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Co-expression of native and introduced genes reveals cryptic regulation of HMG CoA reductase expression in *Arabidopsis*

Edward B. Re[1], Daniel Jones[2] and R. Marc Learned[1,*]
[1]*Section of Molecular and Cellular Biology, University of California, Davis, CA 95616, USA, and*
[2]*Facility for Advanced Instrumentation, University of California, Davis, CA 95616, USA*

Summary

In eukaryotes, all isoprenoid compounds share a common precursor, mevalonic acid, whose synthesis is catalyzed by the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase. As one step towards a better understanding of the role that this enzyme plays in coodinating isoprenoid biosynthesis in plants, *Arabidopsis thaliana* HMG CoA reductase was ectopically expressed in transgenic *Arabidopsis* plants. By using this molecular genetic approach, several novel and fundamental observations have been made regarding isoprenoid biosynthesis in *Arabidopsis*. First, it was demonstrated that the overexpression of authentic *Arabidopsis* HMG CoA reductase is not sufficient to alter the bulk synthesis and accumulation of the abundant end products of the plant isoprenoid pathway. Second, active transcription of the transgene appears to co-activate and deregulate expression of the native gene, resulting in a striking elevation of HMG CoA reductase mRNA levels. Finally, although very high levels of HMG CoA reductase mRNA were expressed in these transgenic plants, only modest increases in enzyme activity could be detected. Taken together, these data suggest that HMG CoA reductase expression is regulated at multiple levels in plants as well as animals, and they provide a foundation for elucidating the molecular mechanisms for mevalonate regulation in *A.thaliana*.

Introduction

In eukaryotes, all isoprenoid compounds, including sterols, dolichol and ubiquinone, share a common precursor, isopentenyl pyrophosphate, that is derived ultimately from acetyl coenzyme A. Mevalonic acid is the key intermediate in the biosynthetic pathway leading to the synthesis of the biologically active isoprene unit, and the significance of the mevalonic acid synthesis in animal cells has been revealed by two lines of research. First, the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34) catalyzes the NADPH-dependent reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) to mevalonic acid (MVA) in a reaction that represents the overall rate-limiting step in the sterol biosynthetic pathway. Second, the amount of HMG CoA reductase has been found to be regulated by sterol and non-sterol derivatives of mevalonic acid at the level of transcription (Liscum *et al.*, 1983; Osborne *et al.*, 1985), translation (Nakanishi *et al.*, 1988; Peffley and Sinensky, 1985) and enzyme stability (Chun and Simoni, 1992; Correll and Edwards, 1994; Nakanishi *et al.*, 1988; Roitelman and Simoni, 1992). In addition, HMG CoA reductase enzyme activity is modulated directly by reversible phosphorylation of the protein (Beg *et al.*, 1978; Omkumar *et al.*, 1994; Sato *et al.*, 1993) and by allosteric effectors (Roitelman and Shechter, 1984). This multivalent regulation provides a sensitive control mechanism that responds to the levels of serum cholesterol and coordinates isoprenoid metabolism to ensure the availability of both sterol and non-sterol products (Goldstein and Brown, 1990).

In higher plants, mevalonic acid serves as the precursor for a much broader array of isoprenoid compounds that play a variety of roles in the metabolism, growth and development of the plant (Figure 1). These additional branches in the isoprenoid biosynthetic pathway give rise to a number of unique products, including growth regulators (such as cytokinin, gibberellin and abscisic acid), photosynthetic pigments, phytotoxins, phytoalexins, and a variety of specialized terpenoids. In fact, there are currently more than 10000 isoprenoid compounds that have been identified from higher plants. However, even though the regulated biosynthesis of these metabolites is thought to be essential to the normal processes of plant growth and development, the mechanisms that control isoprenoid biosynthesis are currently poorly understood and there is no definitive experimental eidence to identify either the major rate-determining steps or the key control points in the pathway (Gray, 1987).

It is likely, however, that the control of mevalonate synthesis plays an important role in coordinating isoprenoid synthesis in plants as well as animals, and studies of HMG CoA reductase regulation in plants suggest that the enzyme activity responds to a variety of external stimuli, including light (Brooker and Russell, 1975, 1979; Wong *et al.*, 1982), plant growth regulators (Bach and Lichtenthaler, 1984; Brooker and Russell, 1979; Nishi and

Received 30 August 1994; revised 26 January 1995; accepted 2 February 1995.
*For correspondence (fax +1 916 752 3085).

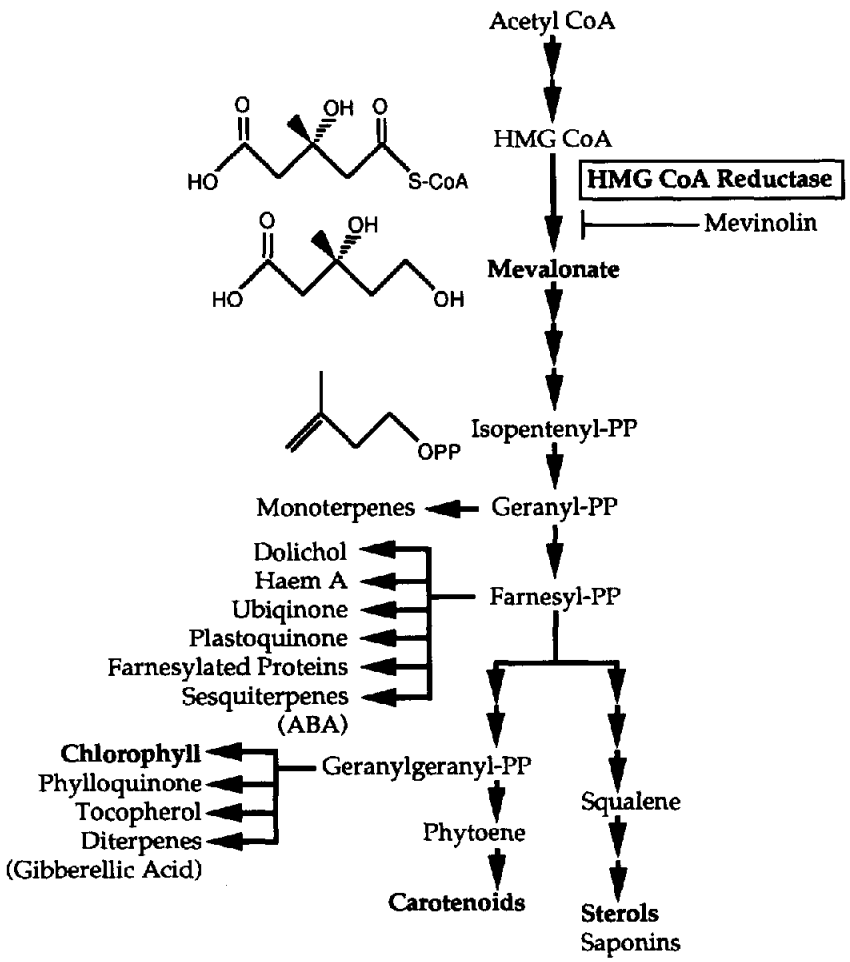


Figure 1. The isoprenoid biosynthetic pathway in plants.

Tsuritani, 1983; Russell *et al.*, 1985), sterols (Russell and Davidson, 1982), and wounding and plant pathogens (Choi *et al.*, 1992; Oba *et al.*, 1985; Stermer and Bostock, 1987; Yang *et al.*, 1991). Furthermore, there are marked variations in the levels of HMG CoA reductase activity at different stages of development and in different plant tissues (Garg and Douglas, 1983; Narita and Gruissem, 1989). More recently, the genes encoding HMG CoA reductase have been cloned from a variety of different plant species (Aoyagi *et al.*, 1993; Burnett *et al.*, 1993; Caelles *et al.*, 1989; Choi *et al.*, 1992; Chye *et al.*, 1991; Genschik *et al.*, 1992; Learned and Fink, 1989; Narita and Gruissem, 1989; Park *et al.*, 1992), and studies are presently underway in a number of laboratories to elucidate their regulation.

As one step towards a better understanding of the role that HMG CoA reductase plays in isoprenoid biosynthesis in plants, we constructed a chimeric HMG CoA reductase gene consisting of the full-length *Arabidopsis thaliana HMG1* cDNA fused to the cauliflower mosaic virus (CaMV) 35S promoter, and introduced this construct into *Arabidopsis* by *Agrobacterium*-mediated transformation. By using this molecular genetic approach, we can alter the levels of enzyme expression, and consequently, the amounts of mevalonic acid produced in these transgenic plants. Ectopic overexpression of HMG CoA reductase has allowed us to examine two fundamental questions regarding isoprenoid biosynthesis in *Arabidopsis*. First, we wanted to test whether this enzyme is a rate-limiting step in the pathway, and in particular, whether certain isoprenoid end products would accumulate when HMG CoA reductase is overproduced. Second, by replacing the *HMG1* promoter with the CaMV 35S promoter, we have uncoupled promoter-dependent transcriptional control of *HMG1* expression from post-transcriptional regulation, and have begun to evaluate the relative contributions of these various control mechanisms to the regulated synthesis of HMG CoA reductase in *A. thaliana*.

Results

Ectopic expression of the HMG1 *gene confers mevinolin resistance in transgenic* Arabidopsis thaliana

The *Arabidopsis HMG1* coding sequences were placed under the transcriptional control of the highly active CaMV

Figure 2. Construction of a CaMV 35S:HMG CoA reductase fusion gene.
The *HMG1* cDNA was placed under the transcriptional control of the promoter and terminator from the cauliflower mosaic virus 35S gene, and the chimeric 35S:*HMG1* gene transferred into wild-type *A. thaliana* (ecotype No-0) by *Agrobacterium*-mediated transformation. *Arabidopsis HMG1* coding sequences are designated by the thick black box; the thinner black boxes represent untranslated regions of the *HMG1* transcription unit. CaMV sequences are indicated by stippled boxes, and polylinker sequences are represented by thin lines. Key regulatory sequences and their positions are indicated above the map.

35S promoter to study the role that HMG CoA reductase plays in controlling isoprenoid biosynthesis in plants. The resulting construct includes the 5′ and 3′ untranslated regions of the *HMG1* mRNA, as well as the entire HMG CoA reductase coding sequence, with translational initiation being directed by the AUG initiation codon used in the native *HMG1* mRNA (Figure 2). Consequently, we predict that the protein encoded by the CaMV 35S:*HMG1* transgene is identical to the native *Arabidopsis* enzyme, and exhibits both the catalytic activity associated with the carboxy-terminal domain, as well as the targetting and structural properties conferred upon the protein by the amino-terminal transmembrane domain. Recent studies are consistent with the synthesis of HMG CoA reductase as a mature, unprocessed polypeptide that is inserted co-translationally into the endoplasmic reticulum where it resides as an integral membrane protein (Enjuto *et al.*, 1994).

Using *Agrobacterium*-mediated transformation (Valvekens *et al.*, 1988), the CaMV 35S:*HMG1* fusion gene was introduced into root explants derived from wild-type *A. thaliana* (No-0 ecotype), and independent transformants belonging to the collection of regenerated plants were assigned labels using the designation No-T43. As a means of identifying lines of transgenic plants that overexpress HMG CoA reductase, these kanamycin-resistant transformants were subjected to a secondary screen to test their ability to grow in the presence of mevinolin, a potent and specific inhibitor of the enzyme (Alberts *et al.*, 1980). We reasoned that increased levels of HMG CoA reductase in the transgenic plants would confer resistance to mevinolin and that this phenotype could be scored by examining the growth of these plants on media containing a range of inhibitor concentrations.

As shown in Figure 3, the growth of wild-type *Arabidopsis* plants is dramatically affected by the inclusion of mevinolin in the growth medium. In particular, the elongation of the primary root in young seedlings (Figure 3b), as well as the development of the root system in older plants (Figure 3a), is severely inhibited in the presence of even very low concentrations of mevinolin. Furthermore, the growth and development of the foliar tissue is also inhibited in these plants, albeit at higher concentrations of the drug (data not shown). In the presence of 75 nM mevinolin, the appearance of the primary set of leaves is delayed, and the rosette leaves are smaller and tend to accumulate anthocyanin pigments. At the highest concentrations of mevinolin tested (100 nM), both root growth (Figure 3) and leaf development are nearly completely inhibited by the drug. In all cases, the effects of mevinolin on plant growth could be reversed by the addition of 1 mM mevalonic acid to the media (data not shown), indicating that the growth inhibition is a consequence of the highly specific action of mevinolin on HMG CoA reductase.

The growth of 14 transgenic *Arabidopsis* lines was also monitored over a range of mevinolin concentrations by measurement of root elongation. Based on these data, the progeny of independently transformed plants could be separated into two categories. Although the growth of wild-type and all transgenic plant lines was identical in the absence of inhibitor, many of the transgenic plants also displayed the same sensitivity to mevinolin as No-0 plants. Root elongation in these transformed plants was indistinguishable from wild-type *Arabidopsis* grown at the same concentration of inhibitor (data not shown). In contrast, two transformed lines, T43.06 and T43.29, exhibited an increased tolerance for the inhibitor, as evidenced both by increased root elongation (Figure 3a and b) and more vigorous foliar growth on media supplemented with mevinolin. Despite this reduced sensitivity, the growth of these transgenic plants was clearly affected by the presence of mevinolin in the nutrient medium, and even at low concentrations of the inhibitor root growth was reduced compared with incubation in the absence of the drug. These results are consistent with the prediction that expression from the CaMV 35S:*HMG1* transgene in the transformed plants would result in elevated levels of a mevinolin-sensitive HMG CoA reductase enzyme, and that the corresponding increase in activity would reduce the sensitivity of these plants to the inhibitor.

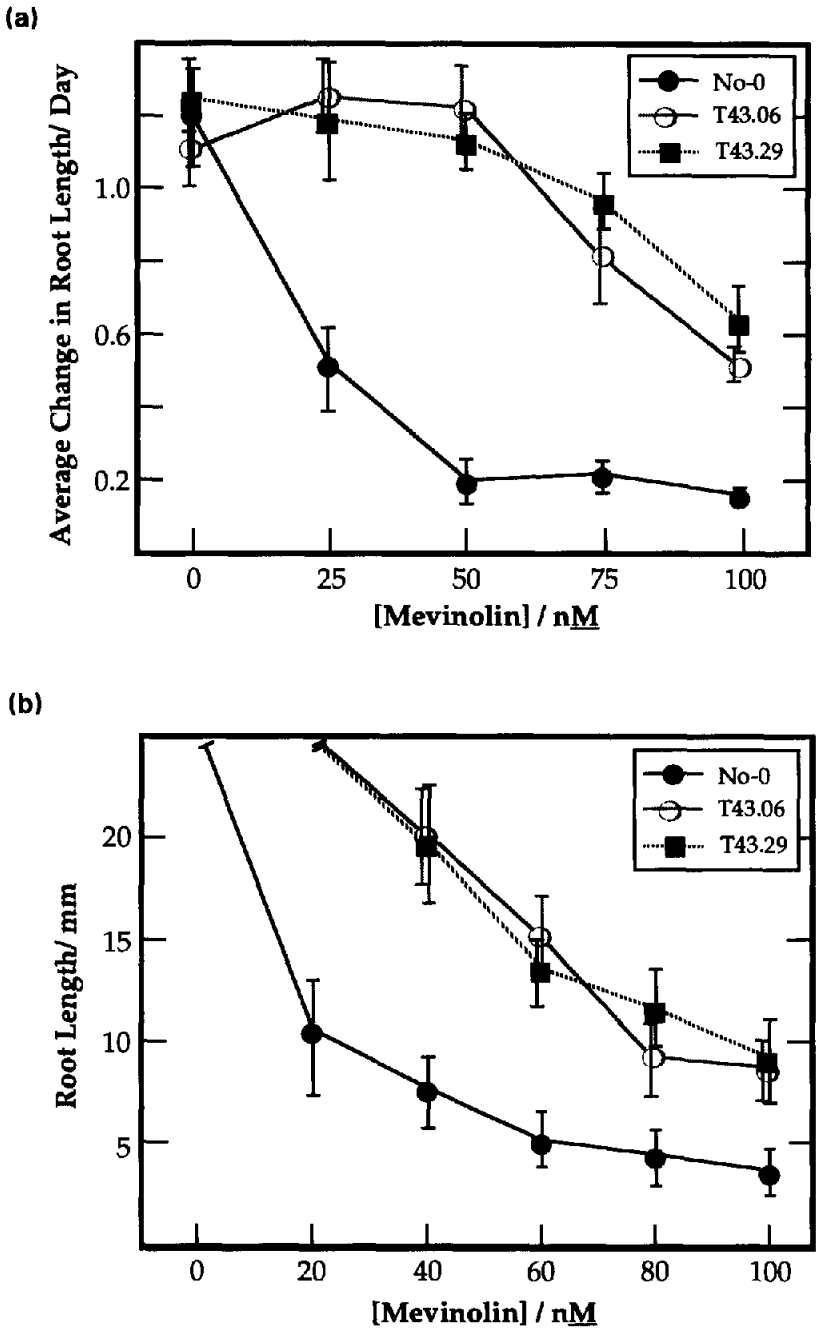


Figure 3. Inhibition of *Arabidopsis* root growth by mevinolin, a specific inhibitor of HMG CoA reductase.
Wild-type and transgenic *Arabidopsis* plants were grown on GM agar supplemented with various concentrations of mevinolin at 20°C under continuous light. Culture plates were placed in a vertical orientation to allow roots to elongate along the surface of the agar and root growth was monitored daily by measuring root length using a micrometer.
(a) Seeds were sown initially on GM agar and allowed to grow for 4 days in the absence of inhibitor. Healthy individuals were then transferred to the test media containing mevinolin and returned to the growth chamber. Root elongation in individual plants was measured for several days and the average change in root length over time is expressed as fractional growth normalized to the root length measured at the time of transfer.
(b) Seeds were sown directly on media containing mevinolin and grown in a vertical orientation in culture plates at 22°C under continuous light. Root elongation of individual plants was monitored daily for 6 days after imbibition using a micrometer.

HMG CoA reductase enzyme activity in transgenic Arabidopsis *plants*

In order to address directly the possibility that HMG CoA reductase activities were elevated in the transgenic *Arabidopsis* lines, extracts were prepared from the leaves of both transgenic and wild-type plants and assayed for the presence of HMG CoA reductase enzyme activity. As shown in Figure 4, approximately threefold higher levels of HMG CoA reductase were observed in the extracts prepared from T43.06 and T43.29 plants compared with extracts from other transgenic lines or from wild-type *Arabidopsis*. This elevated enzyme activity was found only in extracts from transgenic plants that exhibited resistance to mevinolin, whereas extracts prepared from the mevinolin-sensitive transgenic plants contained levels of HMG CoA reductase that were identical to untransformed wild-type *Arabidopsis* (data not shown). Furthermore, the synthesis of mevalonate in extracts from both wild-type and transgenic plants could be abolished by inclusion of mevinolin in the HMG

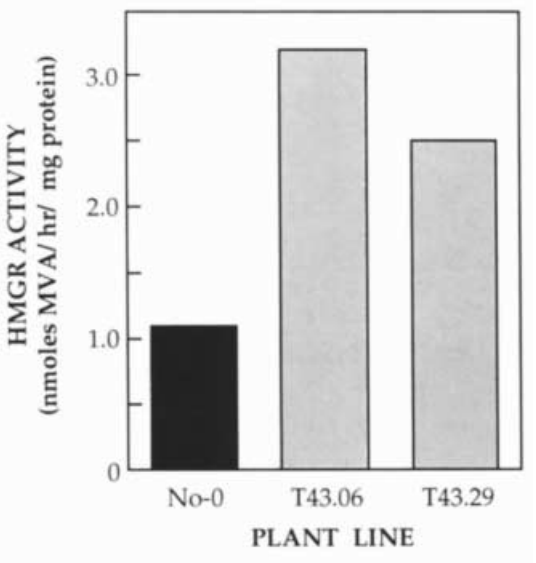


Figure 4. HMG CoA reductase activity in extracts prepared from wild-type and transgenic *Arabidopsis*.
HMG CoA reductase enzyme activity was measured in extracts prepared from mature No-0 wild-type (black bar), T43.06 (stippled bar) and T43.29 (stippled bar) *Arabidopsis* plants. HMG CoA reductase activity is normalized to the total protein concentration present in these crude fractions. These results represent the average of at least two independent assays.

Table 1. Representative values for HMG CoA reductase activity in cell extracts from wild-type and transgenic *Arabidopsis* plants

Plant line	HMG CoA reductase activity (nmol MVA h^{-1} mg^{-1} protein)		% inhibition
	Control	+50 μM mevinolin	
No-0 (WT)	1.14	0.06	96
	1.21	0.06	95
No-T43.06	3.15	0.06	98
	3.10	0.05	98
No-T43.29	2.65	0.05	98
	2.46	0.04	98

CoA reductase assay (Table 1), indicating that a single pool of mevinolin-sensitive enzyme was being expressed in all the plant lines tested. These data establish a direct correlation between an increased level of assayable HMG CoA reductase enzyme in plant extracts and the mevinolin resistance observed in the intact plants.

Analysis of HMG1 *mRNA in wild-type and transgenic* Arabidopsis

The chimeric CaMV 35S:*HMG1* gene introduced into the T43 series of transformants is a transcriptional fusion between the CaMV 35S promoter and the full-length *Arabidopsis HMG1* cDNA, and consequently, the 35S promoter is expected to direct high levels of expression from the *HMG1* transgene. In order to compare the steady-state

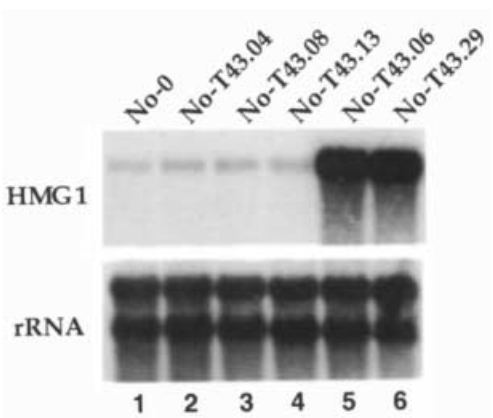


Figure 5. *HMG1* expression in wild-type and transgenic *Arabidopsis*.
Total RNA was isolated from 14-day-old mature *Arabidopsis* plants grown on sterile GM under continuous light, and 5 μg samples were fractionated by agarose gel electrophoresis. The sources of the RNA were as follows: lane 1, wild-type *Arabidopsis* (No-0 ecotype); lane 2, No-T43.04; lane 3, No-T43.08; lane 4, No-T43.13; lane 5, No-T43.06; lane 6, No-T43.29. Blot hybridization analysis was carried out using ^{32}P-labeled DNA probes derived from the *Arabidopsis HMG1* cDNA. As an internal control, rRNA was detected using radish rDNA as a probe. The intensity of the radioactive signals visualized by autoradiography were quantified using a Millipore BioImage System.

levels of *HMG1* mRNA present in wild-type and transgenic plants, RNA isolated from transgenic plants was analyzed by blot hybridization using an *HMG1* gene-specific probe.

As shown in Figure 5, many of the transgenic plant lines, including T43.04, T43.08 and T43.13 (lanes 2–4, respectively), contained levels of *HMG1* mRNA that were indistinguishable from wild-type No-0 plants. Not surprisingly, these lines correspond to the mevinolin-sensitive class of transgenic plants. In contrast, the levels of *HMG1* mRNA in the mevinolin-resistant plant lines, T43.06 and T43.29, were approximately 40-fold higher than the levels observed in wild-type *Arabidopsis* (Figure 5, lanes 5 and 6). Thus, the transformants that contain elevated HMG CoA reductase enzyme levels and are resistant to mevinolin all express high levels of *HMG1* mRNA. Taken together, these results indicate that at least some of this RNA is ultimately converted into catalytically active enzyme. However, the increase in HMG CoA reductase activity in these transgenic plants is relatively modest compared with the increase in *HMG1* mRNA synthesis (Figure 4), suggesting that enzyme activity might be limited by mechanisms other than RNA availability.

Induction of the native HMG1 *gene in plants expressing the 35S:*HMG1 *transgene*

Because the transcripts from the native and transgenic *HMG1* loci are almost exactly the same size, the mRNA derived from the two genes cannot be distinguished by blot hybridization analysis. Consequently, an S1 nuclease protection assay was developed to allow us to discriminate

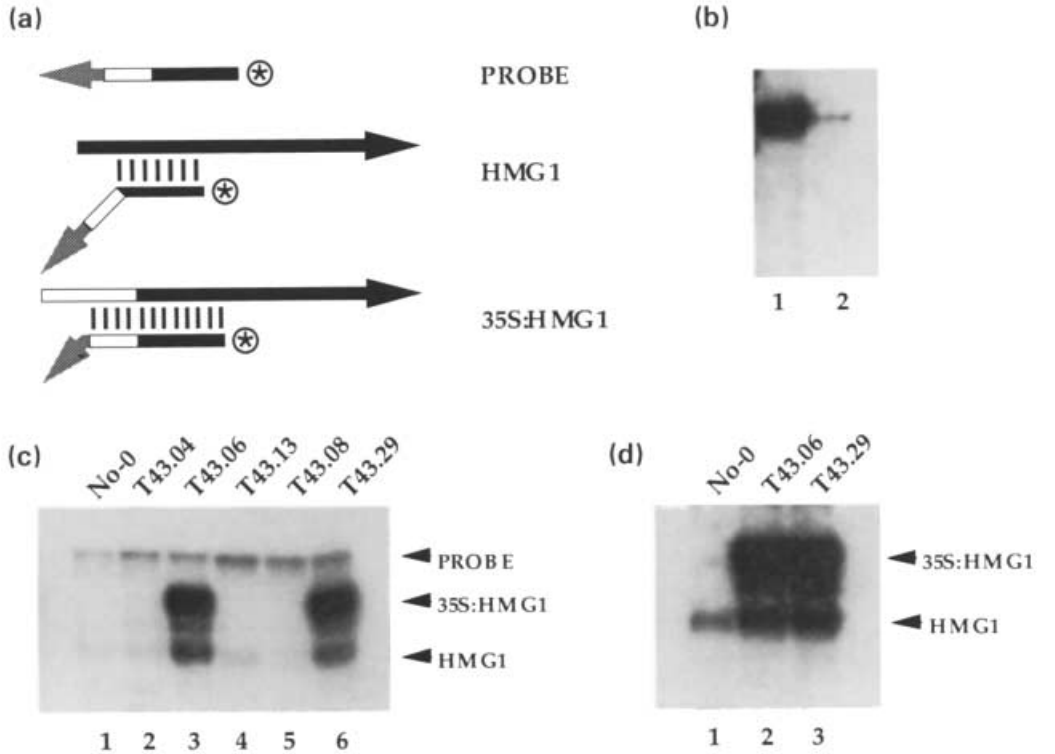


Figure 6. S1 nuclease analysis of RNA transcribed from the *HMG1* and 35S:*HMG1* genes.
(a) An S1 nuclease protection assay was carried out using a ^{32}P-labeled single-stranded DNA probe that can hybridize to RNA derived from either the native *HMG1* gene or the 35S:*HMG1* transgene. This 51 base oligonucleotide includes 30 nt that are complementary to the *HMG1* mRNa (black) and can anneal to both species, 11 nt that are complementary to sequences present only in the 35S:*HMG1* fusion gene (white), and 10 nt that do not hybridize to either RNA (stippled). Following hybridization and treatment with S1 nuclease, the native transcript will protect a 31 nt fragment, whereas the RNA transcribed from the transgene will protect a 41 nt fragment.
(b) Control reactions were carried out using the oligonucleotide probe in the absence of S1 nuclease (lane 1) or in the absence of *Arabidopsis* RNA (lane 2). Following treatment, the reaction products were fractionated on a sequencing gel and the dried gel exposed to X-ray film. In this experiment, a small amount of full-length probe resisted degradation by S1 nuclease and can be detected near the top of the panel.
(c) Total RNA was isolated from 14-day-old mature *Arabidopsis* plants grown on sterile GM under continuous light, and 5 μg samples were hybridized to the ^{32}P-labeled oligonucleotide probe as described in Experimental procedures. Following S1 nuclease digestion, the reaction products were fractionated on a sequencing gel and the dried gel exposed to X-ray film. The sources of the RNA were as follows: lane 1, wild-type *Arabidopsis* (No-0 ecotype); lane 2, No-T43.04; lane 3, No-T43.06; lane 4, No-T43.13; lane 5, No-T43.08; lane 6, No-T43.29. The sizes of the predicted products are indicated by the arrows to the side.
(d) Prolonged exposure of an S1 nuclease protection assay to X-ray film (approximately 5 days at −80°C with an intensifying screen). The sources of the RNA were as follows: lane 1, wild-type *Arabidopsis* (No-0 ecotype); lane 2, No-T43.06; lane 3, No-T43.29.

between these two classes of mRNA (illustrated in Figure 6a). This assay takes advantage of the fact that the transcript derived from the CaMV 35S:*HMG1* transgene contains a short stretch of nucleotides at the 5′ end of the mRNA that is not encoded in the untranslated leader of the native *HMG1* gene. A synthetic oligonucleotide probe was designed that is complementary to the RNA transcript and that hybridizes with the *HMG1* sequence, as well as the region present only in the 35S:*HMG1* chimeric gene. As a result, a tripartite oligonucleotide probe hybridizes efficiently to RNA derived from both the native and transgenic *HMG1* loci, but the size of the S1 nuclease-resistant fragments will depend on the specific RNA to which the probe anneals. In this way, the *HMG1* mRNAs transcribed from the two different genes can be monitored and distinguished from one another using the same end-labeled probe.

As shown in Figure 6(b), control reactions containing the oligonucleotide probe and yeast tRNA yield no protected fragments following treatment with S1 nuclease (lane 2). When total RNA isolated from wild-type *Arabidopsis* (No-0) is hybridized with the probe and treated with S1 nuclease, a discrete 30 nt fragment corresponding to the native *HMG1* transcript is protected from degradation (Figure 6c, lane 1 and Figure 6d, lane 1). Similarly, only RNA transcribed from the native gene was detected in extracts prepared from the mevinolin-sensitive transgenic plants T43.04, T43.08, T4.13 (Figure 6c, lanes 2, 4 and 5), suggesting that the CaMV 35S:*HMG1* transgenic present in these plant lines is not actively expressed. In contrast, when RNA–DNA hybrids formed between the end-labeled oligonucleotide probe and the RNA isolated from the T43.06 or T43.29 transformants are digested with S1 nuclease, two fragments appear to be protected from nuclease degradation (Figure 6c, lanes 3 and 6; Figure 6d, lanes 2 and 3).

The smaller of these fragments co-migrates with the DNA fragment protected from digestion by hybridization with RNA from wild-type *Arabidopsis*, and corresponds to mRNA derived from the native *HMG1* gene. The larger nuclease-resistant fragment exhibits the mobility predicted for the RNA transcribed from the CaMV 35S:*HMG1* transgene that contains the additional non-coding leader sequence. As expected, the levels of *HMG1* expression from the transgenic detected in the nuclease protection assay are considerably greater than the levels of *HMG1* expression from the native gene in wild-type *Arabidopsis*. Based on this high resolution assay, the levels of native *HMG1* mRNA also appear to be elevated in these transgenic plants relative to wild-type. This surprising result was obtained for all transgenic lines overexpressing HMG CoA reductase, suggesting that this was not a random event, but a reflection of some physiological control of *HMG1* gene expression.

Constitutive expression of HMG CoA reductase genes in transgenic Arabidopsis

Previous studies indicated that HMG CoA reductase enzyme activity was higher in etiolated pea seedlings, than in light-grown green seedlings (Russell *et al.*, 1985), and suggested the possibility that MVA synthesis is regulated in response to changing light conditions. By taking advantage of the availability of molecular probes, we have recently examined the molecular basis of this control by studying the photoregulation of HMG CoA reductase gene expression (Learned *et al.*, manuscript submitted). Using a gene-specific probe, we have shown that HMG CoA reductase mRNA can be detected in *Arabidopsis* plants grown under a variety of light conditions, and that the *HMG1* gene is expressed in both the light and dark. However, *HMG1* is expressed preferentially in etiolated and dark-adapted plants, with high levels of *HMG1* mRNA accumulating in plants deprived of light.

In order to study the effects of light on the expression of the chimeric CaMV 35S:*HMG1* gene, we analyzed *HMG1* RNA levels from transgenic plants grown in continuous light or continuous darkness for 5 days. Using the S1 nuclease protection assay described above, *HMG1* RNA transcribed from both the native and the transgene can be monitored simultaneously in the same reaction. In wild-type *Arabidopsis* maintained in the dark, *HMG1* mRNA accumulates to approximately fivefold higher levels than comparable plants grown under continuous light (Figure 7a, lanes 1 and 2). As predicted, only the 30 nt fragment is protected from S1 digestion by the native *HMG1* transcript (Figure 7b, lanes 4 and 5). These results are in agreement with previous observations (Enjuto *et al.*, 1994; Learned, unpublished results), and indicate that light plays a role in controlling the expression of the *HMG1* gene in *Arabidopsis*.

In contrast, S1-resistant fragments corresponding to transcripts from both the native and transgenic *HMG1* loci were generated when RNA isolated from either T43.06 or T43.29 plants was analyzed by nuclease protection. Furthermore, the levels of *HMG1* transcripts derived from both the transgene and the native gene remained constant under the different light conditions (Figure 7a, lanes 3–6). These results are consistent with the idea that the regulation of *HMG1* gene expression by light in wild-type plants is mediated, at least in part, at the level of transcription and is controlled by sequences in the *HMG1* promoter. Although we anticipated that the CaMV 35S promoter would direct high level constitutive expression from the *HMG1* cDNA, both the native and transgenic *HMG1* loci appear to be constitutively expressed in the T43.06 and T43.29 transformants (Figure 7b, lanes 6–9). This unexpected result suggests that the presence of a transcriptionally active CaMV 35S:*HMG1* transgene disrupts the light-mediated suppression of *HMG1* expression.

Isoprenoid analysis of transgenic plants overexpressing HMG CoA reductase

In addition to our interest in studying the regulation of HMG CoA reductase gene expression in higher plants, we wanted to test whether overexpression of HMG CoA reductase in plants, and the resulting increase in mevalonic acid synthesis, would alter the accumulation of isoprenoid end products. In normal, healthy plants, the bulk of the mevalonic acid is converted primarily into three families of compounds, the phytosterols, carotenoids and chlorophylls, although less abundant isoprenoid compounds are also produced. As a preliminary examination of the effects of ecoptic HMG CoA reductase expression on isoprenoid biosynthesis, we compared the steady-state levels of the most abundant isoprenoids in wild-type *Arabidopsis* with the isoprenoids that accumulate in transgenic plants carrying the CaMV 35S:*HMG1* chimeric gene. Based on the results described in the previous sections, the two independent transformants that overexpress HMG CoA reductase, T43.06 and T43.29, were selected for further analysis. As shown in Figure 8(a), the levels of total carotenoid and chlorophyll pigments are the same in wild-type and transgenic *Arabidopsis*. Furthermore, analysis of the neutral lipids extracted from the leaves of these plants by gas chromatography reveals similar profiles for all three of the plant lines that were examined (Figure 8b). Furthermore, the major peaks were identified by mass spectroscopy, and those corresponding to isoprenoid compounds are labeled according to their position in the elution profile. The most abundant leaf sterol was eluted from the column at position 824, and exhibits a mass spectrum consistent

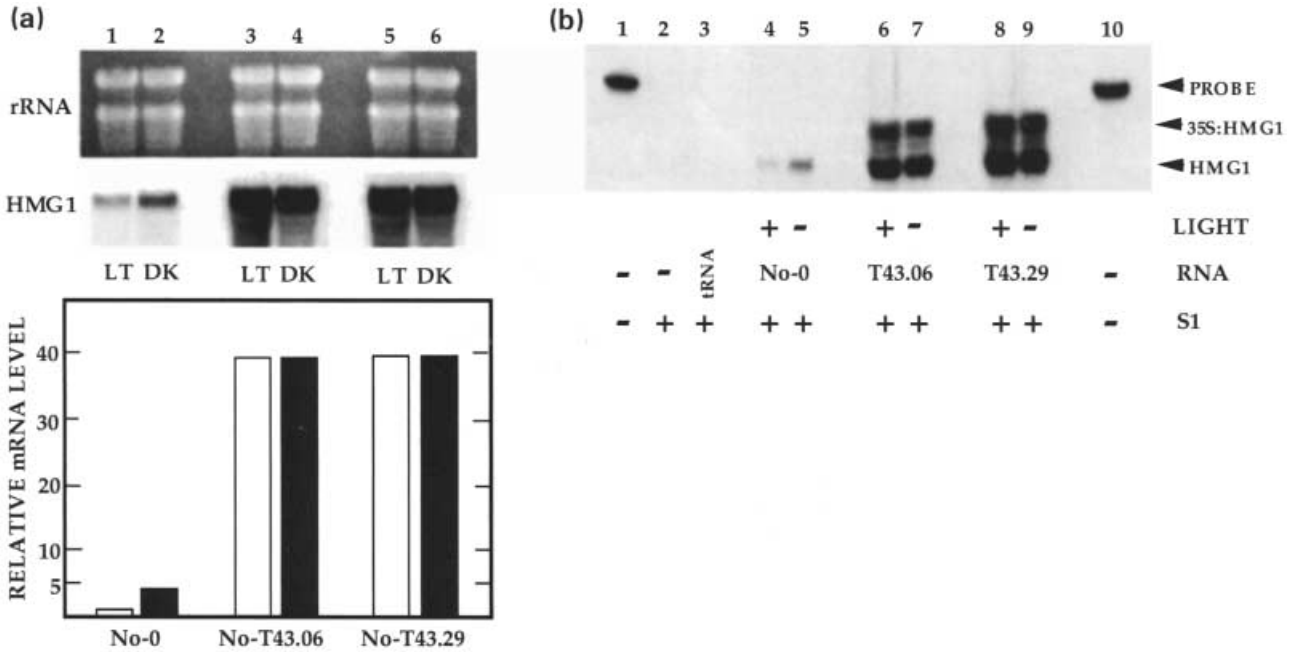


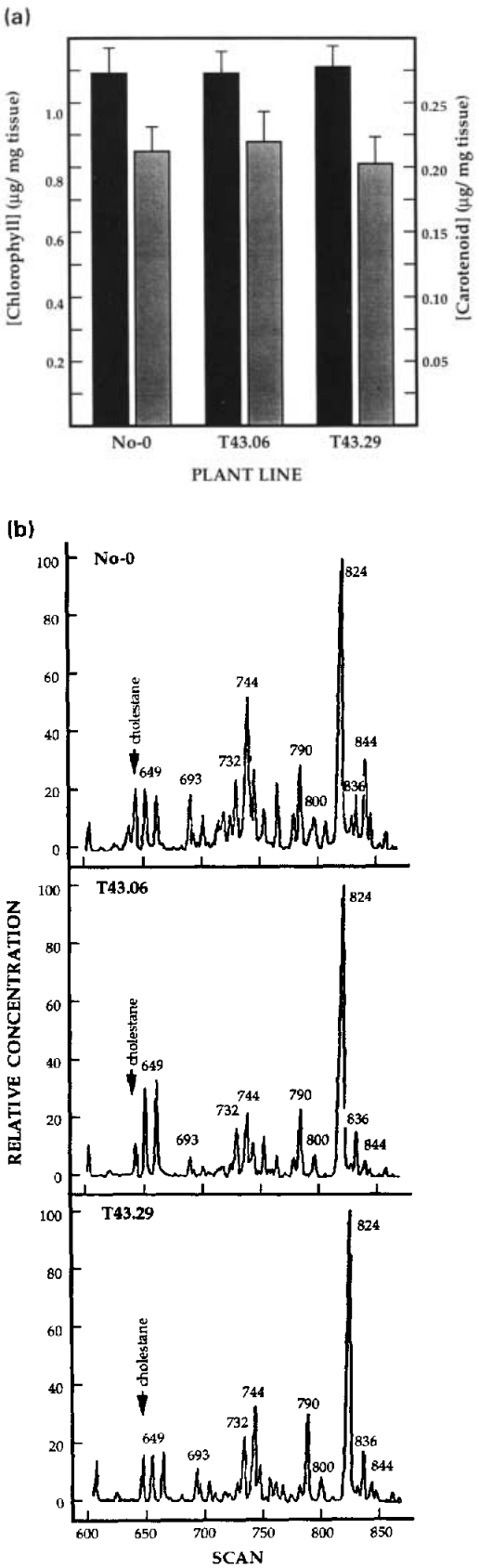
Figure 7. Effects of light on *HMG1* mRNA levels in wild-type and transgenic *Arabidopsis*.
(a) Total RNA was isolated from mature *Arabidopsis* plants grown under continuous light (lanes 1, 3 and 5) and from plants adapted to darkness for 24 h (lanes 2, 4 and 6). The sources of the RNA were as follows: lanes 1 and 2, wild-type *Arabidopsis* (No-0 ecotype); lanes 3 and 4, No-T43.06.; lanes 5 and 6, No-T43.29. RNA samples were prepared as described and 5 μg samples were fractionated by agarose gel electrophoresis. As a control for sample preparation, the agarose gel was stained with acridine orange and the rRNA visualized using UV illumination. *HMG1* mRNA was detected by blot hybridization using a uniformly labeled *HMG1* cDNA probe, and the levels were quantified using a Fuji BAS 1000 phosphorimager.
(b) Total RNA was isolated from mature *Arabidopsis* plants grown under continuous light (lanes 4, 6 and 8) and from plants adapted to darkness for 24 h (lanes 5, 7 and 9). The sources of the RNA were as follows: lanes 4 and 5, wild-type *Arabidopsis* (No-0 ecotype); lanes 6 and 7, No-T43.06; lanes 8 and 9, No-T43.29. The S1 nuclease protection assay was carried out as described, the products fractionated on a sequencing gel, and the dried gel exposed to X-ray film. Control reactions were carried out in the absence of S1 nuclease (lanes 1 and 10), in the absence of RNA (lane 2) or in the presence of yeast tRNA (lane 3). Predicted products are labeled and indicated by arrows.

with that for sitosterol. Spectra for several other peaks (693, 728, 744, 790 and 844) also share features of this sterol. Although there is some variation in the relative amounts of some of these isoprenoids in lipid extracts from the different plant lines, there is no indication from these data that sterols or sterol precursors are accumulating in transgenic plants that overexpress HMG CoA reductase. These results indicate that the modest increase in the levels of HMG CoA reductase observed in these transgenic plants is not sufficient to perturb the controlled synthesis of these isoprenoid end products, and that any increase in mevalonic acid production can be accommodated by the normal regulatory mechanisms that operate in *Arabidopsis*.

Discussion

In order to better understand the regulatory mechanisms that control isoprenoid biosynthesis in higher plants, the CaMV 35S promoter was used to direct high-level, constitutive transcription from the *Arabidopsis HMG1* cDNA as a means of overexpressing HMG CoA reductase in transgenic *Arabidopsis* plants. Analysis of 14 plant lines independently transformed with the CaMV 35S:*HMG1* chimeric gene revealed two distinct classes of transformants. The CaMV 35S:*HMG1* transgene is not actively expressed in most of the transgenic plants we studied (Figure 6), and consequently does not contribute to the pool of active enzyme. Furthermore, both the expression of the native *HMG1* gene (Figures 5 and 6) and HMG CoA reductase activity (data not shown) in these transgenic plants are indistinguishable from those in their wild-type counterpart.

In contrast, the second class of transgenic plant exhibits elevated levels of *HMG1* gene expression, and studies of these plants have provided insight into some of the mechanisms that may control HMG CoA reductase expression in higher plants. In addition, the effects of HMG CoA reductase overexpression on isoprenoid biosynthesis were monitored by measuring the levels of the major isoprenoid compounds that accumulate in the transgenic plants. Although the rate-limiting steps in plant isoprenoid biosynthesis have not been identified, mevalonic acid production has been assumed to represent the major metabolic bottleneck, as it does in animal cells. Therefore, we reasoned that by increasing the amount of HMG CoA reductase, the availability of mevalonic acid for isoprenoid



synthesis would no longer be a limiting factor. The question of flux through the isoprenoid pathway is an important element in our understanding of its control, and must be addressed by a systematic and rigorous measurement of biosynthetic rate. However, the overall flux through a pathway from substrate to end product is difficult to measure accurately, particularly for a highly branched pathway such as isoprenoid biosynthesis.

Consequently, in this study we exploited a molecular genetic approach as a means of perturbing isoprenoid metabolism by isolating transformed plants that overexpress HMG CoA reductase. We used both an *in vitro* assay, the direct measurement of MVA production, and an *in vivo* assay, the monitoring of mevinolin-resistant growth, to confirm that higher levels of HMG CoA reductase were being expressed in transgenic plant lines. Surprisingly, we found that in these transgenic plants, the steady-state levels of the three major isoprenoid end products, namely, phytosterols, carotenoids and chlorophyll, are the same as in wild-type plants, despite the fact that the transgenic plants contain measurably higher levels of HMG CoA reductase enzyme activity and presumably larger internal pools of mevalonic acid. Interestingly, similar results were obtained in studies of cultured mammalian cells. Despite the fact that UT-1 cells express extremely high levels of HMG CoA reductase, the total cell cholesterol content in these cells was actually reduced compared with the parental CHO cell line (Chin *et al.*, 1982). In contrast, expression of the hamster HMG CoA reductase in transgenic tobacco plants resulted in moderate increases in both enzyme activity and sterol accumulation, although the levels of other isoprenoid end products were not affected (Chappell *et al.*, 1991). Based on results from these various studies, it would appear that increasing the levels of HMG CoA reductase is not sufficient to alter the accumulation of the abundant isoprenoids derived from mevalonic acid.

The simplest interpretation of our results is that mevalonic acid production is not the only limiting reaction in the isoprenoid pathway and that control of the metabolic

Figure 8. Effects of ectopic HMG CoA reductase expression on isoprenoid synthesis in *Arabidopsis*.

(a) *Arabidopsis* plants were grown for 10–12 days on GM agar at 20°C under continuous light. The photosynthetic pigments were extracted from individual plants and the levels of total carotenoids (stippled) and chlorophyll (solid black) were measured as described in Experimental procedures. At least eight individual plants from each line were assayed in two independent experiments, and the values averaged as shown.

(b) Lipids were extracted from No-0, No-T43–06 and No-T43–29 plants grown for 10 days on GM under continuous light, and separated by gas chromatography using a DB-5 capillary column. Mass spectra of major peaks in the gas chromatograms were obtained and searched against the NBS mass spectrum library. Isoprenoid identities were confirmed by the presence of molecular ions and $[M\text{-}H_2O]^+$ peaks in the mass spectra. Major peaks corresponding to isoprenoid compounds are indicated by scan position. Cholestane was added to the lipid extract as an internal standard and is designated by the arrow.

flux through the pathway may either be restricted by the availability of reducing equivalent or substrate (acetate carbon), or mediated by the catalytic activity of enzymes at other steps in the pathway. The regulation of these genes alone may be sufficient to control isoprenoid biosynthesis, even in the presence of constitutively expressed HMG CoA reductase. Experiments to address the possibility of this type of coordinate control in *Arabidopsis* are currently underway in our laboratory. Alternatively, because neither end products nor intermediates appear to accumulate as a result of HMG CoA reductase overexpression, it is possible that isoprenoid homeostasis in plants is maintained by regulating both catabolic and anabolic reactions. In either case, we suggest that in plants, as in animals, a sensitive regulatory mechanism operates to maintain the appropriate cellular balance of isoprenoids under changing physiological conditions, and that these controls may be implemented at multiple steps within the pathway.

Although isoprenoid biosynthesis seems to be largely unchanged in transgenic plants that overexpress HMG CoA reductase, by replacing the *HMG1* promoter with the CaMV 35S promoter, we have uncovered a number of interesting features of HMG CoA reductase regulation. In particular, we have uncoupled promoter-dependent transcriptional control of *HMG1* expression from post-transcriptional regulation, and have begun to study some of the previously cryptic control mechanisms that regulate HMG CoA reductase expression in *Arabidopsis*. In these transgenic plants, *HMG1* mRNA is efficiently and constitutively expressed under control of the CaMV 35S promoter, accumulating to levels more than 40-fold higher than in wild-type plants grown under the same conditions. Strikingly, these transgenic plants accumulated at most three times the HMG CoA reductase activity of wild-type plants, suggesting the possibility of a maximum threshold of enzyme activity. Consequently, we speculate that in the transgenic plant lines T43.06 and T43.29, HMG CoA reductase expression is controlled at a level other than RNA synthesis, and that some post-transcriptional mechanism must be implemented in order to limit the enzyme activity in these plants. We are exploiting plant transformation to examine further the possibility that *Arabidopsis* HMG CoA reductase is regulated by a multivalent control mechanism, as it is in animal cells (Goldstein and Brown, 1990), and that covalent modification, translational efficiency or enzyme stabilty contribute to regulation of the plant mevalonate pathway. In addition, because we have been unable to raise specific antibodies against either bacterially expressed HMG CoA reductase or synthetic peptides conjugated to carrier proteins (Re, unpublished results), we also introduced an epitope-tag into the *HMG1* coding sequence as a means of monitoring quantitative changes in the levels of HMG CoA reductase protein in transgenic plants containing the tagged alleles.

The transgenic *Arabidopsis* plants we constructed contain both the native *HMG1* gene and the CaMV 35S:*HMG1* fusion gene, and consequently, the pattern of *HMG1* gene expression in these plants reflects the activity of both genes. There are a number of cases that have been documented in higher plants in which the co-expression of two such genes has resulted in aberrant expression of the native gene, and examples of both co-suppression (De Carvalho *et al.*, 1992; Fray and Grierson, 1993; Napoli *et al.*, 1990) and co-activation (Hirel *et al.*, 1992) have been reported. Therefore, we examined the influence of the CaMV 35S:*HMG1* gene fusion on expression from the native *HMG1* locus. Surprisingly, our data include the novel observation that not only is the chimeric *HMG1* gene driven by the CaMV 35S promoter expressed constitutively, but that the expression of the native gene is also deregulated compared with wild-type plants, resulting in atypically high levels of *HMG1* mRNA in transgenic plants grown under continuous light. The molecular basis for this induction is not clear, but appears to be correlated with active expression of the CaMV 35S:*HMG1* transgene. One possible rationale for this phenomenon is that ectopic expression of HMG CoA reductase may initiate a series of changes in cellular metabolism that in turn directly influence the regulation of the native *HMG1* gene. However, it is important to note that despite the apparent release of the native *HMG1* gene from the normal controls that regulate its expression, the overall increase in HMG CoA reductase enzyme activity is relatively small. Furthermore, increased production of mevalonic acid is not an adequate explanation for co-activation since inclusion of mevalonic acid in the nutrient media does not affect *HMG1* expression in a similar fashion (Learned, unpublished results). It seems most likely, therefore, that the increase in native *HMG1* mRNA levels in these plants is a consequence of the *HMG1* mRNA being expressed constitutively at high levels from the transgene. We speculate that under these conditions, the post-transcriptional regulatory machinery may override normal transcriptional controls, including those involved in light-mediated suppression of gene expression, resulting in deregulated accumulation of *HMG1* mRNA in the transgenic plants. Clearly, additional experiments will be necessary to determine the molecular mechanism that is responsible for co-activation of the *HMG1* genes.

Ectopic overexpression of HMG CoA reductase has allowed us to address two fundamental questions regarding isoprenoid biosynthesis in *Arabidopsis*. First, we demonstrated that the overexpression of authentic *Arabidopsis* HMG CoA reductase is not sufficient to alter the bulk synthesis and accumulation of some of the abundant end products of the plant isoprenoid pathway. Second, by replacing the *HMG1* promoter with the CaMV 35S promoter, we have uncoupled promoter-dependent transcriptional control of *HMG1* expression from post-transcriptional regu-

lation. Co-expression of native and transgenic *HMG1* genes results in novel and striking patterns of HMG CoA reductase expression. These results are consistent with the idea that both transcriptional and post-transcriptional devices may contribute to *HMG1* gene regulation in *Arabidopsis*, and that HMG CoA reductase gene expression is subject to multivalent control in higher plants, just as it is in animal cells. We are now in a position to evaluate the contributions of these various control mechanisms to the regulated synthesis of HMG CoA reductase in *A. thaliana*.

Experimental procedures

*Construction of the chimeric CaMV 35S:*HMG1 *gene*

The full-length *HMG1* cDNA previously isolated from *Arabidopsis* (Learned and Fink, 1989) was transferred from pBluescript S/K to the *Bam*HI and *Sal*I sites of pDH51 (Pietrzak *et al.*, 1986), placing the *HMG1* cDNA between the promoter and terminator elements from the 35S gene from CaMV. The resulting construct includes the entire *HMG1* coding sequence (amino acids +1 to +592), as well as 78 bp of the untranslated leader and approximately 250 bp of the 3′ untranslated region. Translational initiation is directed by the AUG initiation codon used in the native *HMG1* mRNA. The chimeric 35S:*HMG1* gene was then transferred as an *Eco*RI fragment to the binary plant transformation vector pBIN19 (Bevan, 1984) for introduction into *Arabidopsis*. The resulting plasmid was designated as pML43 and the details of the promoter fusion are shown in Figure 2.

Plant transformation and regeneration

The binary vector plasmid containing the chimeric 35S:*HMG1* gene was introduced by direct transfection into LBA4404, a disarmed strain of *Agrobacterium tumefaciens* (Hoekma *et al.*, 1983). Transformation of *Arabidopsis* roots (No-0 ecotype) and regeneration of kanamycin-resistant calli into fertile plants was carried out using the procedure of Valvekens *et al.* (1988). T_1 seeds obtained from self-fertilization of the primary transformants were grown asceptically on germination media (GM) supplemented with 25 μg ml^{-1} kanamycin under continuous light and scored for resistance to the antibiotic. Kanamycin-resistant plants were transferred to soil and the T_2 seeds resulting from self-fertilization were harvested. Using this method, 14 independently transformed plant lines were identified for further analysis.

Plant growth conditions

In preparation for planting, seeds were surface-sterilized by treatment with $NaClO_3$ (1.3% solution in water), washed extensively with sterile water, and vernalized overnight in the dark at 4°C. Transgenic plants were grown asceptically on GM in a controlled growth chamber under a continuous light cycle at 22°C.

The growth characteristics of wild-type and transgenic *Arabidopsis* plants were examined by sowing sterilized seeds on to the media surface, and then placing the Petri plate in a vertical orientation in the growth chamber. After germination, the roots elongate along the surface of the agar in a vertical fashion. Measurements of root growth were made daily using a hand-held micrometer.

Isolation of RNA and blot hybridization analysis

Total RNA was prepared from whole plant tissue by grinding in liquid nitrogen and extraction of the nucleic acids as described (Nagy *et al.*, 1988). Following covalent modification by treatment with glyoxal (McMaster and Carmichael, 1977), RNA was fractionated by agarose gel electrophoresis and blotted using 10 × SSC to Nytran$^+$ membranes (Schleicher and Schuell) by capillary transfer. Following transfer, the RNA was covalently cross-linked to the membrane by UV irradiation. Hybridization to ^{32}P-labeled probes was performed in 5 × SSC, 20 nM KPO_4/pH 7.4, 50% formamide, 100 mg ml^{-1} denatured herring sperm DNA, 0.1% sodium dodecyl sulfate (w/v), 10% dextran sulfate for 14–24 h at 42°C (Ausubel *et al.*, 1987). The membrane was then washed twice with 2 × SSC/0.1% SDS at room temperature, and twice with 0.1 × SSC/0.1% SDS at 65°C for 15 min. The relative amounts of mRNAs detected by hybridization were determined by densitometric scanning of the autoradiograms using a Millipore BioImage system. Radiolabeled probes were prepared by random priming of gel-purified DNA fragments (Feinberg and Vogelstein, 1983). The probe for *HMG1* mRNA was a 2.3 kb full-length *Arabidopsis HMG1* cDNA (Learned and Fink, 1989). This fragment is gene-specific and shows no cross-hybridization with the *Arabidopsis HMG2* gene under the conditions described (data not shown). The 1.0 and 1.6 kbp fragments containing the 18S and 25S coding sequences from the radish rDNA repeats (Delseny *et al.*, 1993) were used as probes for rRNA.

S1 nuclease protection of Arabidopsis *RNA*

High-resolution analysis of RNA was performed by S1 nuclease protection using total *Arabidopsis* RNA and a synthetic oligonucleotide probe. This oligonucleotide consisted of the 51 base sequence 5′-**GA GAG GAG AGA GGA GAG AGG TGG TGA GGT G** GC AAT GAA GTC *GAC CCT AGA T*-3′, which includes 30 nt complementary to both the *HMG1* and 35S:*HMG1* mRNAs (bold characters), 11 nt that are complementary only to the mRNA derived from the 35S:*HMG1* transgene (underlined), and a 10 nt tail that is not complementary to either mRNA (italics). Radiolabeled probe was prepared by end-labeling the oligonucleotide with T4 polynucleotide kinase (US Biochemicals) and [γ-^{32}P]ATP (Weaver and Weissman, 1979). Hybridization to *Arabidopsis* RNA and digestion with S1 nuclease were carried out as described (Sharp *et al.*, 1980). Following degradation of the RNA–DNA hybrid molecules, the reaction products were fractionated on a 16% polyacrylamide gel containing 8 M urea (Maxam and Gilbert, 1980) and exposed to X-ray film. This high-resolution S1 nuclease assay using the tripartite oligonucleotide probe allows us to distinguish between mRNAs derived from the native *HMG1* gene and from the 35S:*HMG1* transgene.

HMG CoA reductase enzyme activity

Arabidopsis seeds were surface-sterilized and grown asceptically on GM under a continuous light cycle at 22°C. After 3 weeks of growth, plant tissue was harvested at 4°C and a 16 000 ***g*** cell-free homogenate isolated according to the protocol of Bach *et al.* (1986). The reaction mixture and assay conditions used were similar to those described by Ong *et al.* (1991). The standard HMG CoA reductase assay system consisted of 100 mM Na_2PO_4, pH 7.5, 10 mM EDTA, 5 mM DTT, 12 mM glucose 6-phosphate, 0.5 units glucose 6-phosphate dehydrogenase, 3 mM NADPH and 30 mM [^{14}C]HMG CoA (0.1 mCi) in a final volume of 100 μl. The

assay was initiated by adding an aliquot of a homogenate and incubating at 37°C for 30 min. Reactions were terminated by adding 25 μl of 2 M HCl plus 10 mM mevalonolactone. Samples were incubated an additional 30 min at 37°C and then chromatographed on QAE–Sephadex columns. Eluates were collected directly into scintillation vials and 17.5 ml of Ecolite (ICN) scintillant added. Blanks contained the same reagents as the samples except the termination mixture was added prior to the homogenate. Protein levels were determined for each sample using a Coomassie dye-binding assay reagent (Bradford, 1976). Bovine serum albumin was used as a standard and blank samples contained the same reagents as found in the homogenate.

Chlorophyll and carotenoid determinations

Chlorophyll and carotenoid determinations were performed on plants harvested after 10–12 days of growth on GM under a continuous light cycle at 22°C. In the case of chlorophyll determinations, whole plants were immersed in DMF and stored in the dark for approximately 24 h at 4°C prior to spectroscopic examination. The total chlorophyll levels were calculated as described by Moran (1982).

Carotenoid levels were measured in extracts prepared by homogenizing plants in 80% acetone (v/v), followed by centrifugation at 12 000 ***g*** for 10 min. Absorbance values obtained from the supernatants at 662, 645 and 470 nm were used to determine total carotenoid levels, as described in Lichtenthaler (1987).

Lipid extraction and analysis

Wild-type and transgenic plants were grown for 10 days on GM under conditions described previously. Tissue from each line was harvested and a wet weight determined (approximately 1 g). Total lipids were extracted by freezing the tissue in liquid nitrogen and grinding it into a fine powder using a mortar and pestle. A chloroform:methanol:formic acid (10:10:1 by vol.) mixture was added and the samples incubated at −20°C for 12–20 h. The extracts were centrifuged at 4000 ***g*** for 10 min at 4°C and the supernatants decanted off into new tubes. The remaining pellet was re-extracted by adding a chloroform:methanol:water (5:5:1 by vol.) mixture and incubating at room temperature for 10 min. The sample was centrifuged as described above and the supernatants from the two extractions combined.

In preparation for analysis by GC-MS, solvent extracts were evaporated to dryness under a stream of nitrogen gas, and subsequently dissolved in 100 μl of dichloromethane containing 100 ng of cholestane for use as an internal standard. The tubes were vortexed for 15 s to ensure mixing, and the solvent evaporated at room temperature under a stream of nitrogen until a final volume of 40 μl remained. The extracts were transferred to amber autosampler vials with tapered 100 μl inserts and analyzed using GC-MS.

Sterols were determined using a Hewlett Packard 5890 gas chromatograph (Hewlett Packard, Palo Alto, CA) interfaced with a Trio-2 mass spectrometer (VG Masslab, Altrincham, UK). A Hewlett Packard model 7673A autosampler delivered splitless injections of 2 ml on to a 15 meter DB-5 capillary column (0.25 mm I.D., 0.25 mm film) using helium as carrier gas at a linear velocity of 25 cm sec^{-1}. The injector was held at 275°C, and the transfer line between the gas chromatograph and the mass spectrometer was maintained at 300°C. The column temperature was programmed from its initial value of 150°C (1 min hold) at a rate of 10°C/min^{-1} to 300°C (25 min hold). Electron ionization mass spectra were obtained using 70 eV electrons, and mass spectra were acquired over the range of *m/z* 40–500 at 1 scan sec^{-1}. Mass spectra of major peaks in the chromatograms were searched against the NBS mass spectrum library provided with the VG 11/250J data acquisition system. Sterol identities were confirmed by the presence of molecular ions and $[M\text{-}H_2O]^+$ peaks in the mass spectra.

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