt*	*% dry wt*	*% dry wt*	*% dry wt*	*μg g^{-1} dry wt*
14–2						
Apical buds	0.14	0.006	0.69	0.03	0.08	trace
Top leaves	0.13	0.005	0.72	0.12	0.12	8.0
Middle leaves	0.18	0.020	1.12	0.16	0.23	8.0
Bottom leaves	0.22	0.026	0.72	0.18	0.18	53.0
Stem	0.04	0.002	n.d.	0.01	n.d.	n.d.
14–8						
Apical buds	0.76	0.15	0.48	0.14	0.06	13.0
Top leaves	0.46	0.12	0.65	0.32	0.14	trace
Middle leaves	0.77	0.25	1.07	0.48	0.21	34.0
Bottom leaves	1.19	0.68	0.83	0.41	0.19	41.0
Stem	0.36	0.16	n.d.	trace	n.d.	n.d.

studies of radiolabeled mevalonate incorporation into sterols by pea cotyledons. These investigators observed that, during the initial stages of germination when cotyledons do not synthesize sterols, radioactivity from mevalonate preferentially accumulated in the sterol biosynthetic precursor cycloartenol. However, in tissues actively synthesizing sterols, radioactivity was more equally distributed between cycloartenol and 24-methylene cycloartenol.

Whether the sterol methyltransferase is a rate-limiting step for sterol biosynthesis in plants remains an open question. The apparent limiting nature of the methyltransferase has been observed only under conditions of HMGR overexpression. How limiting this enzyme is under normal conditions of mevalonate biosynthesis remains to be determined. Another reason for cautious interpretation is that two routes for cycloartenol incorporation into sterols have been proposed. One is the methyltransferase route discussed above. The alternative proposes a demethylation at carbon 4 to generate norcycloartenol (Grunwald, 1975). The former route is considered the primary route because various downstream intermediates have been detected, whereas downstream intermediates for the latter route have been much more difficult to detect (Heupel et al., 1986). It is nonetheless possible to consider the existence of this alternative pathway as a means to explain some of the marginal increases in the level of the 24-methyl and 24-ethyl sterols.

The Benveniste laboratory has used an alternative approach to isolate mutants in the isoprenoid biosynthetic pathway and have observed similar findings. These investigators selected UV-mutagenized, haploid protoplasts for growth in the presence of sterol biosynthetic inhibitors (Maillot-Vernier et al., 1989; Schaller et al., 1991). The sterol inhibitors used, both of which inhibit steps relatively late in the pathway, are a triazole compound that inhibits the demethylation of obtusifoliol and an *N*-alkyl morpholine that inhibits the isomerization of cycloeucalenol to obtusifoliol. The sterol composition of the selected tobacco mutants was dramatically altered and exhibited very significant increases in the proportion of cyclopropylsterols (intermediates in the sterol biosynthetic pathway). It is interesting that the extra sterol accumulating in the LAB1–4 mutant accumulates predominately in sterol esters associated with novel cytoplasmic lipid droplets (Gondet et al., 1994). Further studies of the LAB1–4 mutant have demonstrated that the phenotype of the mutation does not require continuous selection with the demethylase inhibitor, but the altered sterol composition is attenuated in regenerated plants and dampened over generations (Maillot-Vernier et al., 1991). Genetic studies also indicated that the alteration in sterol composition segregated as a single semidominant mutation (Maillot-Vernier et al., 1991), and biochemical analysis suggested that the enzyme activities up to HMG-CoA synthase may not be enhanced, but the HMGR activity was increased approximately 3-fold (Gondet et al., 1992). Whether the LAB1–4 mutation directly or indirectly effects the reductase or other downstream biosynthetic enzyme activities or some regulatory functions remains to be determined.

A second conclusion from our work is that an enhanced cytosolic form of HMGR activity alone is not sufficient to enhance the production of other isoprenoids such as the phytol chain of Chl, carotenoids, tocopherol, and sesquiterpenes. That a cytosolic form of the enzyme may not enhance the accumulation of chloroplastic isoprenoids may not be surprising, given that a complete pathway may be compartmentalized to the chloroplast and cytosolic precursors may not freely permeate into the plastid. A logical extension of this interpretation is that there might be an endogenous HMGR isozyme targeted to the plastid just for the synthesis of plastidic isoprenoids. Consistent with this notion, Brooker and Russell (1975) measured a small but significant level of HMGR enzyme activity associated with an organellar fraction enriched for chloroplasts by differential centrifugation. More recently, multiple HMGR genes have been isolated from numerous plant tissues. At least in the case of potato, the expression of specific HMGR genes has also been correlated with the induced accumulation of steroids or sesquiterpenoids (Choi et al., 1992).

The inability of the enhanced reductase activity to direct more carbon into sesquiterpene phytoalexins cannot be explained at this time. One possibility is that other downstream enzymes are limiting, similar to the situation discussed above for the sterol methyltransferase and sterol biosynthesis. Alternatively, but more speculatively, it may be that the hamster reductase becomes associated with a metabolic channel dedicated to sterols but not with channels dedicated to nonsterol isoprenoids. More experimental evidence is obviously needed to distinguish between such possibilities.

NOTE

While this paper was in review, Re et al. (1995) reported that the overexpression of an authentic Arabidopsis HMGR gene was not sufficient to alter the synthesis or accumulation of bulk end-product isoprenoids in Arabidopsis.

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