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Antisense Expression of *hmg1* from *Arabidopsis thaliana* Encoding 3-hydroxy-3-methylglutaryl Coenzyme A Reductase, Reduces Isoprenoid Production in Transgenic Tobacco Plants

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Summary

With the aim of studying the production of chloroplast isoprenoid derivatives, chimeric gene constructs comprising partial and full-length forms of the *Arabidopsis thaliana hmg1* gene, encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), in sense and antisense forms were fused to a chloroplast transit peptide (CTP) sequence from a pea ribulose bisphosphate carboxylase/oxygenase (Rubisco) small subunit gene. These plasmids were placed downstream from the constitutive CaMV 35S promoter and introduced into tobacco (*Nicotiana tabacum* cv. Xanthi) by *Agrobacterium*-mediated transformation. Southern and northern blot analysis confirmed the transformation and transcription of the respective constructs within transgenic tobacco plants. In addition total and chloroplast specific HMGR enzyme activities, and the levels of chlorophyll, carotenoids and total sterols were analyzed. Results of these analyses suggest that neither partial nor full-length sense constructs had any effect on chloroplast isoprenoid production. However, the corresponding antisense constructs decreased general isoprenoid levels. The significance of these results is discussed.

Key words: Arabidopsis thaliana, Nicotiana tabacum cv. Xanthi, antisense, chloroplast transit peptide, HMGR isoforms, isoprenoids, metabolons.

Introduction

Isoprenoids are a numerous and heterogeneous group of compounds (e.g. sterols, phytols, quinones, cytokinins, gibberellins, essential oils, phytoalexins, pigments, etc.) with a variety of important roles in plant physiology. The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; EC. 1.1.1.34) catalyzes the NADPH-dependent reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) into (3*R*)-mevalonic acid. Subsequently, mevalonate is phosphorylated and decarboxylated to yield isopentenyl pyrophosphate (IPP), which is utilized as the building block for all

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isoprenoids. (Gray, 1987; Bach et al., 1990; Gershenzon and Croteau, 1990, 1993; McGarvey and Croteau, 1995). The biosynthesis of plant isoprenoids occurs in several subcellular compartments. In particular, HMGR activity has most frequently been reported to be associated with the endoplasmic reticulum (ER) (Bach et al., 1990; Campos and Boronat, 1995; Denbow et al., 1996), but has also been detected in the mitochondrial membrane (Brooker and Russell, 1975; Suzuki and Uritani, 1976; Yu-Ito et al., 1982) and in plastids (Brooker and Russell, 1975; Wong et al., 1982; Arebalo and Mitchell, 1984; Ramachandra and Das, 1986; Wilson and Russell, 1992).

The possibility of an independent and complete isoprenoid pathway in chloroplasts has been controversial because the location of the early biosynthetic steps prior to IPP production is still unknown. The proposal of an independant chloroplast pathway was recently reinforced by: 1) demostration of a minor effect of the HMGR inhibitor mevinolin on the synthesis of chloroplast isoprenoids, in contrast to the strong inhibition of cytoplasmic isoprenoid synthesis; 2) the detection of HMGR enzyme activity in isolated and intact chloroplasts; and 3) the presence of all necessary enzymes to synthesize acetyl-CoA from Calvin cycle intermediates in chloroplasts (Gray, 1987; Gershenzon and Croteau, 1990, 1993).

Despite these results, two different models of subcellular compartmentation of IPP synthesis are currently under discussion. One model involves the independant synthesis of IPP in each compartment. The other proposes the synthesis of IPP exclusively in the cytoplasm followed by distribution to different intracellular locations as required (Gray, 1987). A third, and conciliatory, possibility arose after finding that plastid isoprenoids are synthesized from plastid-derived IPP in very young barley leaf tissues. However, mature leaf chloroplasts import the necessary IPP from the cytoplasm (Heintze et al., 1990). Thus, the subcellular location of IPP synthesis may change during development according to the metabolic requirements of the developmental stage (McCaskill and Croteau, 1995).

In most plants HMGR is encoded by small gene families (Stermer et al., 1994; Weissenborn et al., 1995) and differential gene expression, in response to several stimuli, results in the presence of different HMGR isoforms. Some of these isoforms may be channeled to separate subcellular pathways for specific isoprenoid biosynthesis (Stermer et al., 1994). In addition, HMGR activity in plants is regulated by a number of different factors such as plant growth regulators, wounding, pathogen attack, phosphorylation, light, calcium-calmodulin, and proteolytic degradation (Gray, 1987; Bach et al., 1990; Gershenzon and Croteau, 1990, 1993; Stermer et al., 1994). HMGR is also under strong feedback regulation as demonstrated by overexpression of the *hmg1* gene of *Arabidopsis thaliana* localized to the ER of the same plant. In spite of the high level of the respective mRNA, the synthesis and accumulation of isoprenoids remained almost unchanged (Re et al., 1995). In contrast, the transformation of tobacco with the *hmg1* gene of *Hevea brasiliensis* under control of the CaMV 35S promoter increased the level of total sterols by up to 6-fold (Schaller et al., 1995). Also, the expression of a truncated *hmgr* gene from hamster in transgenic tobacco plants resulted in both high enzyme activity and increased levels of total sterols even though the levels of chlorophyll and carotenoids were not affected (Chappell et al., 1995).

In *A. thaliana,* HMGR is encoded by two differentially expressed genes, *hmg1* and *hmg2* (Caelles et al., 1989; Enjuto et al., 1994). The *hmg1* mRNA was detected in all tissues (Enjuto et al., 1994) and encodes an enzyme anchored at the amino terminus in the ER membrane (Enjuto et al., 1994; Campos and Boronat, 1995). This gene encodes a novel HMGR isoform with an extended N-terminal region that does not have a role in targeting the enzyme to plastids (Lumbreras et al., 1995). Recently, the suppression of *hmg1* gene expression in response to illumination was reported (Learned, 1996). These results are consistent with the concept of metabolic channels, or arrays of isoenzymes, independently regulated and specifically dedicated to the production of particular isoprenoids (Chappell, 1995). Hypothetically, the compartmentalization of HMGR toward specific metabolic units (e.g. chloroplast), may result in an increased production of defined isoprenoids (e.g. carotenoids and chlorophyll).

With the aim of studying the production of the chloroplast isoprenoids of tobacco, we constructed chimeric plasmids with a Rubisco chloroplast transit peptide sequence (CTP) fused to the *hmg1* gene of *A. thaliana* in the sense or antisense orientation (Learned and Fink, 1989). In order to avoid the self-targeting of the HMGR enzyme to endoplasmic reticulum, we used either the whole *hmg1* gene or a truncated gene, without the intramembranal domain which potentially directs the synthesized protein to specific subcellular compartments. The results obtained following expression of these constructs in transgenic tobacco plants are discussed in this work.

Materials and Methods

Biological material

Plasmid pKAH5 (kindly supplied by Dr. Luis Herrera-Estrella, CINVESTAV-Irapuato) is a binary vector having the 35S CaMV promoter up-stream to the nucleotide sequence coding for the transit peptide of the rubisco small subunit from *Pisum sativum* (Cashmore, 1983), ending with a single *Bam*HI site which allows in-frame subcloning with any structural gene digested with the same endonuclease. Transgenic plant selection was carried out by kanamycin resistance given by neomycin phosphotransferase II (*nptII*) gene expression driven by nopaline synthase promoter. Plasmid pUC119-*hmg1*, with the HMGR-cDNA from *A. thaliana* was kindly supplied by Dr. Gerald Fink (Learned and Fink, 1989). Plasmid cloning and propagation were conducted in *Escherichia coli* DH5α (Hanahan, 1983) using LB medium (Herrera-Estrella and Simpson, 1988). Bacterial selection was by ampicillin (Ap^r), streptomycin (Str^r), and spectinomycin (Spc^r) resistance genes. The *Agrobacterium* strain pGV 2260 used for transformation (Deblaere et al., 1985) was grown in YEB medium (Herrera-Estrella and Simpson, 1988).

Plants of *Nicotiana tabacum* cv. Xanthi were maintained *in vitro* by vegetative propagation on MS medium without plant growth regulators.

Plasmid constructs

Primers for PCR amplification were designed according to the target nucleotide sequences of the *hmg1* gene from *A. thaliana,* with a *Bam*HI restriction site incorporated on the 5′-end of each oligo

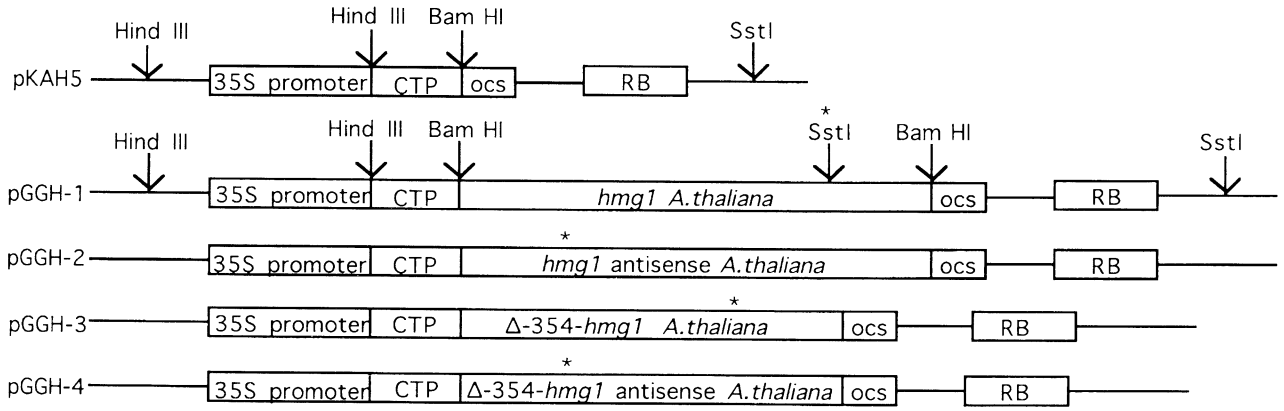

Fig. 1: Organization of chimeric HMGR genes. The *hmg1* and Δ-354-*hmg1* cDNAs from *A. thaliana* were cloned in sense and antisense orientations in the *Bam*HI site of the pKAH5 vector. The internal *Sst*I site (*) at the end of the *hmg1* gene was used to establish the orientation of the respective constructs. Names of the plasmids are at the left of all constructs. The 35S CaMV promoter (**35S promoter**), the chloroplast transit peptide (**CTP**), the octopine synthase 3′-end (**ocs**), the right border (**RB**) and some restriction site sequences are also indicated.

for adequate subcloning into the pKAH5 plasmid vector. The designed oligonucleotides were synthesized on an Applied Biosystems Synthesizer. The oligo 5′-GG**GGATCC**CATGGATCTCCGTCGG-3′ (the *Bam*HI site is indicated in bold in all oligos) was used to amplify the whole *hmg1* gene (with intact intramembrane domains); oligo 5′-GG**GGATCC**CATGGATCTCCGTCGG-3′ primed the PCR reaction of a *hmg1* fragment starting from nucleotide +354 encoding a truncated HMGR protein without the initial 118 amino acids (Δ-354-*hmg1*) corresponding to the intramembrane domains. In both cases, the second oligo-5′-GGG**GGATCC**-TCATGTTGTTGTTGT-3′ primed the PCR reaction from the 3′-end of the *hmg1* gene. PCR reactions were carried out in a GeneAmp PCR System 9600 (Perkin Elmer) using 30 cycles at 92 °C/1.5 min, 50 °C/2 min plus 72 °C/2 min extension. Reactions were carried out in 100 µL with 0.2 µmol · L^{-1} of each specific primer, 250 µmol · L^{-1} of deoxynucleotides, 200 ng of pUC119-*hmg1* DNA, and 3 units of Taq polymerase.

PCR products were subcloned into the pCR II plasmid (Invitrogen Corporation), the resulting pCR II-HMGR and pCR II-Δ-354-HMGR plasmids were digested independently with *Bam*HI and the respective HMGR fragments were ligated to the pKAH5 vector. The CTP sequence was fused to sense and antisense HMGR sequences (Fig. 1); the joining regions between CTP and sense HMGR sequences (pGGH-1, pGGH-3) were confirmed to be in frame by dideoxy sequencing (Sequenase Kit) using the Sanger procedure (Sanger et al., 1977) with a primer for the CaMV 35S promoter; antisense constructs (pGGH-2, pGGH-4) were also confirmed by sequence analysis.

Transformation procedures

Agrobacterium tumefaciens strain pGV 2260 (Deblaere et al., 1985) was transformed with the pKAH5 vector and chimeric constructs (pGGH 1-4), by electroporation (Bio-Rad Gene Pulser) at 200 µFD, 2.5 kV and 200 ohms. Transformants were grown at 28 °C in YEB semisolid medium with rifampicin (100 mg/L) and carbenicillin (100 mg/L), selection was carried out with streptomycin (100 mg/L). Selected clones were verified by colony hybridization using the *hmg1* gene labelled by Random Primer Extension (Dupont: NEN Life Science Products) as a probe.

Pieces of tobacco (*N. tabacum* var. Xanthi) leaves, were immersed in 1/10 MS medium and 1/10 of respective bacterial suspension cultured overnight at 28 °C (10^{-} cells per milliliter as measured at 600 nm) was added for 15 min; excess liquid was blotted with sterile filter paper and explants were placed on MS medium with benzyl amino purine (BAP) (0.5 mg/L); after 48 hour they were transferred to fresh MS/BAP medium containing claforan (500 mg/L, kindly supplied by Roussell laboratories) to eliminate *Agrobacterium* contamination and kanamycin (150 mg/L) for selection of transformed plants, until shoot regeneration occurred. Shoots were removed and transferred to a 16 hour light/8 hour dark photoperiod at 25 °C in the same MS medium without BAP for further development and root formation.

Different plasmids were used for 2–3 transformation procedures. Ten to twelve independent plants were regenerated each time. An average of 20–30 independent transgenic plants per plasmid were transferred to the greenhouse for further growth.

PCR and nucleic acid hybridization procedures

Plant DNA extraction was carried out according to Gordom-Kamm et al. (1990) with the following modifications: after precipitation with ammonium acetate and isopropyl alcohol, the pellet was resuspended in TE buffer for RNAse digestion during 30 min at 37 °C; proteins were extracted with phenol/chloroform (1 : 1) and DNA in aqueous phase was precipitated with 10 mol · L^{-1} ammonium acetate and 1 volume of isopropyl alcohol. After washing with 70 % ethanol at −20 °C, the pellet was resuspended in TE and the DNA concentration was measured at 260 nm in a spectrophotometer.

Detection of a 600 bp fragment of the *nptII* gene in transgenic plants was done by PCR amplification of genomic DNA using *nptII* specific primers (5′-TATTCGGCTATGACTGGGCA-3′ and 5′-GCCAACGCTATGTCCTGATA-3′). The reaction was carried out in a GeneAmp PCR System 9600 (Perkin Elmer) using 30 cycles at 94 °C/1 min, 55 °C/2 min plus 72 °C/3 min extension, reactions were carried out in 100 µL with 0.2 µmol · L^{-1} of each specific primer, 250 µmol · L^{-1} of deoxynucleotides, 200 ng of plant DNA, and 3 units of Taq polymerase.

Southern blots were carried out after digestion of genomic DNA with *Bam*HI and *Hin*dIII restriction enzymes. DNA samples were separated by electrophoresis through 1 % agarose gels in TAE buffer and capillary blotted onto Hybond-N (Amersham) membranes with 0.4 mol · L^{-1} NaOH according to standard procedures (Sambrook et

al., 1989). A four hour prehybridization and 16–20 hour hybridization were carried out at 65 °C in 1 % crystalline BSA (Sigma), 1 mmol · L^{-1} EDTA, 0.5 mol · L^{-1} $NaHPO_4$, pH 7.2, and 7 % SDS (Ausubel et al., 1987), with gentle agitation. The probes used were a 1.678 Kbp full-CaMV 35S promotor fragment isolated from pKAH5 following *Hin*dIII digestion and the 1.776 Kbp full-size *hmg1* gene from *A. thaliana* restricted with *Bam*HI from pGGH-1. Both fragments were isolated from an agarose gel with a DNA Purification Kit (BIO-RAD), labelled with α-^{32}P dCTP by the Random Primer Extension System Kit (Dupont: NEN Life Science Products) and purified by spin dialysis (Sambrook et al., 1989). Filters were washed twice at 65 °C in 0.5 % BSA, 1 mmol · L^{-1} EDTA, 40 mmol · L^{-1} $NaHPO_4$, pH 7.2, and 5 % SDS for 10 min. The X-ray films (X-OMAT Kodak) were exposed for 1–2 days at –70 °C.

Northern blots were carried out with total RNA isolated from transformed plant leaves by the procedure of Schuler and Zielinski (1989). Twenty µg of total RNA was separated on a 1 % agarose/formaldehyde gel in MOPS buffer and transferred to Hybond-C (Amersham) membranes with 20X SSC following standard procedures (Sambrook et al., 1989). Prehybridization, hybridization, washing and exposure procedures were as described for Southern blots using the same *hmg1* labelled probe.

HMGR enzyme assay and isoprenoid analysis

Fully expanded leaves from only primary transgenic plants were used for these analyses. Tobacco chloroplasts were isolated from five g of leaves, which had been sliced into small pieces and homogenized in 40 mL of extraction buffer [343 mmol · L^{-1} sorbitol, 0.4 mmol · L^{-1} KCl, 0.04 mmol · L^{-1} EDTA, 2 mmol · L^{-1} HEPES buffer, pH 7.8, and 10 mmol · L^{-1} β-mercaptoethanol] (Cerovic and Pleniscar, 1984), using a Waring Blender at full speed for 10 seconds. The homogenate was filtered through two layers of cheese cloth and centrifuged at 400 g_n for 10 min. The supernatant was centrifuged at 3,000 g_n for 10 min and the pellet was resuspended in resuspension buffer (330 mmol · L^{-1} sorbitol, 10 mmol · L^{-1} KCl, 1 mmol · L^{-1} EDTA, 1 mmol · L^{-1} $MgCl_2$, 1 mmol · L^{-1} $MnCl_2$, 1 % BSA, 50 mmol · L^{-1} HEPES buffer, pH 7.9, and 10 mmol · L^{-1} β-mercaptoethanol) (Cerovic and Pleniscar, 1984). The chloroplast suspension obtained was separated on a discontinuous sucrose gradient consisting of 3 mL–60 % sucrose, 4 mL–45 % sucrose, 3 mL–30 % sucrose all of which were in 0.1 mol · L^{-1} Tris-HCl (pH 7.2) with fresh 10 mmol · L^{-1} β-mercaptoethanol. 2.5 mL of chloroplast suspension was added on the top of the gradients and centrifuged in a Beckman SW-40 rotor at 12,000 rpm for 2 hour at 4 °C. The chloroplast-containing fraction (lower band) was collected, diluted with extraction buffer and centrifuged at 10,000 g_n for 10 min.

Plastids were osmotically ruptured by resuspension in 20 mL 0.1 mol · L^{-1} Tris-HCl (pH 7.0) with fresh 10 mol · L^{-1} β-mercaptoethanol, and centrifuged at 15,000 g_n for 15 min. The membrane pellet was resuspended in 0.1 mL 0.1 mol · L^{-1} potassium phosphate buffer (pH 7.0) with 10 mmol · L^{-1} dithiothreitol and used to assay HMGR activity (Brooker and Russell, 1975). Total and chloroplast HMGR activities were determined as previously described by Chappell et al. (1995).

Carotenoids and chlorophyll were extracted from one g of tobacco leaves previously frozen with liquid nitrogen and finely ground in a cold mortar. 50 mg of butylated hydroxytoluene (BHT, Sigma) was added to the ground tissue extracted with acetone-hexane (1 : 1) until the tissue became white. Organic solvent fractions were collected, mixed, and evaporated. The pigments were resuspended in pure methanol for absorbency measurements at 665.2, 652.4, and 470 nm. The total amounts of carotenoids and chlorophyll were calculated as described by Lichtenthaler (1987). Lyophilized leaf samples were used for total sterols analysis by GC as previously described by Chappell et al. (1995).

Results of total and chloroplast HMGR activities, and total chlorophyll and carotenoid contents were analyzed by the Tukey test (SAS program) with a significance of 0.05 %.

Results

Transformation of tobacco plants with pKAH5 and pGGH 1–4 plasmid constructs was confirmed by the presence of a 600 bp fragment corresponding to the *nptII* gene that was observed in all cases after PCR amplification of genomic DNAs (Fig. 2 A). The non-transformed control plant gave no amplification (Fig. 2 A, lane c). A Southern blot of transgenic and non-transgenic genomic DNA samples digested with *Hin*dIII–*Bam*HI and hybridized with a 1670 bp *Hin*dIII fragment containing the CaMV 35S promoter isolated from the pKAH5 vector showed the expected bands with the exception of the non-transformed control plants (Fig. 2 B, lane c). Hybridization was also seen to a 1776 bp fragment corresponding to the whole *hmg1* gene (with intramembrane domains) in pGGH-1 and pGGH-2 plant samples (Fig. 2 C, lanes 1 and 2 respectively) and to a 1422 bp fragment corresponding to the Δ-354-*hmg1* gene fragment (without intramembrane domains) in pGGH-3 and pGGH-4 plant samples (Fig. 2 C, lanes 3 and 4 respectively), thus confirming the presence of the corresponding constructs. Non-transformed and pKAH5 vector transformed plants (Fig. 2 C, lanes c and v) did not show any of these bands, as expected. Three additional bands were detected in all cases, suggesting the presence of at least three HMGR genes in *N. tabacum* cv. Xanthi (Fig. 2 C).

Northern blots hybridized with the *hmg1* probe showed mRNA transcripts corresponding to the whole *hmg1* gene from pGGH-1 and the Δ-354-*hmg1* gene from pGGH-3 (Fig. 3), both of which are in the sense orientation and regulated by the CaMV 35S promoter. On the other hand, RNA samples from pKAH5 vector transformed plants, those corresponding to antisense constructs pGGH-2 and pGGH-4 and from control non-transformed plants (Fig. 3, lanes v, 2, 4 and c respectively) showed only a faint signal after a longer exposure time (data not shown). In order to check the amount of RNA loaded on the gel, the *hmg1* probe was removed from the membrane and the membrane was re-hybridized with a labelled actin-cDNA (Fig. 3, ACTIN). Results show the expected band with approximately the same intensity in all cases.

Total and chloroplast HMGR enzyme activities as well as chlorophyll and carotenoids were assayed in plant extracts from independent control and transformed tobacco plants. Plots in Fig. 4 represent the highest, intermediate and lowest values of the respective assays of all studied plants. It is important to note that a similar tendency of the corresponding values is found in all lines of transformed plants. For example, the lowest value of total HMGR activity in pGGH-1 (Fig. 4 A) was higher than the lowest value of the control plant. This also occurs with the intermediate and high values and with the different transformed plants. Although the chloroplast HMGR activity (Fig. 4 B) was much less compared to the total HMGR activity, the high and intermediate values followed the same tendency. A similar behaviour was

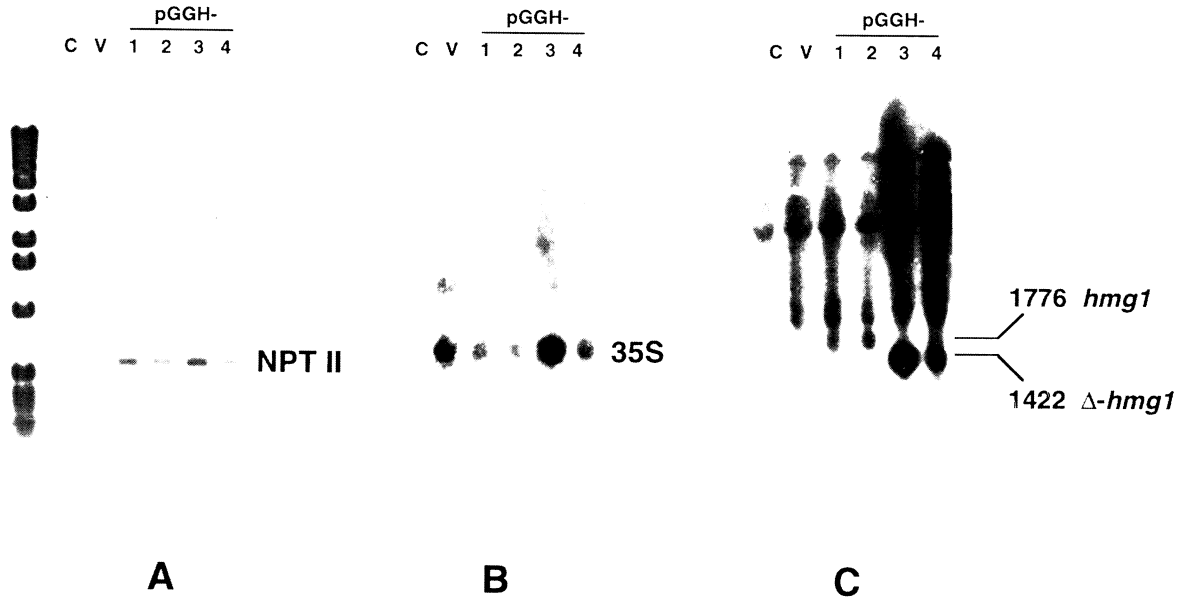

Fig. 2: Confirmation of transgenic tobacco plants was carried out by PCR and Southern blot analysis. Amplified PCR products from genomic DNA samples of transformed plants were separated by electrophoresis in an 1% agarose gel and stained with ethidium bromide (**A**), the 600 bp *npt*II gene fragment amplified with specific oligos was present in plants transformed with the pKAH5 vector (**v**), and with the different plasmids (**pGGH 1–4**). Regenerated, but non-transformed control plants (**c**) did not show the *npt*II fragment, 1 Kb DNA ladder marker is shown at the left side. Autoradiographs of Southern blot from *Bam*HI-*Hin*dIII digested genomic DNA samples hybridized with labelled *Hin*dIII (full-CaMV 35S promoter) fragment (**B**), showed the respective (**35S**) 1678 bp fragment only in vector and pGGH 1–4 transformed plants. Hybridization with the 1.776 Kbp *hmg1* full size fragment labelled probe (**C**) showed a 1776 bp full size *hmg1* fragment (**1776 *hmg1***) in samples from pGGH-1 and pGGH-2 plants, a 1422 bp truncated *hmg1* fragment (**1442 Δ-*hmg1***) in samples from pGGH-3 and pGGH-4 plants, and at least three putative tobacco HMGR genes in all samples including control and vector transformed plants.

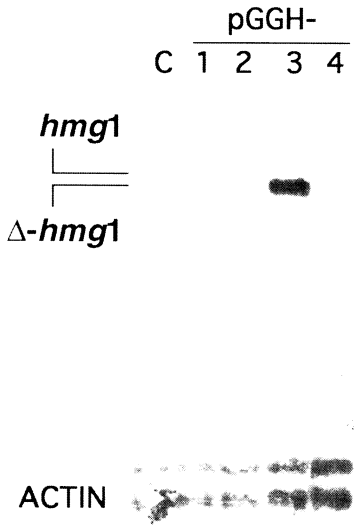

Fig. 3: Transcription of chimeric gene constructs was confirmed by northern blot analysis. RNA samples from control (**c**) and transgenic plants were hybridized with the 1.7 Kbp *hmg1* full size fragment labelled probe. The HMGR transcript was detected clearly in sense constructs, the full size transcript (***hmg1***) was detected in pGGH-1 plants and the truncated transcript (**Δ-*hmg1***) was detected in pGGH-3 plants. After overexposure of the autoradiogram, some bands were detected in vector (**v**), antisense pGGH-2 and pGGH-4 constructs and control (**c**) plants (data not shown). The same membrane was stripped and hybridized with labelled actin cDNA (**ACTIN**) as control for the amount of RNA loaded in all cases.

Table 1

A

	HMGR Activity (μKat)	
	Total	Chloroplast
Control	41.129 ± 6.67	0.137 ± 0.051
pGGH-1	79.423 ± 21.28 *	0.960 ± 0.475
pGGH-2	54.843 ± 10.90	0.244 ± 0.096
pGGH-3	61.616 ± 12.79	0.229 ± 0.101
pGGH-4	51.514 ± 10.65	0.169 ± 0.062

B

	Total isoprenoids (μg/g fresh weight)	
	Chlorophyll	Carotenoids
Control	1076.20 ± 102.58	188.39 ± 20.90
pGGH-1	1271.49 ± 126.09	237.40 ± 28.56
pGGH-2	978.27 ± 103.74	150.67 ± 19.41 *
pGGH-3	1113.07 ± 130.88	162.77 ± 16.33
pGGH-4	853.67 ± 138.69 *	135.75 ± 19.48 *

C

	Total sterol (μg/g dry weight)	Cholesterol (%)	Campesterol (%)	Stigmasterol (%)	β-Sitosterol (%)
Control	86.4	13.32 (15)	12.8 (15)	40.4 (47)	20.0 (23)
pGGH-1	90.4	15.68 (17)	7.6 (8)	38.8 (43)	28.4 (31)
pGGH-2	36.4	5.96 (16)	3.2 (9)	22.4 (62)	4.8 (13)
pGGH-3	71.2	5.80 (8)	8.8 (13)	32.8 (46)	23.6 (33)
pGGH-4	45.2	6.84 (15)	6.4 (14)	24.8 (55)	7.2 (16)

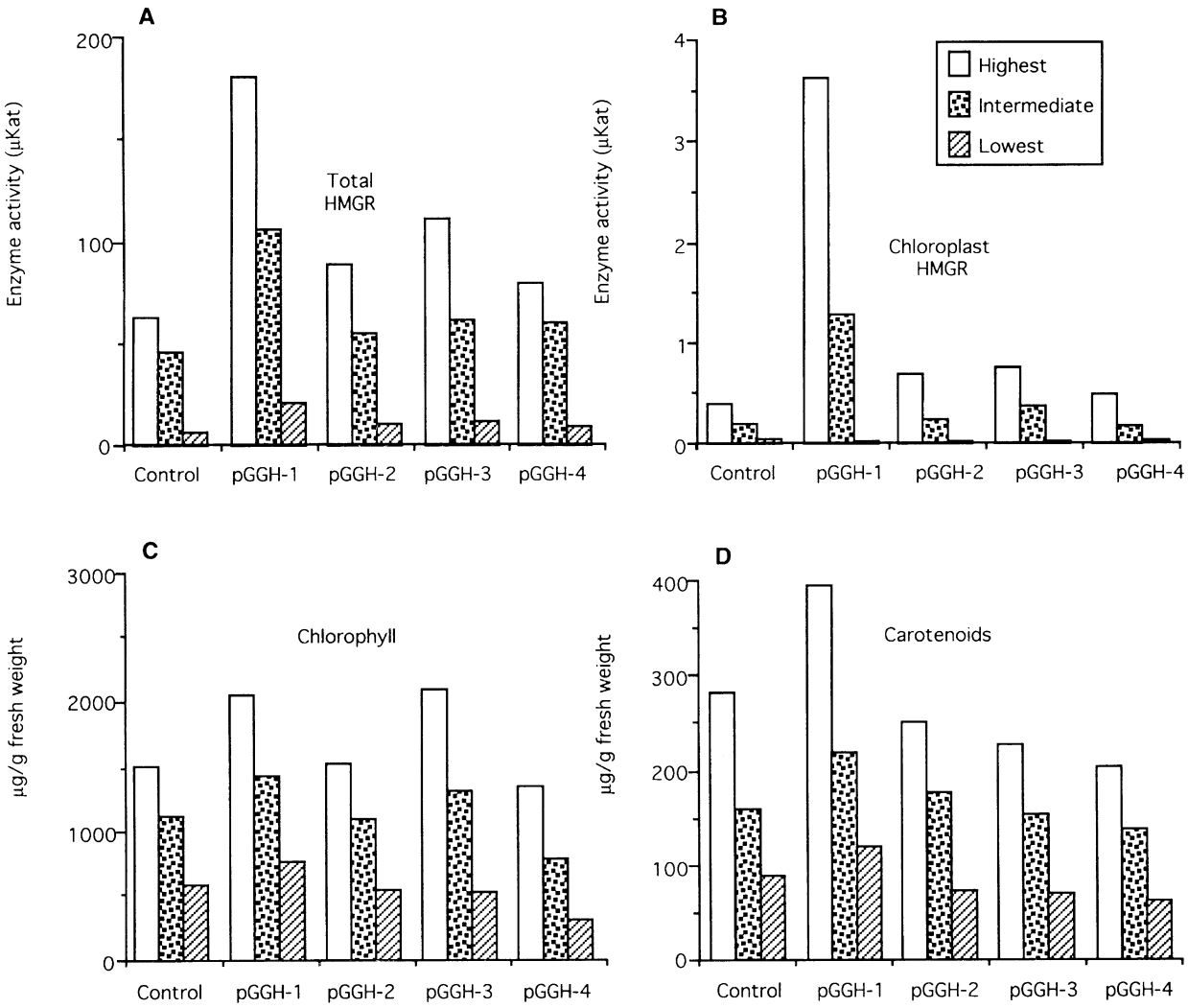

Fig. 4: Plots of different assays from control and transgenic plants. Data from three independent plants with the highest, intermediate and lowest values of the respective assays are plotted. Each bar shows the value of a different plant in total (**A**) and chloroplast (**B**) HMGR assay as well as for chlorophyll (**C**) and carotenoids (**D**).

observed in the chlorophyll (Fig. 4 C) and carotenoid analysis plots (Fig. 4 D).

When all data were considered, total HMGR activity (Table 1 A) was approximately 200–300 fold higher than chloroplast activity, with the exception of pGGH-1 plants for which the total/chloroplast HMGR ratio was 82. Although an increase in chloroplast HMGR activity of almost 100 % was detected in pGGH-1 extracts (sense whole *hmg1* gene), only the increase of total HMGR activity was significant according to the Tukey test. Despite this increase in HMGR activity neither chlorophyll or carotenoids from pGGH-1 plants showed a significant increase (Table 1 B). Total and chloroplast HMGR activity, as well as chlorophyll and carotenoids, from pGGH-3 plants (sense Δ-*hmg1* gene) showed no changes. In contrast, a decrease of chlorophyll and carotenoids in pGGH-2 plants (antisense whole *hmg1* gene) and pGGH-4 plants (antisense Δ-*hmg1* gene) was observed with respect to control plants (non-transformed and pKAH5 vector plants). These values were statistically significant with exception of chlorophyll from pGGH-2 plants.

Sterol analysis uncovered similar results (Table 1 C). pGGH-1 and pGGH-3 plants (sense, whole and Δ-*hmg1* genes respectively) had normal amounts of total sterols with small differences in specific sterols. However, pGGH-2 and pGGH-4 plants (antisense, whole and Δ-*hmg1* genes) had lower amounts of total and specific sterols, with respect to the non-transformed and pKAH5 vector plants. Although statistical analysis was not applied to these data, the decrease in sterol content correlates with the other assayed isoprenoids.

Finally, the phenotypes of some transgenic tobaccos were slightly different from controls. The following alterations were observed: 1) a decrease in flowering time in some plants containing any *hmg1* gene construction, and 2) failure to form seeeds and reduction in height of some antisense *hmg1* transformed plants.

Discussion

Results obtained in transformed tobacco plants with the sense construction pGGH-1 showed high levels of *A. thaliana hmg1* mRNA (Fig. 3) and a significant increase in HMGR activity in the respective plant extracts. However, we were not able to discriminate whether this increase was due to the chimeric HMGR or to the endogenous tobacco HMGR as a physiological response of the plant. In any case, this increase was not reflected in an alteration of chlorophyll, carotenoids, or total sterol levels, suggesting that the increased HMGR activity could be related with the production of other isoprenoids different from those assayed in this work (e.g. gibberellins, essential oils, sesquiterpenes and others). Failure to modify chloroplast isoprenoids by use of chimeric HMGRs may be due to several reasons, such as HMGR inactivation by mutation during the cloning procedure. Although the correct frame between CTP and sense HMGR constructs, as well as DNA fragments corresponding to the enzyme active site, was confirmed by nucleotide sequencing, we can not discard the possibilities of mutations close to the carboxy-end of the enzyme introduced during cloning or transformation procedures, which should have little or no effect on the enzyme activity. Also, a putative interference of targeting signals from the intramembrane domains of the whole *hmg1* gene with the added CTP sequence may have occurred. However, similar results were obtained in plant extracts containing pGGH-3 lacking the intramembrane domains. The possibility that all sense CTP-HMGR constructs would not be properly targeted to the chloroplast is low due to the already proven efficiency of the added CTP in targeting foreign proteins to the chloroplast (Van den Broeck et al., 1985; Schreier et al., 1985; Cashmore et al., 1985; Kuntz et al., 1986; Wasmann et al., 1986). No physiological failures seem to be involved, such as rate limiting enzymatic steps following the HMGR reaction, since the phenotype and development of most of the transgenic plants was normal, with exception of those mentioned. A more plausible explanation for these results could be the biochemical regulation of the excessive amount of chimeric HMGR such as feed-back regulation. This possibility is supported by similar results obtained after transformation of *A. thaliana* with the whole *hmg1* gene under control of the CaMV 35S promoter (Re et al., 1995). On the other hand, the increase in the level of total sterols of up to 6-fold reported after transformation of tobacco plants with the *hmg1* from *Hevea brasiliensis* (Schaller et al., 1995) or with the truncated hamster-HMGR gene, both under control of the CaMV 35S promoter (Chappell et al., 1995), suggests that the HMGR enzyme encoded by the *hmg1* gene of *A. thaliana* is more susceptible to, or under a different biochemical regulation than the other two enzymes. In addition the HMGR from animal sources could escape from plant regulatory systems.

The biochemical evidence for HMGR activity in isolated chloroplasts from *Pisum sativum* (Brooker and Russell, 1975; Wong et al., 1982; Wilson and Russell, 1992), *Nepeta cataria* (Arebalo and Mitchell, 1984), and *Parthenium argentatum* (Ramachandra and Das, 1986) suggest clear differences between the plastid and cytoplasmic enzymes, thus supporting the idea of independent proteins rather than just different locations of the same protein. Besides the differences in enzyme characteristics, there is some evidence for the regulation of HMGR activity in plants by reversible phosphorylation (Budde and Chollet, 1988; Gershenzon and Croteau, 1993; Stermer et al., 1994). Indeed, the catalytic domain of the HMGR enzyme from the *hmg1* gene of *A. thaliana* expressed in *E. coli* was reversibly inactivated by a *Brassica oleracea* HMGR kinase in a cell-free system (Dale et al., 1995). Thus, we can not exclude the possibility that the foreign HMGR encoded by pGGH-1 and pGGH-3 plasmids could be regulated by a phosphorylation/dephosphorylation mechanism. Also, different protein phosphatases from those already described in plant cells, such as PP1 and PP2A, seem to be responsible for phosphorylating regulatory mechanisms of chloroplast enzymes like phosphoribulokinase and glucose-6-phosphate dehydrogenase. Other post-translational and light-mediated modifications of chloroplast enzymes appears to be exerted by a thiol-disulfide exchange (Mackintosh et al., 1991). None of these regulatory mechanisms were investigated in the present work and we can not exclude the possibility that either the HMGR from the pGGH-1 construction resulted in an increase of other tobacco HMGR enzymes not related to carotenoid or sterol production, or that the HMGR enzymes from pGGH-1 and pGGH-3 could be down regulated, before or after, reaching the chloroplast. Unfortunately, immunodetection of inactive chimeric HMGR was not feasible because the enzyme from *A. thaliana* is not antigenic (Bach et al., 1990).

Southern analysis indicated sequence homology between the *hmg1* gene from *A. thaliana* with some (at least three) corresponding genes in the genome of *N. tabacum* vs. Xanthi (Fig. 2), which is in agreement with previous reports (Weissenborg et al., 1995). The existence of several HMGR genes in one plant is common since there are two known genes in *A. thaliana* (Caelles et al., 1989; Monfar et al., 1990) and *Oryza sativa* (Nelson et al., 1994), two or more isogenes in *Lycopersicon esculentum* (Narita and Gruissen, 1989; Yang et al., 1991; Weissenborn et al., 1995), at least three genes in *Hevea brasilensis* (Chye et al., 1992), three genes in *Solanum tuberosum* (Choi et al., 1992) with a subfamily of genes containing at least 9 members (Bhattacharyya et al., 1995), four genes in *Triticum aestivum* (Aoyagi et al., 1993) and a complex multigene family in pea (Monfar et al., 1990; Weissenborn et al., 1995). Specific HMGR isogenes are differentially expressed during development and in response to stress conditions (Narita and Gruissem, 1989; Yang et al., 1991; Choi et al., 1992, 1994; Chye et al., 1992; Aoyagi et al., 1993; Burnett et al., 1993; Nelson et al., 1994) but, ultimately, the efficiency of gene expression will depend on mRNA processing, transcript stability, nucleocytoplasmic transport, translation efficiency, and protein modification and half life. In this context, posttranscriptional regulatory mechanisms play an important role in the control of the gene expression and the antisense strategy has been shown to be a valuable tool for this purpose (Meyer and Saedler, 1996).

The similarity of about 75 % of the polypeptide encoded by the *hmg1* gene from *A. thaliana* with the *hmg1* gene reported for *Nicotiana sylvestris,* and the higher conservation level (88 % identity and 97 % similarity) in the putative catalytic site regions (Genschik et al., 1992), could explain the

successful inhibition of translation of the tobacco HMGR-RNAs by chimeric antisense *hmg1* RNAs (Fig. 3) transcribed from pGGH-2 and pGGH-4. However, neither total or chloroplast HMGR enzyme activity decreased in tobacco extracts transformed with any antisense construction (pGGH-2 and pGGH-4), but the levels of chlorophyll and carotenoids (Table 1 B) as well as total and specific sterols (Table 1 C) from these transgenic tobaccos were reduced. These intriguing results could be explained by considering that antisense *hmg1* RNAs may have inhibited the translation of only some specific tobacco HMGR-RNAs related with production of the assayed isoprenoids. It is unknown at present time if degradation of sense-antisense duplexes (Flavell, 1994) by RNAses (Matzke and Matzke, 1995) or by RNAse H-like endogenous enzymes (Meyer and Saedler, 1996) is occurring as previously reported.

On the other hand, both kinds of assayed isoprenoids (carotenoids and sterols) are derived from quite similar and specific condensation reactions of long and hydrophobic isoprenoid precursors such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) respectively. Whether this kind of condensation reaction requires the same and particular HMGR enzyme is also unknown. No transit peptide-like sequences have been reported for the HMGR plant genes isolated suggesting that either this enzyme has a particular and unknown transit peptide or that one is not required. Results in this work with antisense constructs (pGGH-2 and pGGH-4) could be explained by the existence of a putative specific HMGR isoform(s) involved in the initial steps of membrane-bound isoprenoid condensation reactions occurring outside of the chloroplast. Due to the hydrophobic environment required for these condensation reactions, they must be carried out in appropriate locations or as part of metabolic channels or «metabolons» (Chappell, 1995) where membrane-bound reactions may be involved. Such regions were recently proposed for different segments of the endoplasmic reticulum (ER) (Staehelin, 1997). Hence, if some enzymes such as HMGR could use another mechanism of membrane translocation is unknown at the moment. This idea is not so speculative since there are proteins anchored in the outer chloroplast membrane, containing a non-cleavable transit peptide, which are integrated into this membrane by a pathway distinct from that of other plastid proteins (Salomon et al., 1990; Li et al., 1991, 1992).

These latest considerations do not discard the possibility of early HMGR-dependent biosynthetic steps leading to IPP either outside or inside the chloroplast (Heintze et al., 1990; McCaskill and Croteau, 1995). Our results with antisense constructs show that the mevalonate-dependent pathway plays an important role in the production of carotenoids and chlorophylls. Alternative pathways to produce isoprenoid compounds, independent of the HMGR reaction, should also be considered here. Regarding this concept and taking into account the endosymbiotic origin of chloroplasts (Weeden, 1981; Yuan et al., 1994), the existence of a novel pathway for IPP formation from glyceraldehyde 3-phosphate/pyruvate, where the HMGR reaction is unnecessary, has been recently reported in bacteria (Rohmer et al., 1993), green alga (Schwender et al., 1996) and in higher plants (Lichtenthaler et al., 1997). Thus, it is possible that both HMGR-dependent and independent pathways could be functionally active in chloroplasts.

Finally, some of our chimeric sense-HMGR transgenic plants (pGGH-1 and pGGH-3) showed a decrease in flowering time and some antisense-HMGR transgenic plants (pGGH-2 and pGGH-4) showed a reduction in height and absence of seeds. These results may indicate a metabolic alteration. Whether such alterations involved HMGR-related isoprenoid production (e.g. steroids and/or gibberellins) or was due to gene inactivation by DNA integration after the transformation procedure is unknown.

There are still many important questions to be answered about the regulation of HMGR enzyme activity in plants, such as the intracellular location of HMGR isoenzymes. Answering these questions will help to elucidate how these enzymes may be regulated at biochemical or molecular levels such as posttranslational and signal transduction mechanisms. Because HMGR activity has an important role in so many aspects of plant metabolism, a better understanding of its regulation is necessary to alter plant metabolism for crop improvement or production of valuable plant metabolites in the future.

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References

AOYAGI, K., A. BEYOU, K. MOON, L. FANG, and T. ULRICH: Isolation and characterization of cDNA encoding wheat 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Physiol. *102*, 623–628 (1993).

AREBALO, R. E. and E. D. MITCHELL: Cellular distribution of 3-hydroxy-3-methylglutaryl coenzyme A reductase and mevalonate kinase in leaves of *Nepeta cataria*. Phytochemistry *23*, 13–18 (1984).

AUSUBEL, F. M., R. BRENT, R. E. KINGSTON, D. D. MOOR, J. G. SEIDMAN, J. A. SMITH, and K. STRUHL: Current Protocols in Molecular Biology. John Wiley & Sons, New York, USA (1987).

BACH, T. J., T. WEBER, and A. MOTEL: Some properties of enzymes involved in the biosynthesis and metabolism of 3-hydroxy-3-methylglutaryl-CoA in plants. In: TOWER, G. H. N. and H. A. STAFFORD (eds.): Biochemistry of the Mevalonic Acid Pathway to Terpenoids, Recent Advances in Phytochemistry, Vol. *24*, pp. 1–82. Plenum Press, London, New York (1990).

BHATTACHARYYA, M. K., N. L. PAIVA, R. A. DIXON, K. L. KORTH, and B. A. STERMER: Features of the *hmg1* subfamily of genes encoding HMG-CoA reductase in potato. Plant Mol. Biol. *28*, 1–15 (1995).

BROOKER, J. D. and D. W. RUSSELL: Subcellular localization of 3-hydroxy-3-methylglutaryl coenzyme A reductase in *Pisum sativum* seedlings. Arch. Biochem. Biophys. *167*, 730–737 (1975).

BUDDE, R. J. A. and R. CHOLLET: Regulation of enzyme activity in plants by reversible phosphorylation. Physiol. Plant. *72*, 435–439 (1988).

Burnett, R. J., I. E. Maldonado-Mendoza, T. D. McKnight, and C. L. Nessler: Expression of a 3-hydroxy-3-methylglutaryl coenzyme A reductase gene from *Camptotheca acuminata* is differentially regulated by wounding and methyl jasmonate. Plant Physiol. *103,* 41–48 (1993).

Caelles, C., A. Ferrer, L. Balcells, F. G. Hegardt, and A. Boronat: Isolation and structural characterization of a cDNA encoding *Arabidopsis thaliana* 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Mol. Biol. *13,* 627–638 (1989).

Campos, N. and A. Boronat: Targeting and topology in the membrane of plant 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Cell *7,* 2163–2174 (1995).

Cashmore, A. R., L. Szabo, M. Timko, A. Kausch, G. van den Broeck, P. Schreier, H. Bohnert, L. Herrera-Estrella, M. van Montagu, and J. Schell: Import of polypetides into chloroplasts. Bio/technology *3,* 803–808 (1985).

Cashmore, A. R.: Nuclear genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. In: Kosuge, T., C. P. Meredith, and H. Hollander (eds.): Genetic Engineering of Plants. An Agricultural Perspective, pp. 29–38. Plenum Press, New York (1983).

Cerovic, Z. G. and P. M. Pleniscar: An improved procedure for the isolation of intact chloroplast of high photosynthetic capacity. Biochem. J. *223,* 543–545 (1984).

Chappell, J.: Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. *46,* 521–547 (1995).

Chappell, J., F. Wolf, J. Proulx, R. Cuellar, and C. Saunders: Is the reaction catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase a rate-limiting step for isoprenoid biosynthesis in plants? Plant Physiol. *109,* 1337–1343 (1995).

Choi, D., R. M. Bostock, S. Avdiushko, and D. F. Hildebrand: Lipid-derived signals that discriminate wound- and pathogen-responsive isoprenoid pathway in plants: Methyl jasmonate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3-methylglutaryl coenzyme A reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. Proc. Natl. Acad. Sci. USA *91,* 2329–2333 (1994).

Choi, D., B. L. Ward, and R. M. Bostock: Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic acid. Plant Cell *4,* 1333–1344 (1992).

Chye, M. L., C. T. Tan, and N. H. Chua: Three genes encode 3-hydroxy-3-methylglutaryl-coenzyme A reductase in *Hevea brasiliensis: hmg1* and *hmg3* are differentially expressed. Plant Mol. Biol. *19,* 473–484 (1992).

Dale, S., M. Arró, B. Becerra, N. G. Morrice, A. Boronat, D. G. Hardie, and A. Ferrer: 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,* 506–513 (1995).

Deblaere, R., B. Bytebier, H. De Greve, F. Deboeck, J. Schell, M. van Montagu, and J. Leemans: Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. Nucleic Acid Res. *13,* 4777–4788 (1985).

Denbow, C. J., S. Lang, and C. L. Cramer: The N-terminal domain of tomato 3-hydroxy-3-methylglutaryl-CoA reductases. J. Biol. Chem. *271,* 9710–9715 (1996).

Enjuto, M., L. Balcells, N. Campos, C. Caelles, M. Arró, and A. Boronat: *Arabidopsis thaliana* contains two differentially expressed 3-hydroxy-3-methylglutaryl coenzyme A reductase genes, which encode microsomal forms of the enzyme. Proc. Natl. Acad. Sci. USA *91,* 927–931 (1994).

Flavell, R. B.: Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA *91,* 3490–3496 (1994).

Genschik, P., M. C. Criqui, Y. Parmentier, J. Marbach, A. Durr, J. Fleck, and E. Jamet: Isolation and characterization of a cDNA encoding a 3-hydroxy-3-methylglutaryl coenzyme A reductase from *Nicotiana sylvestris.* Plant Mol. Biol. *20,* 337–341 (1992).

Gershenzon, J. and R. Croteau: Regulation of monoterpene biosynthesis in higher plants. In: Tower, G. H. N. and H. A. Stafford (eds.): Biochemistry of the Mevalonic Acid Pathway to Terpenoids, Recent Advances in Phytochemistry, Vol. *24,* pp. 99–160. Plenum Press, London, New York (1990).

Gershenzon, J. and R. Croteau: Terpenoid biosynthesis: The basic pathway and formation of monoterpenes, sequiterpenes, and diterpenes. In: Moore, T. S. Jr. (ed.): Lipid Metabolism in Plants, pp. 339–388. CRC, Boca Raton, Tokyo (1993).

Gordon-Kamm, W. J., T. M. Spencer, M. L. Mangano, T. R. Adams, R. J. Daines, W. G. Start, J. V. O'Brien, S. A. Chambers, W. R. Adams, N. G. Willetts, T. B. Rice, C. J. Mackey, R. W. Krueger, A. P. Kaush, and P. G. Lemaux: Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell *2,* 603–618 (1990).

Gray, J. C.: Control of isoprenoid biosynthesis in higher plants. Adv. Bot. Res. *14,* 25–91 (1987).

Hanahan, D.: Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. *166,* 557–580 (1983).

Heintze, A., J. Görlach, C. Leuschner, P. Hoppe, P. Hagelstein, D. Schulze-Siebert, and G. Schultz: Plastidic isoprenoid synthesis during chloroplast development. Change from metabolic autonomy to a division-of-labor stage. Plant Physiol. *93,* 1121–1127 (1990).

Herrera-Estrella, L. and J. Simpson: Foreign gene expression in plants. In: Shaw, C. H. (ed.): Plant Molecular Biology, A Practical Approach, pp. 131–160. IRL Press, Oxford, England (1988).

Kuntz, M., A. Simons, J. Schell, and P. H. Schreier: Targeting of proteins to chloroplasts in transgenic tobacco by fusion to mutated transit peptide. Mol. Gen. Genet. *205,* 453–460 (1986).

Learned, R. M.: Light suppress 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression in *Arabidopsis thaliana.* Plant Physiol. *110,* 645–655 (1996).

Learned, R. M. and G. R. Fink: 3-hydroxy-3-methylglutaryl-coenzyme A reductase from *Arabidopsis thaliana* is structurally distinct from the yeast and animal enzymes. Proc. Natl. Acad. Sci. USA *86,* 2779–2783 (1989).

Li, H. M., T. Moore, and K. Keegstra: Targeting of proteins to the outer envelope membrane uses a different pathway than transport into chloroplasts. Plant Cell *3,* 709–717 (1991).

Li, H. M., T. D. Sullivan, and K. Keegstra: Information for targeting to the chloroplastic inner envelope membrane is contained in the mature region of the maize *Bt1*-encoded protein. J. Biol. Chem. *267,* 18999–19004 (1992).

Lichtenthaler, H. K., J. Schwender, A. Disch, and M. Rohmer: Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. FEBS Lett. *400,* 271–274 (1997).

Lichtenthaler, H. K.: Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods in Enzymol. *148,* 350–382 (1987).

Lumbreras, V., N. Campos, and A. Boronat: The use of an alternative promoter in the *Arabidopsis thaliana* HMG1 gene generates an mRNA that encodes a novel 3-hydroxy-3-methylglutaryl coenzyme A reductase isoform with an extended N-terminal region. Plant J. *8,* 541–549 (1995).

Mackintosh, C., J. Coggins, and P. Cohen: Plant protein phosphatases. Subcellular distribution, detection of protein phosphatase 2C and identification of protein phosphatase 2A as the major quinate dehydrogenase phosphatase. Biochem. J. *273*, 733–738 (1991).

Matzke, M. A. and A. J. M. Matzke: How and why do plants inactivate homologous (trans) genes? Plant Physiol. *107*, 679–685 (1995).

McCaskill, D. and R. Croteau: Monoterpene and sesquiterpene biosynthesis in glandular trichomes of peppermint (*Mentha × piperita*) rely exclusively on plastid-derived isopentenyl diphosphate. Planta *197*, 49–56 (1995).

McGarvey, D. J. and R. Croteau: Terpenoid metabolism. Plant Cell *7*, 1015–1026 (1995).

Meyer, P. and H. Saedler: Homology-dependent gene silencing in plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. *47*, 23–48 (1996).

Monfar, M., C. Caelles, L. Balcells, A. Ferrer, F. G. Hegardt, and A. Boronat: Molecular cloning and characterization of plant 3-hydroxy-3-methylglutaryl coenzyme A reductase. In: Tower, G. H. N. and H. A. Stafford (eds.): Biochemistry of the Mevalonic Acid Pathway to Terpenoids, Recent Advances in Phytochemistry, Vol. *24*, pp. 83–97. Plenum Press, London, New York (1990).

Narita, J. O. and W. Gruissem: Tomato hydroxymethylglutaryl-CoA reductase is required early in fruit development but not during ripening. Plant Cell *1*, 181–190 (1989).

Nelson, A. J., P. W. Doerner, Q. Zhu, and C. J. Lamb: Isolation of a monocot 3-hydroxy-3-methylglutaryl coenzyme A reductase gene that is elicitor-inducible. Plant Mol. Biol. *25*, 401–412 (1994).

Ramachandra, A. R. and V. S. R. Das: Partial purification and characterization of 3-hydroxy-3-methylglutaryl coenzyme A reductase from leaves of guayule (*Parthenium argentatum*). Phytochemistry *25*, 2471–2474 (1986).

Re, B. E., D. Jones, and R. M. Learned: Co-expression of native and introduced genes reveals cryptic regulation of HMG CoA reductase expression in *Arabidopsis*. Plant J. *7*, 771–784 (1995).

Rohmer, M., M. Knani, P. Simonin, B. Sutter, and H. Sahm: Isoprenoid biosynthesis in bacteria: a novel pathway for the early steps leading to isopentenyl diphosphate. Biochem. J. *295*, 517–524 (1993).

Russell, D. W.: 3-hydroxy-3-methylglutaryl-CoA reductases from pea seedlings, Methods in Enzymol. *110*, 26–40 (1985).

Salomon, M., K. Fischer, U. I. Flügge, and J. Soll: Sequence analysis and protein import studies of an outer chloroplast envelope polypeptide. Proc. Natl. Acad. Sci. USA *87*, 5778–5782 (1990).

Sanger, F., S. Nicklen, and A. R. Coulson: DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463–5467 (1977).

Sambrook, J., E. F. Fritsch, and T. Maniatis: Molecular Cloning. A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, New York (1989).

Schaller, H., B. Grausem, P. Benveniste, M. L. Chye, C. T. Tan, Y. H. Song, and N. H. Chua: Expression of the *Hevea brasiliensis* (H. B. K.) Müll. Arg. 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 in tobacco results in sterol overproduction. Plant Physiol. *109*, 761–770 (1995).

Schreier, P. H., E. A. Seftor, J. Schell, and H. J. Bohnert: The use of nuclear-encoded sequences to direct the light-regulated synthesis and transport of a foreign protein into plant chloroplasts. EMBO J. *4*, 25–32 (1985).

Schuler, M. A. and R. E. Zielinski: RNA isolation from light- and dark-grown seedlings. In: Methods in Plant Molecular Biology, pp. 89–96. Academic Press Inc., San Diego, Toronto (1989).

Schwender, J., M. Seemann, H. K. Lichtenthaler, and M. Rohmer: Biosynthesis of isoprenoids (carotenoids, sterols, prenyl side-chains of chlorophylls and plastoquinone) via a novel pyruvate/glyceraldehyde 3-phosphate non-mevalonate pathway in the green alga *Scenedesmus obliquus*. Biochem. J. *316*, 73–80 (1996).

Staehelin, L. A.: The plant ER: a dynamic organelle composed of a large number of discrete functional domains. Plant J. *11*, 1151–1165 (1997).

Stermer, B. A., G. M. Bianchini, and K. L. Korth: Regulation of HMG-CoA reductase activity in plants. J. Lipid Res. *35*, 1133–1140 (1994).

Suzuki, H. and I. Uritani: Subcellular localization of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and other membrane-bound enzymes in sweet potato roots. Plant Cell Physiol. *17*, 691–700 (1976).

Van de Broeck, G., M. P. Timko, A. P. Kausch, A. R. Cashmore, M. van Montagu, and L. Herrera-Estrella: Targeting of a foreign protein to chloroplasts by fusion to the transit peptide from the small subunit of ribulose 1,5-bisphosphate carboxylase. Nature *313*, 358–363 (1985).

Wasmann, C. C., B. Reiss, S. G. Bartlett, and H. J. Bohnert: The importance of the transit peptide and the transported protein for protein import into chloroplasts. Mol. Gen. Genet. *205*, 446–453 (1986).

Weeden, N. F.: Genetic and biochemical implications of the endosymbiotic origin of the chloroplast. J. Mol. Evol. *17*, 133–139 (1981).

Weissenborn, D. L., C. J. Denbow, M. Laine, S. S. Lang, Z. Yang, X. Yu, and C. L. Cramer: HMG-CoA reductase and terpenoid phytoalexins: molecular specialization within a complex pathway. Physiol. Plant. *93*, 393–400 (1995).

Wilson, T. M. and D. W. Russell: Localization, partial purification and regulation of pea plastid HMG-CoA reductase. Biochem. Biophys. Res. Comm. *184*, 530–537 (1992).

Wong, R. J., D. K. McCormack, and D. W. Russell: Plastid 3-hydroxy-3-methylglutaryl coenzyme A reductase has distinctive kinetic and regulatory features: properties of the enzyme and positive phytochrome control of activity in pea seedlings. Arch. Biochem. Biophys. *216*, 631–638 (1982).

Yang, Z., H. Park, G. H. Lacy, and C. L. Cramer: Differential activation of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes by wounding and pathogen challenge. Plant Cell *3*, 397–405 (1991).

Yu-Ito, R., K. Oba, and I. Uritani: Some problems in the assay method of HMG-CoA reductase activity in sweet potato in the presence of other HMG-CoA utilizing enzymes. Agric. Biol. Chem. *46*, 2087–2091 (1982).

Yuan, J., R. Henry, M. McCaffery, and K. Cline: Sec A homolog in protein transport within chloroplasts: evidence for endosymbiont-derived sorting. Science *266*, 796–798 (1994).