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# Expression of the *Hevea brasiliensis* (H.B.K.) Müll. Arg. 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase 1 in Tobacco Results in Sterol Overproduction

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**A genomic fragment encoding one (HMGR1) of the three 3-hydroxy-3-methylglutaryl coenzyme A reductases (HMGRs) from *Hevea brasiliensis* (H.B.K.) Müll. Arg. (M.-L. Chye, C.-T. Tan, N.-H. Chua [1992] Plant Mol Biol 19: 473–484) was introduced into *Nicotiana tabacum* L. cv xanthi via *Agrobacterium* transformation to study the influence of the *hmg1* gene product on plant isoprenoid biosynthesis. Transgenic plants were morphologically indistinguishable from control wild-type plants and displayed the same developmental pattern. Transgenic lines showed an increase in the level of total sterols up to 6-fold, probably because of an increased expression level of *hmg1* mRNA and a corresponding increased enzymatic activity for HMGR, when compared with the level of total sterols from control lines not expressing the *hmg1* transgene. In addition to the pathway end products, campesterol, sitosterol, and stigmasterol, some biosynthetic intermediates such as cycloartenol also accumulated in transgenic tissues. Most of the overproduced sterols were detected as steryl-esters and were likely to be stored in cytoplasmic lipid bodies. These data strongly support the conclusion that plant HMGR is a key limiting enzyme in phytosterol biosynthesis.**

The enzyme HMGR, which converts 3-hydroxy-3-methylglutaryl-CoA to mevalonate, is involved in sterol biosynthesis in most eukaryotic cells. In addition to sterols it is involved in the biosynthesis of a vast array of isoprenoids, playing important physiological roles. In plants some isoprenoids are ubiquitously present as growth regulators (ABA, GAs, and cytokinins), as compounds involved in photosynthesis or respiration (Chls, tocopherols, plastoquinone, carotenoids, and ubiquinone), and as compounds involved in posttranslational modifications of proteins (farnesol, geranylgeraniol, and dolichol derivatives). In several plant families (*Asteraceae* and especially *Euphorbiaceae*), isoprenoids (triterpenes and rubber) may accumulate in specialized vessels: laticifers. Natural rubber, which is a *cis*-1,4-polyisoprene of high molecular weight ($4 \times 10^6$) (Westall, 1968), is obtained from the latex of *Hevea brasiliensis* (H.B.K.) Müll. Arg. HMGR, which has been shown to be present in the pelleted fraction of centrifuged latex, is presumably involved in rubber biosynthesis (Hepper and Audley, 1969; Sipat, 1982, 1985). To better understand the regulation and expression of genes implicated in rubber biosynthesis, cDNA and genomic clones encoding *H. brasiliensis* HMGRs have been isolated and characterized (Chye et al., 1991a, 1992). Sequence analysis revealed that these clones fall into three different members of a small gene family: *hmg1*, *hmg2*, and *hmg3*. Northern blot analysis indicated that *hmg1* is more highly expressed in laticifers than in leaves, suggesting strongly that one (*hmg1*) of the three *hmg* genes in *H. brasiliensis* is specifically involved in rubber biosynthesis.

To further examine the function and the regulation of the *hmg1* gene, stable transformation of *Nicotiana tabacum* L. cv xanthi with the latter has been performed. In a first approach, an *hmg1* genomic fragment lacking the *hmg1* promoter was placed downstream of a CaMV 35S promoter to determine whether a typically *H. brasiliensis hmg* gene may be expressed in a plant unable to synthesize rubber (laticifer cells are absent in tobacco) and influence isoprenoid biosynthesis within it.

In recent years, arguments have been made in favor of a limiting role of HMGR in plant sterol biosynthesis (Bach, 1986, 1987; Chappell et al., 1991; Gondet et al., 1992). Such a conclusion was supported in part by the isolation and characterization of a tobacco mutant (LAB1–4) that was shown to overproduce sterols; in this mutant, most of the overproduced sterols were steryl-esters stored in the cells as cytoplasmic lipid droplets (Maillot-Vernier et al., 1991; Gondet et al., 1994). In addition, leaf or callus tissues from the mutant contained approximately a 3-fold higher HMGR activity when compared with wild-type tissues (Gondet et al., 1992).

Sterols appeared to be attractive targets to study the effects of the expression of a foreign *hmg* gene on cellular metabolism because of the above data. Therefore, we investigated the sterol content of the progeny of transgenic

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Abbreviations: CaMV, cauliflower mosaic virus; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase (EC 1.1.1.34; mevalonate-$NADP^+$-oxidoreductase).

plants regenerated from tobacco leaf discs infected with an *Agrobacterium tumefaciens* strain carrying a *H. brasiliensis hmg1* genomic fragment under control of the CaMV 35S promoter.

In this paper, we describe *hmg1* transgenic tobacco lines that overaccumulate sterols. A thorough study of these plants show that they possess biochemical traits (sterol overproduction, accumulation of biosynthetic intermediates as steryl-esters, presence of cytoplasmic lipid droplets) very similar to those previously identified in the tobacco mutant LAB1–4.

Our results provide a strong argument in favor of a limiting role of HMGR in plant sterol biosynthesis.

## MATERIALS AND METHODS

### Plasmid Constructs

A 3.8-kb *Eco*RI fragment containing the *Hevea brasiliensis* (H.B.K.) Müll. Arg. *hmg1* gene (nucleotides −20 to +3740 [Chye et al., 1991a]) was cloned into the *Eco*RI site of pMON9818 (Cuozzo et al., 1988), which contains a kanamycin-resistance marker gene for selection in plants and a spectinomycin-resistance marker gene for selection in bacteria. The ligation mixture was used to transform *Escherichia coli* LE 392 (Stratagene). Transformants were selected on Luria broth medium supplemented with spectinomycin (100 μg/mL). Colony hybridization was carried out using a random-primed $^{32}$P-labeled *Hevea hmg1* cDNA (Chye et al., 1991a) probe to identify the LE 392 transformants, which harbored the recombinant plasmid containing the *hmg1* insert. To verify the orientation of the insert in the recombinant plasmid, plasmid DNA extracted from the transformants were digested with *Hin*dIII, which cleaves the *hmg1* gene uniquely after nucleotide +3015 (Chye et al., 1991a) and the vector, just downstream from the *Eco*RI site. Two pMON9818 derivatives, one with the *hmg1* gene inserted downstream from the CaMV 35S promoter in a 5′ to 3′ orientation (pHEV15) and the other, in the opposite orientation (pHEV16), were introduced into *Agrobacterium tumefaciens* strain A208SE by triparental mating using the helper plasmid pRK2013 (Rogers et al., 1988).

### Plant Transformation

pHEV15 and pHEV16 were transferred via *A. tumefaciens* to *N. tabacum* L. var xanthi by leaf disc transformation (Horsch et al., 1985). Transformants were selected on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with kanamycin (100 μg/mL). Twenty primary transformants ($T_1$) were chosen for northern blot analysis. $T_1$ transgenic tobacco plants were selfed and plants from succeeding generations were grown under standard greenhouse conditions as described by Maillot-Vernier et al. (1990).

### Northern Blot Analysis

Twenty micrograms of total RNA isolated from young leaves (Nagy et al., 1988) were denatured at 50°C in the presence of glyoxal, separated by electrophoresis in 1.5% agarose gels, and blotted onto Hybond-N (Amersham) filters. The conditions of blotting, prehybridization, and hybridization were as recommended by the manufacturer. Northern blot analysis was carried out using a random-primed $^{32}$P-labeled *Hevea hmg1* cDNA (Chye et al., 1991a) probe. The blots were washed in 0.1× SSC buffer (Sambrook et al., 1989) containing 0.1% SDS at 65°C. Following hybridization with the $^{32}$P-labeled *Hevea* cDNA probe encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (Chye et al.,1991b), blots were washed in 2× SSC buffer containing 0.1% SDS at room temperature.

### HMGR Enzymatic Assay

Measurements of enzymatic activity for HMGR were performed essentially as described by Bach et al. (1986).

Isolation of membranes from tobacco leaves was carried out according to the method of Gondet et al. (1992). Microsomal proteins were quantified by the Bio-Rad protein assay using BSA as a standard. The standard HMGR assay system consisted of 100 mM $Na_2PO_4$, pH 7.5, 4.2 mM EDTA, 8.4 mM DTE, 100 μg of BSA, 5.1 mM Glc-6-P, 10 milliunits of Glc-6-P dehydrogenase, 600 μM NADPH, 8 mM $NaHCO_3$, and 30 μM (0.1 μCi) (*R,S*)-[3-$^{14}$C]3-hydroxy-3-methylglutaryl-CoA in a final volume of 60 μL. Approximately 300 μg of microsomal proteins were incubated at 37°C for 30 min. Reactions were terminated by adding 10 μL of 37% HCl. Lactonization of samples was then carried out at 37°C for 30 min. Substrate was separated from product (mevalonolactone) by TLC on Merck 60F254 (E. Merck, Darmstadt, Germany) precoated silica plates with diethyl-ether/acetone (3:1) as the developing solvent for two runs. Mevalonolactone was scraped off ($R_f$ = 0.8) and collected into scintillation vials containing 15 mL of liquid scintillation cocktail for aqueous samples (Ready Gel, Beckman). Radioactivity was monitored with a Packard Instrument (Downers Grove, IL) liquid scintillation counter.

### Genetic Analysis of Transformants

Seeds were surface sterilized in a 25% commercial sodium hypochlorite solution for 10 min and rinsed three times with autoclaved distilled water. Axenic seeds were germinated in vitro on an agar-solidified medium containing one-half-diluted macronutrients of Murashige and Skoog (1962). For each generation of seeds, a sample of seedlings was randomly chosen after 2 weeks of germination. One cotyledon per seedling was then excised and plated on Murashige and Skoog medium with 3% Glc, 2 mg/L BA, 0.2 mg/L IAA, and 100 mg/L kanamycin. Generations of cotyledons were scored for kanamycin resistance (development of shoots) or sensitivity (no development of shoots) after 1 month of culture at 25°C with a 16-h light period. Experiments were performed twice. A sample of the corresponding generation of seedlings was also scored for segregation of kanamycin resistance on the germination medium described above.

### Callus Culture

Cultures were generated by plating cotyledons from axenic seedlings or surface-sterilized leaf explants on a callus-inducing medium already described (Maillot-Vernier et al., 1990). To determine the sterol composition of a genotype at the callus level, cotyledon-derived calli were subcultured for 2 months on the medium with or without 100 mg/L kanamycin.

### Extraction and Titration of Total Sterols

About 100 mg of lyophilized material were ground in an extraction mixture of dichloromethane/methanol (2:1) with an Ultra-Turrax homogenizer (Janke and Kundel Ika-Werk, Staufen, Germany). Total lipids were then extracted at 70°C. The dried residue was saponified with 5 mL of a solution of 6% (w/v) KOH in methanol at 90°C for 1 h to release the sterol moiety of steryl-esters. The total sterols obtained after saponification were extracted with 3 volumes of *n*-hexane. The dried residue was then resolved by TLC on Merck 60F254 precoated silica plates. Two runs in dichloromethane led to the separation of 4,4-dimethyl sterols, 4α-methyl sterols, and 4-desmethyl sterols ($R_f$ = 0.20–0.30); the three bands corresponding to the three types of sterols were scraped off and eluted together in dichloromethane to be analyzed as one pool. After the sample was filtered through sodium sulfate and solvent evaporation, the residue was dissolved in toluene and then acetylated for 1 h at 60°C with a mixture of pyridine/acetic anhydride (1:1). After the reagents were evaporated, the sterol derivatives were resolved by TLC. One run in dichloromethane led to the migration of steryl-acetates as a single band, which was scraped off and eluted in dichloromethane. Sterol derivatives were analyzed by GC using a Carlo Erba GC4160 (Erba Science, Paris, France) with a flame ionization detector and a glass capillary column (Wall-Coated Open Tubular, 30 m long, 0.25 mm i.d., coated with DB1; J. and W. Scientific, Folsom, CA) and using $H_2$ as a carrier gas (2 mL/min). The temperature program used a fast increase from 60 to 230°C (30°C/min) and then a slow increase from 230 to 280°C (2°C/min). Amounts of steryl-acetates were quantified with a SP4270 integrator (Spectra Physics France, Les Ulis) using cholesterol as an internal standard. Sterol structures were identified by GC-MS as described elsewhere (Rahier and Benveniste, 1989). The SE was less than 15% for each determination of a sterol amount from the extraction procedure to the analytical step.

### Separate Titration of Free Sterols and Steryl-Esters

The dried residue of total lipid extract was chromatographed on silica plates with dichloromethane as the developing solvent. Free sterols ($R_f$ = 0.2 to 0.3) and steryl-esters ($R_f$ = 0.9) were then treated separately. Steryl-esters underwent a saponification step. Each fraction was then acetylated, and steryl-acetates were identified as described above. When free sterols and steryl-esters were quantified separately, the sum of each was lower than the amount of total sterols determined directly after saponification of the crude extract. This was due to unavoidable losses during the additional steps involved in separation of free sterols and steryl-esters.

### Specific Staining of Intracytoplasmic Lipid Droplets

Calli or leaf epidermis from the plant material used in the experiments described above were stained in a saturated (about 0.3%, w/v) Sudan III solution in 70% (v/v) ethanol. With this dye, lipid droplets appear as orange spherical granules by light microscopic examination.

## RESULTS

### Northern Blot Analysis

Twenty independent kanamycin-resistant *N. tabacum* $T_1$ transformants of pHEV15 and 10 such transformants of pHEV16 were chosen for northern blot analysis. The results of this analysis of five $T_1$ transformants of pHEV15 ($T_{1/A}$, $T_{1/C}$, $T_{1/8}$, $T_{1/9}$, and $T_{1/15}$) and one $T_1$ transformant of pHEV16 are shown in Figure 1. All of the $T_1$ transformants of pHEV15 showed expression of *Hevea hmg1*, which was demonstrated by the presence of a 2.4-kb *hmg1* transcript upon hybridization to a $^{32}$P-labeled *Hevea hmg1* cDNA probe. Transformants $T_{1/A}$, $T_{1/C}$, and $T_{1/9}$ (Fig. 1, lanes 3, 4, and 6) showed higher expression of *hmg1* than transformants $T_{1/8}$ and $T_{1/15}$ (Fig. 1, lanes 5 and 7). The untransformed wild-type tobacco control (Fig. 1, lane 1) and the $T_1$ transformant of pHEV16 (Fig. 1, lane 2), in which the *hmg1* genomic fragment was inserted in the opposite orientation downstream from the CaMV 35S promoter, did not express *hmg1*.

$T_1$ transformants of pHEV15 were selfed and the harvested seeds were sown to obtain $T_2$ transgenic plants. Five independent kanamycin-resistant $T_2$ plants derived from each of five $T_1$ transformants were chosen for northern blot analysis. Figure 2 shows that the expression of the 2.4-kb *Hevea hmg1* transcript was higher in $T_{2/9}$ plants (Fig. 2, lanes 6–10) than in $T_{2/8}$ (Fig. 2, lanes 1–5) or $T_{2/15}$ (Fig. 2, lanes 11–15).

### HMGR Enzymatic Activity in *hmg1* Transgenic Tobacco

HMGR activities were monitored in microsomal fractions obtained from $T_3$ plants from each of the *hmg1* trans-

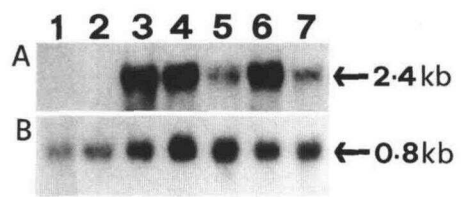


**Figure 1.** Northern blot analysis. Lane 1, Untransformed wild-type tobacco; lane 2, kanamycin-resistant $T_1$ transgenic plant of pHEV16; lanes 3 to 7, kanamycin-resistant transgenic $T_{1/A}$, $T_{1/C}$, $T_{1/8}$, $T_{1/9}$, and $T_{1/15}$ plants, respectively, of pHEV15. A, Blot was hybridized to a $^{32}$P-labeled *hmg1* cDNA probe. The arrow denotes the 2.4-kb *hmg1* transcript. B, Blot was hybridized to a *Hevea* ribulose-1,5-bisphosphate carboxylase small subunit control probe. The arrow denotes the 0.8-kb *rbcS* transcript.

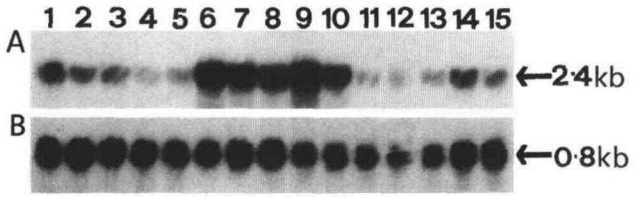


**Figure 2.** Northern blot analysis of $T_2$ kanamycin-resistant transgenic plants obtained by selfing the $T_{1/8}$, $T_{1/9}$, and $T_{1/15}$ plants. Total RNA samples extracted from $T_{2/8-1}$ to $T_{2/8-5}$ plants (lanes 1–5), $T_{2/9-1}$ to $T_{2/9-5}$ plants (lanes 6–10), and $T_{2/15-1}$ to $T_{2/15-5}$ (lanes 11–15) were hybridized. A, *Hevea hmg1* cDNA probe; the arrow denotes the 2.4-kb *hmg1* transcript. B, *Hevea* ribulose-1,5-bisphosphate carboxylase small subunit control probe; the arrow denotes the 0.8-kb *rbcS* transcript.

genic lines displaying a high level of HMGR1 mRNA (lines A, C, and 9 as shown in Fig. 1). Table I shows first that untransformed wild-type and transgenic control tobacco, in which the *hmg1* genomic fragment has been inserted in the opposite orientation downstream from the CaMV 35S promoter, were characterized by the same HMGR activities ($2.0 \pm 0.8$ pmol $min^{-1}$ $mg^{-1}$ protein). Second, transgenic plants from all three lines that were examined had a very strong increase in HMGR activity; they contained 4- to 8-fold higher enzymatic activities than control plants.

### Genetic Analysis

The $T_2$ generations obtained by selfing the $T_1$ primary transformants were scored for kanamycin resistance (Table II). Transgenic lines $T_{2/A}$, $T_{2/8}$, $T_{2/9}$, and $T_{2/15}$ segregated the kanamycin-resistance marker in a ratio of 3 to 1 resistant to sensitive individuals, whereas line $T_{2/C}$ segregated the same character in a ratio of 15 to 1 resistant to sensitive individuals. It is therefore likely that $T_{1/A}$, $T_{1/8}$, $T_{1/9}$, and $T_{1/15}$ contained a single insert, whereas two T-DNA inserts were present in the genome of $T_{1/C}$.

Twenty individuals from lines $T_{2/A}$, $T_{2/C}$, and $T_{2/9}$ were selfed and the resulting $T_3$ progenies were also scored for kanamycin resistance to discriminate the wild-type, heterozygote, and homozygote $T_2$ individuals with respect to the transgene. The results are consistent with a Mendelian segregation of one and two loci in lines $T_{3/A}$ and $T_{3/C}$, respectively (Fig. 3).

**Table I.** *Enzymatic activity for HMGR in membranes isolated from tobacco leaves with a high HMGR1 mRNA level*

Leaf material was taken from the upper third of 6-week-old greenhouse plants.

| Untransformed Wild Type | Transgenic Lines[a] | | | |
|---|---|---|---|---|
| | Control[b] | A | C | 9 |
| $2.0 \pm 0.8$[c] | $2.0 \pm 0.8$ | $7.8 \pm 1.2$ | $11.6 \pm 1.0$ | $15.6 \pm 2.8$ |

[a] HMGR assays were performed with $T_3$ families of transgenic *hmg1* lines A, C, and 9. Segregation analysis indicated 100% kanamycin resistance in these families. [b] Control $T_3$ plants are carrying the *hmg1* genomic fragment inserted downstream from the CaMV 35S promoter in a 3′ to 5′ orientation and were shown to be kanamycin resistant by leaf explant culture on a medium containing 100 mg/L kanamycin. [c] pmol $min^{-1}$ $mg^{-1}$ protein ± SE. Values are the means of three independent assays.

**Table II.** *Segregation analysis of $T_2$ generations obtained by selfing the $T_1$ original hmg1 transformants*

R, Resistant. S, Sensitive.

| Line | No. of Progeny | | R | $\chi^{2a}$ |
|---|---|---|---|---|
| | R | S | | |
| | | | % | |
| $T_{2/A}$ | 54 | 19 | 74 | 0.04 |
| $T_{2/C}$ | 109 | 4 | 96 | 1.42 |
| $T_{2/8}$ | 86 | 28 | 75 | 0.01 |
| $T_{2/9}$ | 47 | 21 | 69 | 1.25 |
| $T_{2/15}$ | 58 | 12 | 83 | 2.30 |

[a] $\chi^2 < 3.84$, $P > 0.05$.

### Biochemical Phenotype of *hmg1* Transgenic Tobacco

Twenty plants (mentioned above) from each $T_2$ generation were scored for a biochemical phenotype by determining the total sterol content of well-expanded leaves. Progeny of a transformant carrying *hmg1* in the 3′ to 5′ orientation downstream from the CaMV 35S promoter was used as a control. Indeed, these latter plants had a total sterol content of approximately 2 to 3 mg/g dry weight (Fig. 3); similar values were obtained for untransformed wild-type *N. tabacum* L. var xanthi plants (data not shown), as reported elsewhere (leaf tissue from wild-type tobacco contains approximately 2.4 mg total sterols $g^{-1}$ dry weight [Maillot-Vernier et al., 1991]). Subsequently, tobacco plants transformed with the *hmg1* genomic fragment in the opposite orientation are referred to as control plants in this work. Plants from the $T_{2/8}$ and $T_{2/15}$ generations showed no remarkable difference in their sterol amount when compared to the control plants, whereas kanamycin-resistant plants but none of the kanamycin-sensitive plants from $T_{2/A}$, $T_{2/C}$, and $T_{2/9}$ contained increased levels of sterols up to 6-fold (Fig. 3). Likewise, plants from 100% kanamycin-resistant $T_3$ families of each of the lines A, C, and 9 were shown to contain higher levels of total sterols than control $T_3$ plants (Fig. 4). In addition, we measured the sterol content of $T_4$ control and *hmg1* plants at various times during their growth in a greenhouse and noticed that the level of sterols in *hmg1* plants constantly increased during development (Fig. 5). Taken together, our results show that the highest levels of sterols are found in transgenic lines displaying the highest HMGR1 mRNA levels (as shown in Fig. 1) and presenting elevated levels of HMGR activities (as shown in Table I).

Finally, it is worth noting that all of the $T_2$, $T_3$, or $T_4$ *hmg1* plants were morphologically similar to control transformed or to untransformed tobacco plants; moreover, they flowered, self-pollinated, and set seed like the wild-type genotype (data not shown).

We have carried out exhaustive investigation of the sterol composition of plants expressing the *hmg1* transgene. GC-MS identification of the different sterols showed that leaf tissue from $T_{3/A}$, $T_{3/C}$, and $T_{3/9}$ accumulated not only

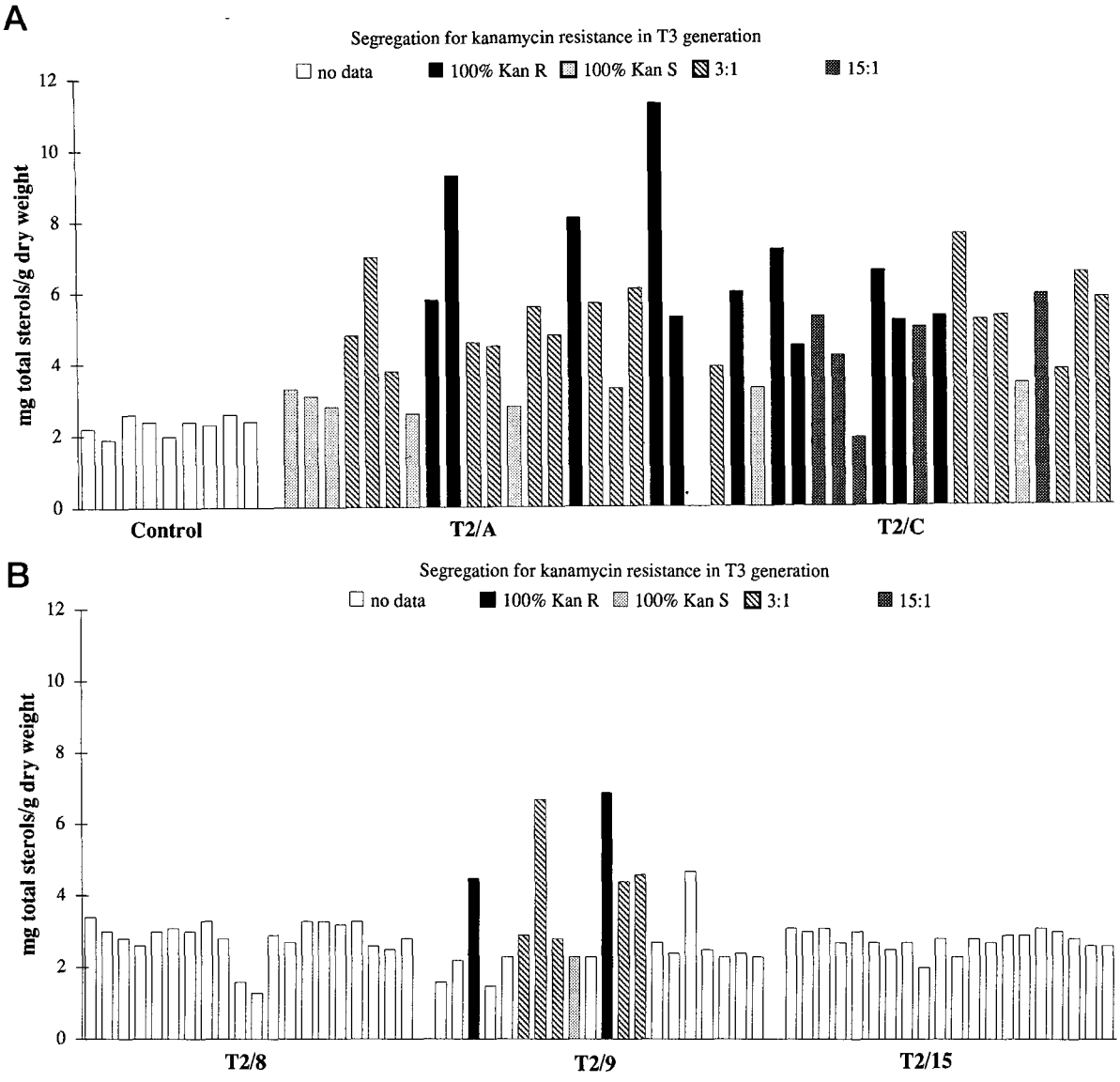


**Figure 3.** Total leaf sterols in individuals from five independent transgenic $T_2$ lines obtained by selfing the $T_1$ primary transformants A and C (A) and 8, 9, and 15 (B) expressing the *hmg1* gene from *H. brasiliensis* under control of the CaMV 35S promoter. Control $T_2$ plants are carrying the *hmg1* genomic fragment inserted downstream from the CaMV 35S promoter in a 3′ to 5′ orientation and were shown to be kanamycin resistant by leaf explant culture on a medium containing 100 mg/L kanamycin. Leaf material was taken from the upper third of 3-month-old greenhouse-grown plants. $T_2$ individuals were selfed, and $T_3$ progenies were scored for kanamycin resistance or sensitivity to determine the genotype of the $T_2$ individuals with respect to the transgene. Each segregation test included at least 50 to 100 individuals. Dotted bars are for 100% kanamycin sensitivity, black bars are for 100% kanamycin resistance, striped bars indicate Mendelian segregation fitting to the ratio of 3 resistant to 1 sensitive individuals, and gray bars indicate Mendelian segregation fitting to the ratio of 15 resistant to 1 sensitive individuals ($\chi^2 < 3.84$, $P > 0.05$). Open bars indicate that no segregation data are available.

$\Delta^5$-sterols (end products of the pathway) but also biosynthetic intermediates such as cycloartenol, 24-methylenecycloartanol, 24-ethylidene-lophenol, obtusifoliol, and $\Delta^7$-sterols (Figs. 6 and 7). In contrast, sterols of control leaf tissues were essentially $\Delta^5$-sterols. Analysis of the different forms of sterols indicated that most of the overproduced sterols were steryl-esters (which migrated in TLC as fatty acid esters at $R_f = 0.9$) in $T_3$ leaf tissue (Table III); in addition, the quantity of steryl-esters from control material was extremely low. Consequently, the concentration of free sterols (sterols that bear a free $\beta$-hydroxyl group at position C3) in $T_3$ leaf tissues became equivalent to that of control leaf tissues (Table III, approximately 2 mg/g dry weight).

To address the question of the intracellular location of the overproduced sterols, we stained petiole epidermis from $T_2$, $T_3$, and $T_4$ plants from the control line and from the lines A, C, and 9 in a saturated ethanolic solution of Sudan III, a lipid-specific dye. Light microscopic observa-

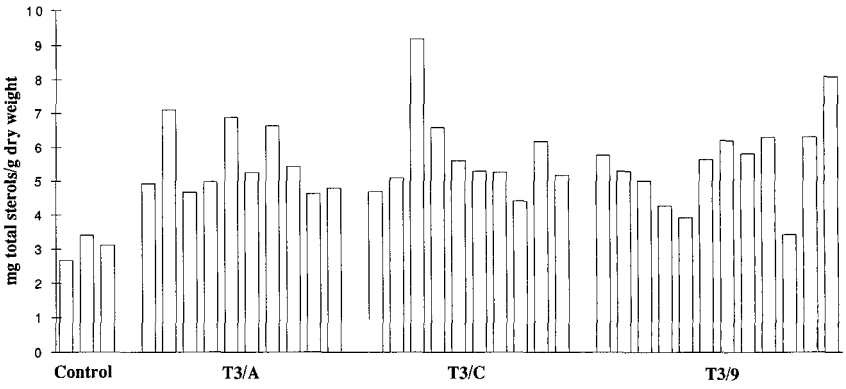


**Figure 4.** Total leaf sterols in individuals from three independent $T_3$ families of transgenic *hmg1* lines A, C, and 9. Segregation analysis indicated 100% kanamycin resistance in these families. Control $T_3$ plants are carrying the *hmg1* genomic fragment inserted downstream from the CaMV 35S promoter in a 3′ to 5′ orientation and were shown to be kanamycin resistant by leaf explant culture on a medium containing 100 mg/L kanamycin. Leaf material was taken from the upper third of 3-month-old greenhouse-grown plants.

tion showed the presence of lipid droplets that appeared as orange spheres in the cytoplasm of the stained cells from the *hmg1* plants (Fig. 8B). The picture contrasted with that of stained control cells (Fig. 8A), which did not contain any comparable lipid inclusions. Furthermore, plants with high levels of sterols (a 6-fold increase compared to the control) contained many more lipid droplets in their leaf epidermis cells than plants with only a slight increase of the sterol amount (a 2-fold increase compared to the control; data not shown).

We also studied the biochemical phenotype of the $T_{3/9}$ line at the differentiation level of a callus. Cotyledon-derived callus cultures were generated, and their sterol composition was determined after 2 months of culture. Again, a striking difference in the sterol amount of control (transgenic calli that are not expressing *hmg1*) and $T_{3/9}$ callus cultures was probably due to the expression of the *hmg1* transgene; $T_{3/9}$ callus cultures had a sterol amount 4-fold that of control cultures (data not shown). Distribution of total sterols in free or esterified forms followed the same pattern as that described for leaf tissues. Most of the overproduced sterols were esterified in $T_{3/9}$ callus culture, and levels of free sterols were equivalent in both control and $T_{3/9}$ callus cultures (data not shown). Finally, we showed by staining in a Sudan III solution cells from a $T_{3/9}$ callus that they contained numerous lipid droplets, whereas cells

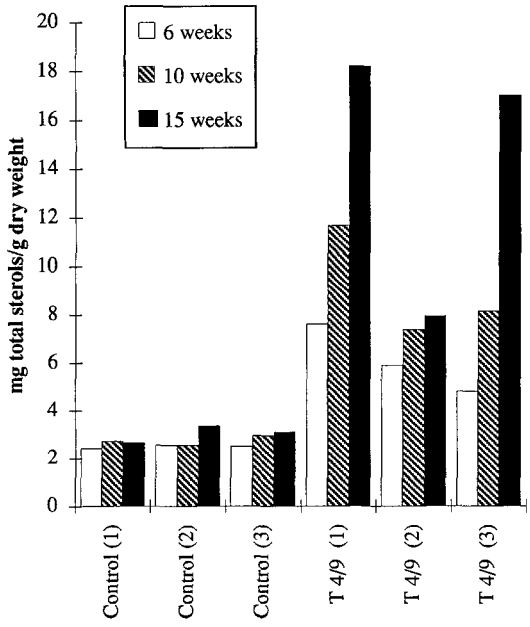


**Figure 5.** Levels of total sterols in three control plants when compared to three $T_{4/9}$ homozygote *hmg1* plants measured at various times during their growth in a greenhouse.

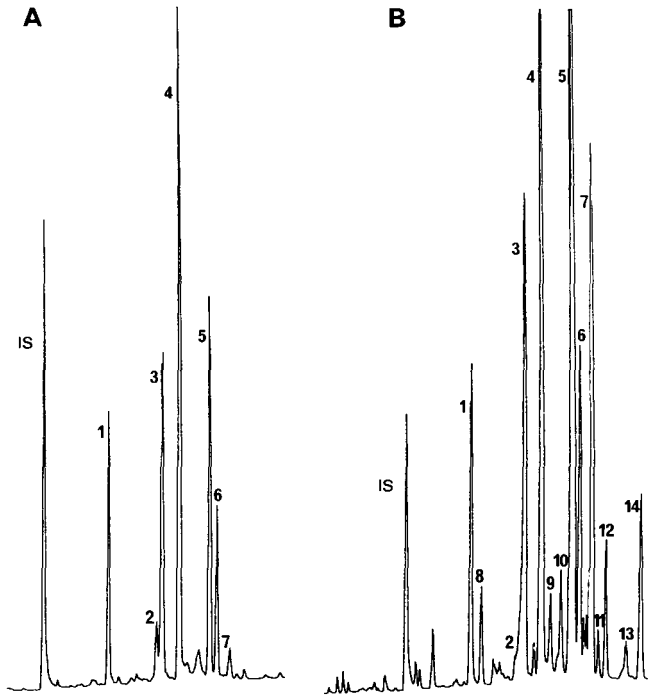


**Figure 6.** GC of total steryl-acetates from leaf tissue of a control plant (A) and a $T_3$ plant from line 9 with a 3-fold increased level of total steryl-acetates compared with the control (B); these data are representative for plants showing an enhanced level of total sterols as quantified in Figures 3 to 5. IS, Internal standard; this peak is 50 ng of cholesterol. Compounds are as follows: 1, cholesteryl-acetate; 2, 24-methylene cholesteryl-acetate; 3, campesteryl-acetate; 4, stigmasteryl-acetate; 5, sitosteryl-acetate; 6, isofucosteryl-acetate; 7, cycloartenyl-acetate; 8, $\Delta^7$-cholestenyl-acetate; 9, obtusifoliyl-acetate; 10, $\beta$-amyrin-acetate; 11, $\Delta^7$-sitostenyl-acetate; 12, $\Delta^7$-avenastenyl-acetate; 13, 24-methylene cycloartanyl-acetate; 14, 24-ethylidene lophenyl-acetate.

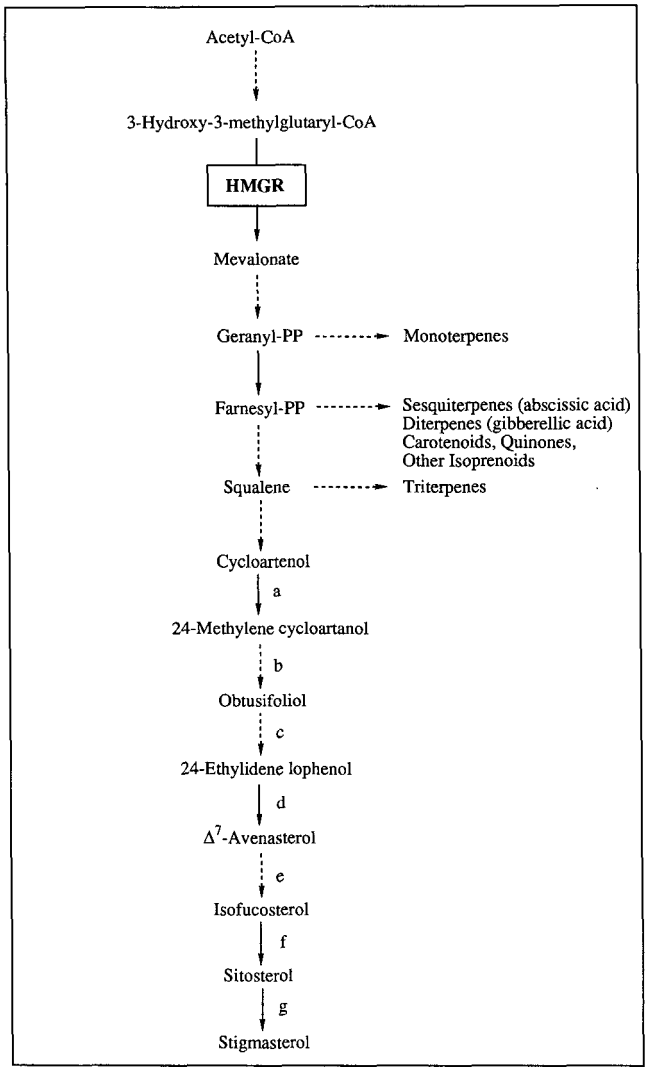


**Figure 7.** Sterol biosynthesis in higher plants. A nonexhaustive pathway for the synthesis of sterols and other isoprenoids is given. The dashed arrows indicate more than one biosynthetic step not shown here. a, C24-methylation; b, C4-demethylation; c, C14-demethylation; d, C4-demethylation; e, C5-desaturation; f, C24(24[1])-reduction; g, C22(23)-desaturation.

from a control callus did not display this feature (data not shown).

## DISCUSSION

The introduction and expression in tobacco of an *H. brasiliensis hmg1* sequence under control of the CaMV 35S promoter results in an altered biochemical phenotype. We have analyzed five independent transgenic tobacco lines expressing *hmg1*. Northern analysis showed that three of the transgenic lines have a high HMGR1 mRNA level, whereas the other two have a weaker expression level. Furthermore, HMGR enzyme assays indicated a strong increase (4- to 8-fold) of the activities in microsomal fractions prepared from transgenic plants with a high level of transcript for the transgene when compared with HMGR activity from untransformed wild-type or transgenic control tobacco plants. Biochemical analysis demonstrated clearly a remarkable increase of the sterol level (up to 6-fold) in transgenic plants from the three lines expressing a high level of HMGR1 mRNA and an elevated HMGR activity, whereas transgenic plants displaying no sterol overproduction phenotype had a much lower level of HMGR1 mRNA. Such a correlation between sterol overproduction and expression of *hmg1* provides a demonstration of the critical role played by HMGR on phytosterol biosynthesis. Our data are consistent with the conclusion that HMGR is a rate-limiting enzyme in sterol synthesis. Moreover, they also strengthen previous suggestions of the regulatory role of HMGR in the branched pathway for isoprenoid synthesis, showing that the control of mevalonate production has a direct effect on the level of sterols in plant tissues. In fact, treatment of plants with a highly specific inhibitor (mevinolin) of HMGR resulted in a lowered accumulation of sterols, whereas levels of other isoprenoids were not affected (Bach, 1986, 1987).

Hata et al. (1987a, 1987b) also reported an increase of the sterol amount in roots of *Medicago sativa* when the plants were supplied with exogenous mevalonate. Likewise, administration of an excess of exogenous mevalonate to *Apium graveolens* cells resulted in an increase of total sterols detected as steryl-esters (Wilkinson et al., 1994). Moreover, a genetic transformation experiment similar to the one that we report here provided evidence that introduction in tobacco of a foreign gene encoding the mammalian HMGR led to a 3- to 5-fold increase in the sterol content of the transgenic plants, compared with untransformed plants, contrasting with a relatively unaltered level of other isoprenoids (sesquiterpenoid phytoalexins and carotenoids) in the same transgenic plants (Chappell et al., 1991). Finally, all of the experimental data indicate that in the plant HMGR is a key limiting step of sterol biosynthesis, as it is in mammalian cells (for review, see Goldstein and Brown, 1990).

Detailed sterol analysis of transgenic *hmg1* plants showed that their biochemical phenotype is almost identical with that of the tobacco mutant LAB1–4 (Maillot-Vernier et al., 1989), which has been screened as a protoplast-derived callus in a UV mutagenesis selection process using resistance to a triazole sterol biosynthesis inhibitor as a scorable phenotype (Maillot-Vernier et al., 1990; Schaller et al., 1992). Sterol profiles of *hmg1* and LAB1–4 plants or leaf-derived calli are not only characterized by an increased amount of pathway end products but also by an accumulation of biosynthetic intermediates, compared with control plant material. As already mentioned in previous reports depicting the phenotype of sterol overproducer mutants such as LAB1–4 (Maillot-Vernier et al., 1991; Schaller et al., 1993), the accumulation of intermediates indicates rate-limiting enzymatic steps in the post-squalene sterol biosynthetic flux. For instance, the accumulation of cycloartenol, 24-methylene-cycloartanol, and 24-ethylidene-lophenol reveals limitations in C24-methylation of cycloartenol, C4-demethylation of 24-methylene-cycloartanol, and C4-demethylation of 24-ethylidene-lophenol (Fig. 7, steps a, b, and

**Table III.** *Levels of free and esterified sterols in tobacco plants transformed with the hmg1 gene*

Detailed sterol and steryl-ester analysis was carried out for five plants randomly chosen among $T_3$ families. Similar results were obtained for each individual of a plant line. A representative set of data is shown for each plant line.

| Sterols | Control[a] | | $T_{3/9}$ | | $T_{3/A}$ | | $T_{3/C}$ | |
|---|---|---|---|---|---|---|---|---|
| | Free sterols | Steryl esters | Free sterols | Steryl esters | Free sterols | Steryl esters | Free sterols | Steryl esters |
| β-Amyrin | 0[b] | 0 | 0 | 0 | 1.5 | 3 | 3 | 2 |
| Cycloartenol | 0.5 | 0 | 7 | 23 | 1 | 7 | 1 | 8 |
| 24-Methylene cycloartanol | 0.5 | 0 | 1 | 3 | 0 | 2 | 0 | 0.5 |
| Obtusifoliol | 0 | 0 | 1 | 5 | 0 | 2 | 0 | 2 |
| 24-Ethylidene lophenol | 0 | 0 | 1 | 4 | 0 | 1 | 0 | 2 |
| $\Delta^7$-Avenastenol | 0 | 0 | 1 | 6 | 0 | 1 | 0 | 1.5 |
| $\Delta^7$-Sitostenol | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 |
| $\Delta^7$-Cholestenol | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| Total of biosynthetic intermediates | 1 | 0 | 12 | 42 | 3 | 18 | 4 | 18 |
| Cholesterol | 9 | 50 | 5 | 2 | 19 | 13 | 11 | 14 |
| 24-Methylene cholesterol | 1 | 0 | 2 | 2 | 0.5 | 1 | 1 | 1 |
| Campesterol | 18 | 10 | 16 | 14 | 15 | 16 | 16 | 16 |
| Isofucosterol | 1 | 0 | 7 | 13 | 2 | 5 | 1 | 4 |
| Sitosterol | 26 | 27 | 23 | 20 | 21 | 30 | 23 | 31 |
| Stigmasterol | 40 | 13 | 35 | 7 | 40 | 17 | 44 | 16 |
| Total of $\Delta^5$-sterols | 99 | 100 | 88 | 58 | 97 | 82 | 96 | 82 |
| Total (μg/g dry wt) | 2015 | 71 | 1700 | 3220 | 1654 | 1256 | 1685 | 952 |

[a] Control $T_3$ plants are carrying the *hmg1* genomic fragment inserted downstream from the CaMV 35S promoter in a 3′ to 5′ orientation. Levels of free and esterified sterols in these control plants are similar to that of untransformed wild-type tobacco (data not shown). [b] Percent of total.

d, respectively) in tobacco. In this respect, recent work identifying and characterizing 24-methylene-cycloartanol-C4-demethylase and 24-ethylidene-lophenol-C4-demethylase from *Zea mays* microsomes indicates a $V_{max}$ value for each enzyme that is relatively low compared to other sterol pathway enzymes from the same material (Pascal et al., 1993).

The similarity of the biochemical phenotypes from the LAB1–4 mutant and from the plants expressing the *hmg1* transgene may demonstrate that the increased amount of sterols in the LAB1–4 mutant results from a modification of the mevalonate production. Thus, results reported in this paper are consistent with the involvement of HMGR in the LAB1–4 phenotype as previously established (Gondet et al., 1992). Although we know that sterol overproduction in transgenic lines is conditioned by the overexpression of a foreign *hmg1* gene, the question of the nature of the LAB1–4 mutation and of its effect on *hmg* expression remains unanswered at this time. Along these lines it has been shown that the expression of the LAB1–4 phenotype depends on

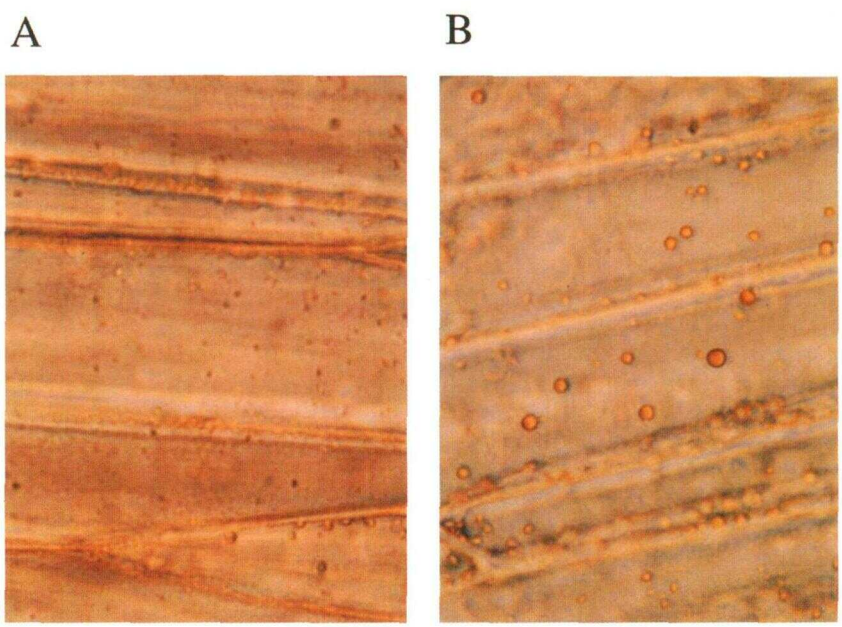


**Figure 8.** Light micrographs (×457) of petiole epidermis cells after staining in a solution of Sudan III. Intracytoplasmic lipid droplets appear as orange spheres. A, Control plants; B, *hmg1* plants.

the differentiation stage of the tissues (Gondet et al., 1994), whereas *hmg1* plants or calli show a similar increase in their sterol levels.

In the case of both *hmg1* transgenic (this paper) and LAB1–4 (Maillot-Vernier et al., 1991) tobacco genotypes, we report an accumulation of sterols with a concomitant esterification of the overproduced metabolites. Consequently, the metabolism of the accumulated sterols in those genotypes should involve one (or more) sterol acyl-transferase(s) (for review, see Dyas and Goad, 1993). Because we have obtained a "LAB1–4-like" phenotype by means of a unique genetic transformation with a functionally determined DNA sequence, namely the HMGR1 genomic sequence from *Hevea*, we suggest that the esterification process (i.e. activity of sterol acyl-transferase[s]) is not limiting in tobacco, certainly as well as the process leading to the formation of lipid droplets in the cells. In the case of the LAB1–4 mutant, the storage of the excess of sterols as esters in cytoplasmic lipid droplets has been related to the regulation of the concentration of free $\Delta^5$-sterols in membranes (Maillot-Vernier et al., 1991; Gondet et al., 1994; Bouvier-Navé and Benveniste, 1995) and this is likely to be a valid conclusion in the case of the transgenic plants described in this report, since sterols are well-known essential constituents of phospholipidic membranes in eukaryotic cells (for review, see Bloch, 1983; Ourisson, 1994).

Finally, the fact that plants expressing *hmg1* possess a developmental pattern and a growth rate identical with wild-type plants raises the question of whether there are changes in the plant isoprenoid composition other than the modification of the sterol content, such as levels of the hormones cytokinins or GAs or the photosynthetic pigments, e.g. carotenoids. In this respect it is of course tempting to presume that HMGR would be the rate-limiting step only for sterol synthesis in plant cells, as already discussed by others (Narita and Gruissem, 1989) and recently debated in review articles (Stermer et al., 1994; Chappell, 1995).

Of wider importance in this article is the fact that the rather specific expression of *hmg1* in laticifers of rubber trees (Chye et al., 1992) would be conditioned only by the specificity of the *hmg1* gene promoter and factors acting on it. In fact, positioning the coding sequence of *hmg1* downstream of the CaMV 35S promoter results in a hybrid gene having a so-called housekeeping function when expressed in tobacco; such a role has indeed been proposed for the *Hevea hmg3*, which shares 77% identity with *hmg1* (Chye et al., 1992), and for the *Arabidopsis thaliana hmg1* (Enjuto et al., 1994), for instance, because of their broad expression spectrum in plants. Whether this is true in *Hevea* can be now answered, since it is possible to introduce foreign genes in this species (Arokiaraj et al., 1994).

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