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# Expression of the Arabidopsis *HMG2* Gene, Encoding 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase, Is Restricted to Meristematic and Floral Tissues

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**The synthesis of mevalonate, which is considered the first rate-limiting step in isoprenoid biosynthesis, is catalyzed by the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; EC 1.1.1.34). In Arabidopsis, HMGR is encoded by two differentially expressed genes (*HMG1* and *HMG2*). The transcriptional activity of the *HMG2* gene was studied after fusing different regions of its 5′ flanking region to the β-glucuronidase (*GUS*) reporter gene and transforming the resulting constructs into tobacco plants. The spatial and temporal expression directed by the *HMG2* promoter in the transgenic plants is consistent with the expression pattern previously established by RNA analysis using an *HMG2*-specific probe. *HMG2* expression is restricted to meristematic (root tip and shoot apex) and floral (secretory zone of the stigma, mature pollen grains, gynoecium vascular tissue, and fertilized ovules) tissues. Deletion analysis of the *HMG2* 5′ flanking region was conducted in transgenic plants and transfected protoplasts. The region containing nucleotides −857 to +64 of the *HMG2* gene was sufficient to confer high levels of expression in both floral and meristematic tissues, although deletion to nucleotide −503 resulted in almost complete loss of expression. Sequences contained within the 5′ transcribed, untranslated region are also important for gene expression. The biological significance of the restricted pattern of expression of *HMG2* is also discussed.**

## INTRODUCTION

Metabolic specialization is a characteristic trait of cell differentiation. Metabolic activities associated with specific cells are essential for growth and survival of plant cells. In some cell types, metabolic specialization may lead to the synthesis of compounds (plant secondary metabolites) that do not always have clear biological significance. Although plant secondary metabolism is mostly related to defense mechanisms or interactions with the environment, the interest in many plant secondary metabolites is due to their biotechnological applications. Among the many metabolic processes reported in plants, isoprenoid metabolism is of special interest not only because of its involvement in the synthesis of compounds that are essential for plant growth and development, but also because plant isoprenoids include many economically important plant secondary products.

Plant isoprenoid biosynthesis is a complex, multibranched pathway. The reactions leading to specific isoprenoid end products emerge from a central pathway in which acetyl coenzyme A (CoA) is converted via mevalonate and isopentenyl diphosphate to the long-chain prenyl diphosphates geranyl diphosphate, farnesyl diphosphate, and geranylgeranyl diphosphate (Figure 1). The synthesis of mevalonate, catalyzed by the enzyme 3-hydroxy-3-methylglutaryl CoA reductase (HMGR; EC 1.1.1.34), is considered the first rate-limiting step in plant isoprenoid biosynthesis (Bach et al., 1990). HMGR from many plant species has been characterized, and its activity has been shown to be regulated by different physiological and environmental stimuli, such as phytohormones, light, wounding, pathogen attack, feedback mechanisms, and endogenous protein factors (for reviews, see Bach et al., 1991a, 1991b; Stermer et al., 1994). Although the intracellular site(s) of mevalonate synthesis has been a matter of controversy for many years (Gray, 1987), recent data support the hypothesis that, in higher plants, the formation of mevalonate occurs solely in the cytosol (Enjuto et al., 1994).

The occurrence of multiple genes encoding HMGR is a general feature of higher plants: two genes are present in Arabidopsis (Caelles et al., 1989; Enjuto et al., 1994), three genes have been reported in *Hevea brasiliensis* (Chye et al., 1992), and four genes are present in tomato (Cramer et al., 1993). Larger HMGR gene families have been reported in wheat (Aoyagi et al., 1993), pea (Monfar et al., 1990; M. Monfar

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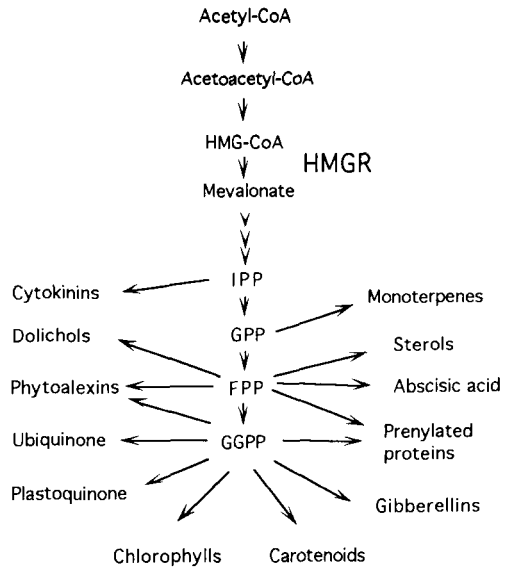


**Figure 1.** A Simplified Pathway for Plant Isoprenoid Biosynthesis.

HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; IPP, isopentenyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate.

and A. Boronat, unpublished results), and potato (Stermer et al., 1994). This is in sharp contrast with animal systems, in which the enzyme is encoded by a single gene. The biological significance of the occurrence of different HMGR isozymes in plants is currently not known, although it probably reflects the great complexity of the isoprenoid pathway in plants. It should be emphasized that the operation of this metabolic pathway is central to the synthesis of the wide range of isoprenoids required in different parts of the plant during the different stages of growth and development.

The pattern of expression of individual genes encoding HMGR has been established in a limited number of plants. In *H. brasiliensis*, three differentially expressed genes encoding HMGR have been identified (*hmg1*, *hmg2*, and *hmg3*). The *Hevea hmg3* gene is constitutively expressed, whereas *hmg1* is expressed predominantly in the laticifers (specialized cells for rubber biosynthesis) and is inducible by ethylene (Chye et al., 1992). In tomato, HMGR is encoded by four genes that are differentially expressed during development and in response to stress (Cramer et al., 1993). The tomato *hmg2* gene is induced in response to wounding and pathogen attack (Cramer et al., 1993). In contrast, tomato *hmg1* gene expression is not induced in response to defense elicitors but is detected in tissues undergoing cell divison (Cramer et al., 1993). The differential expression of two tomato HMGR genes has also been detected during fruit ripening (Gillaspy et al., 1993). Three classes of genes (*hmg1*, *hmg2*, and *hmg3*) have been reported in potato (Choi et al., 1992). The *hmg1* class contains seven or more distinct genes, whereas *hmg2* and *hmg3* contain only one or two genes (Stermer et al., 1994). Specific potato HMGR genes are differentially expressed in response to wounding and pathogen challenge (Yang et al., 1991; Choi et al., 1992). Induction of the *Camptotheca acuminata hmg1* gene by wounding has been reported in transgenic tobacco plants (Burnett et al., 1993).

Arabidopsis contains two differentially expressed HMGR genes, *HMG1* and *HMG2*. *HMG1* mRNA has been detected in all parts of the plant, whereas the presence of *HMG2* mRNA has been shown to be restricted to young seedlings, roots, and inflorescences (Enjuto et al., 1994). Although the two encoded proteins (HMGR1 and HMGR2) have the same structural organization and intracellular location, HMGR2 represents a divergent form of the enzyme that has no counterpart among the plant HMGRs identified (Enjuto et al., 1994). Furthermore, the restricted pattern of expression of the Arabidopsis *HMG2* gene has not been described for any of the other plant HMGR genes reported. Because of the significant divergence of the *HMG2* gene and its restricted pattern of expression, it has been suggested that Arabidopsis *HMG2* might correspond to an HMGR gene form that has not been previously identified in other plant species (Enjuto et al., 1994). Although the specific contribution of each Arabidopsis HMGR isoform to isoprenoid metabolism has yet to be established, the broad expression of *HMG1* suggests that it may encode a housekeeping form of the enzyme, whereas the restricted pattern of expression of *HMG2* might reflect a more specialized role of HMGR2 in the synthesis of specific isoprenoids (Enjuto et al., 1994). To gain further insight into the biological significance of the Arabidopsis HMGR isoforms, we report here the temporal and spatial expression analysis of the *HMG2* gene in transgenic plants.

## RESULTS

### Analysis of *HMG2*–β-Glucuronidase Gene Expression in Transformed Tobacco Plants

Previously, we reported that Arabidopsis *HMG2* mRNA is detected exclusively in young seedlings, roots, and inflorescences (Enjuto et al., 1994). To define the pattern of expression of the Arabidopsis *HMG2* gene more precisely, we generated transgenic tobacco plants containing chimeric genes in which 5′ flanking sequences of the *HMG2* gene (Figures 2 and 3) were fused to the β-glucuronidase (*GUS*)–nopaline synthase terminator cassette in the pBI101.1 vector (Jefferson et al., 1987). The structure of the resulting transcriptional fusions is shown in Figure 3. These constructs were transformed into tobacco using Agrobacterium-mediated transformation.

The primary tobacco transformants and the kanamycin-resistant progeny were analyzed for *GUS* expression using the chromogenic substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) (Jefferson et al., 1987). For each construct, developmental expression was determined in eight to 10

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                                                                GAATTCCAAAAGCCAAAA
-840  TAGGTGTTAACTTATGGTGGATATAAATATAAATCATAGAGAAAATTTTACGGAGATTGA
-780  ATAATTAATACCCGCAAAAGGATCGGGAAACTGAATAGCAAAAAGACGAGAGAGAATGAG
-720  ATTTTACTTCTTTATAATTGAAGTTATGCGAATGTTGTGTATTTTTACAATGAACGGTTT
-660  ATCGTTCTTTATATAGAGATAAAAAAAAGATAAAATTTACTGTGTTGGCATAGTAACATT
-600  TAAATTTTAAACTACATCACTAAATAATTAGCTTTGAAAGAATATATATTTCTCTTAGTT
-540  TTTTTTCTTTTCTTTTTAAGTTATTATTAAGAATGTGGCCAAATTTACCCAAGAGTTAAA
-480  ATTTTGTCCCATCCTTTATATTTTAAGGAAAAAATTAATAGTTTTAGAGATTGATTTTTA
-420  CCATTTCAATTTGGGGGAAAAAAATAACAATCTAAAAGCATTGAAGGTTTGAATTTTTGA
-360  TTCAAAAAGACCCAAAATAAACGCCTCATCTTTAGGTTAATTTTCAGAATAACTATTAAA
-300  AAGTGAATAATTCTTCACTGATCCATGTTGGACTAGTAAGTAGTAACAATCAAATCCATA
-240  ACATTAAATGTGTTGAAAAAAAGAATCAAAAGTAACAAAAACCATAACCCTAAATAATCA
-180  TCCCAAAAGTGGAAATAATAAAAGAAGCAGTTAGATTGTGACGGAGGAGACAAAATCATC
-120  ATCTCTGTTCTTCTTCTTCCTCTCTCACATACGATGATGACCCAACAACCTTGACGTT
 -60  GATTTCGATGTCACACACAACACTATAAATAACACTCTTCACCTATTTTTGTCATCTCAC
  +1  ACTTTGGACTTTGGAGCCCAAAATAATCTCATAACCATTAAAAACAAATCTCCTCCTTCT
 +60  CTCCGGCGTCAATG
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**Figure 2.** Nucleotide Sequence of the 5′ Flanking Region of the Arabidopsis *HMG2* Gene.

Transcription start sites are indicated by arrowheads. The putative *cis*-acting regulatory elements discussed in the text are underlined. Putative TATA boxes are double underlined. The translation start codon is indicated in bold. The GenBank accession number of the updated nucleotide sequence of the *HMG2* gene is L19262.

independent transformants. Staining was quite variable among individual transformants for a given construct, but the qualitative pattern observed was always the same for each construct. *GUS* expression directed by the cauliflower mosaic virus 35S promoter was used as the control.

Different parts of each individual transgenic plant were collected at several developmental stages and analyzed for *GUS* expression. Construct (−857/+64)*HMG2–GUS* (Figure 3) was actively expressed in young seedlings, root tips, shoot apices, and inflorescences. No GUS activity was detected in the elongation zone of the roots, stems, or leaves. The pattern of *GUS* expression observed in the transgenic tobacco plants is consistent with that previously reported for the Arabidopsis *HMG2* gene using a gene-specific probe (Enjuto et al., 1994). These results suggest that the 921-bp EcoRI-HpaII fragment present in the (−857/+64)*HMG2–GUS* construct contains all the *cis* elements required for controlling the expression of the Arabidopsis *HMG2* gene. Thus, a detailed histochemical analysis of GUS activity was undertaken in the (−857/+64)*HMG2–GUS* transgenic tobacco plants.

## Expression of (−857/+64)*HMG2–GUS* Fusions in Developing Seedlings

*GUS* gene expression was analyzed in axenically grown transgenic seedlings ($R_1$ generation). GUS activity was detected from the early stages of seedling development (Figure 4A). High levels of GUS activity were detected in the root tips and in the cotyledons of young seedlings (Figure 4B), and GUS activity in the root tip was restricted to the apical meristem zone (Figures 4B and 4C). Root meristems, developing from the pericycle and initiating lateral roots, also stained intensely even before emerging from the central root (Figure 4D). GUS activity in the cotyledons began to decline after the first leaves appeared, where, in turn, high levels of GUS activity were observed (Figures 4E and 4F). However, detectable levels of GUS

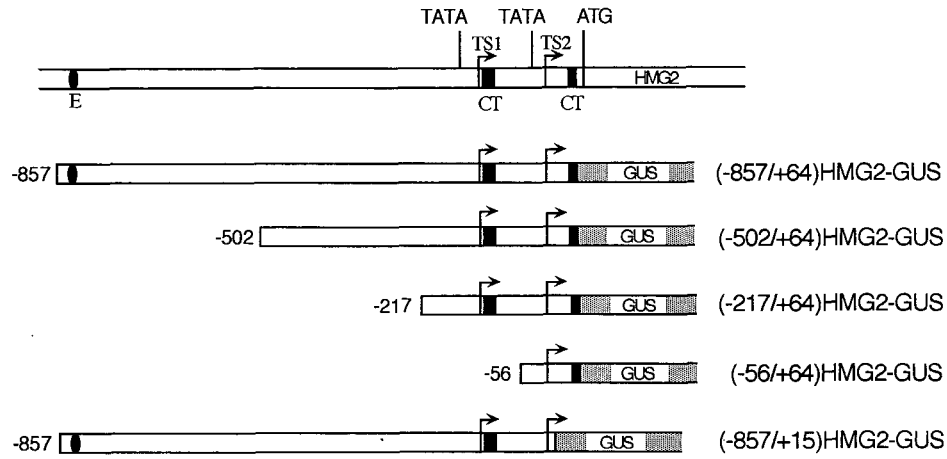


**Figure 3.** Schematic Representation of the Chimeric *HMG2–GUS* Genes Used in This Study.

The acceptor plasmids for all of the *HMG2* promoter fragments tested were pBI221 for the transient expression in protoplasts and pBI101.1 for the stable transformation into tobacco plants. Arrows indicate the transcription start sites. Numbers indicate the distance (in base pairs) from the proximal transcription start site (TS1). The relative positions of the putative regulatory elements discussed in the text are shown: E, sequence having homology with a consensus enhancer; CT, polypyrimidine tracts present in the leader sequence.

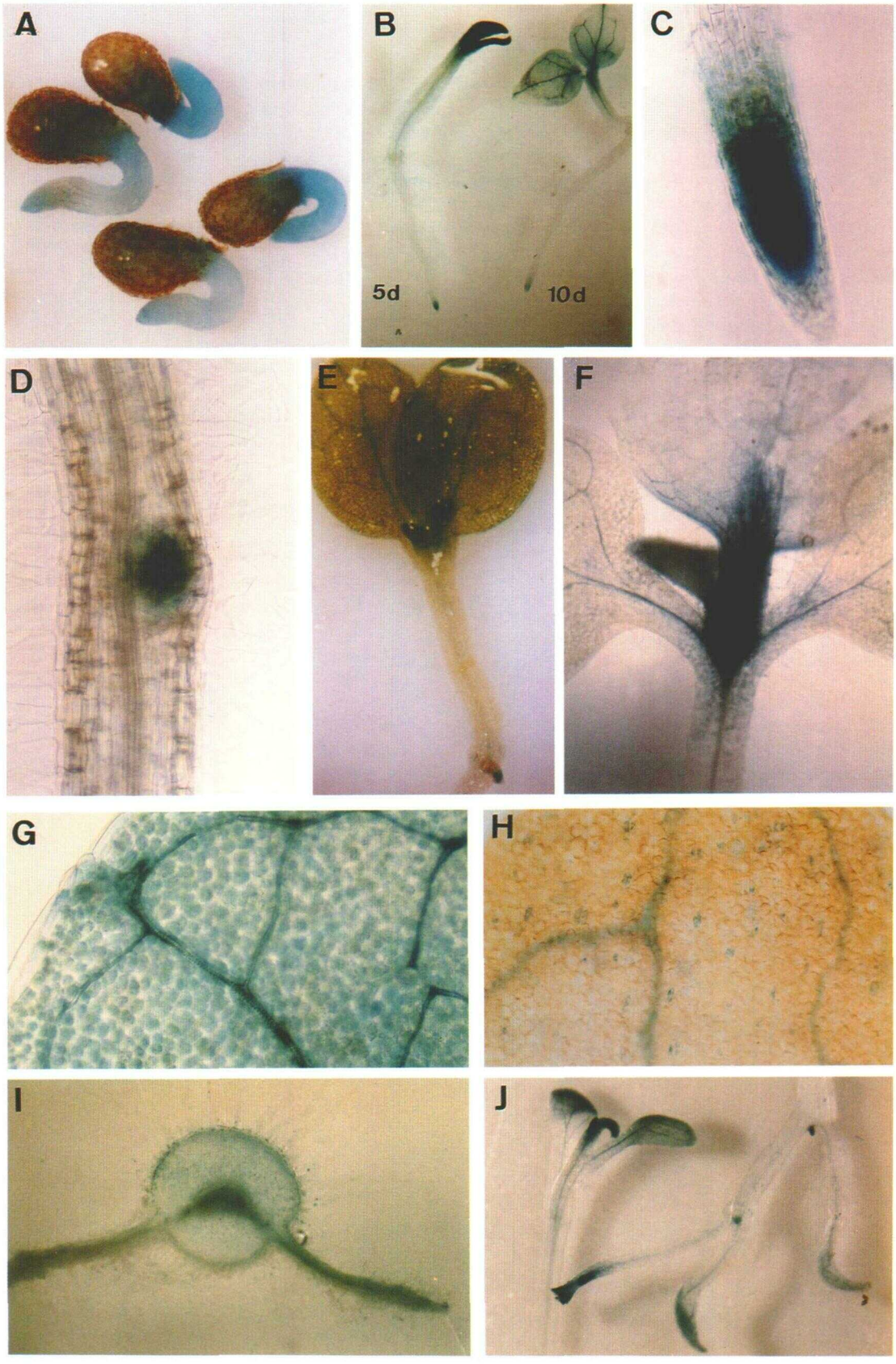


**Figure 4.** Localization of GUS Activity in Transgenic Tobacco Seedlings Transformed with the (−857/+64)*HMG2–GUS* Construct.

activity in the vascular tissue and in the stomatal guard cells of the cotyledons remained for the next 2 to 3 days (Figures 4G and 4H). A significant level of GUS activity was also detected in the vascular tissue and the petiole of young leaves (Figure 4I) but not in older leaves. Etiolated seedlings showed a pattern of *GUS* expression similar to that found in light-grown seedlings (Figure 4J), suggesting that the higher level of *HMG2* mRNA detected in dark-grown seedlings (Enjuto et al., 1994) is not the result of the expression of *HMG2* in other cell types.

### Expression of (−857/+64)*HMG2-GUS* Fusions in the Adult Plant

In adult transgenic plants, GUS activity was detected in meristematic and floral tissues. GUS activity was not detected in either old leaves or stem tissues. Flowers from the primary tobacco transformants were analyzed at the stages described by Nitsch (1970) and are shown in Figure 5A. These stages were chosen because a correlation has been previously established between flower bud development and the steps of androgenesis (Nitsch, 1970). When anthers were analyzed for *GUS* expression, the activity was detected at stages 4 and 5 (Figure 5D). The blue staining observed in the anthers corresponds to the pollen grains, which expressed high levels of GUS activity (Figure 5G). At stages 4 and 5, pollen grains were at the postmitotic stage (Nitsch, 1970). In pollen grains germinated in vitro, GUS activity was also detected in the cytoplasm of the pollen tube (Figure 5H). GUS activity was detected in the upper part of the pistils at developmental stages 3 to 5 (Figure 5C). A longitudinal section through the upper region of the pistil is shown in Figure 5E. GUS staining was detected mainly in the secretory zone of the stigma, located between the papillate epidermis and the parenchyma, which also showed significant GUS activity (Figure 5E). The secretory zone converges into the style as a central region of transmitting tissue surrounded by the cortex and the papillate epidermis (Kandasamy et al., 1990). GUS activity was also present in the vascular system but not in the transmitting tissue (Figure 5E).

GUS activity was detected in the vascular tissue of the gynoecium at both the preanthesis (stage 3) and postanthesis (stages 4 and 5) stages (Figures 5I and 5J). GUS staining was detected in fecundated ovules (Figure 5L) but not in the ovules prior to pollination. Developing seed also showed GUS activity during fruit ripening. The activity was detected until seeds started to brown and the whole fruit began to desiccate. To determine the seed stage at which GUS activity was detected, fruits were collected at different developmental stages and analyzed for GUS activity. In parallel, the stages of embryo development were followed by histological analysis. The detection of GUS activity in the fecundated ovules temporarily correlated with the stages of endosperm development. Figure 5M shows the last stage of seed development at which *GUS* expression was observed; this stage corresponds to the onset of cell divisions leading to the globular embryo stage. GUS activity was not detected either in sepals or in petals at any of the floral stages analyzed.

The response of the *HMG2* gene promoter to wounding was examined in transgenic tobacco plants containing construct (−857/+64)*HMG2-GUS*. No induction of GUS activity (fluorometric assay) was observed in cut leaves maintained in liquid media over a period of time extending from 3 to 72 hr (data not shown). Furthermore, no histochemical GUS staining was detected in the cut leaves after 24 hr of incubation in X-gluc solution (data not shown).

### Analysis of *GUS* Expression Driven by Truncated *HMG2* Promoters in Transgenic Plants and Transfected Protoplasts

To localize promoter regulatory elements involved in the expression pattern previously described, we generated several deletions by removing part of the −857/+64 region (Figure 3) and examined their effect on gene expression both in transgenic plants and transfected protoplasts.

Preliminary results on the functional analysis of the Arabidopsis *HMG1* promoter showed that a regulatory element(s) located within the 5′ untranslated leader sequence is required for gene expression (V. Lumbreras and A. Boronat, unpublished results). The presence of a unique NlaIV restriction site located 12 bp downstream from the main transcription start site (TS1 in Figure 3) of the *HMG2* gene (Enjuto et al., 1994) permitted the generation of construct (−857/+15)*HMG2-GUS*, in which most of the region encoding the untranslated leader sequence was removed (Figure 3). The analysis of this construct in transgenic

**Figure 4.** (continued).

**(A)** Three-day-old germinating seed.
**(B)** Five- and 10-day-old seedlings (5d and 10d, respectively).
**(C)** Root tip of a 6-day-old seedling.
**(D)** *GUS* expression in a secondary initiating root.
**(E)** and **(F)** Aerial part of a 9-day-old seedling and a 3-week-old plant, respectively.
**(G)** and **(H)** Cotyledons of a 6-day-old seedling and a 10-day-old seedling, respectively.
**(I)** Cross-section through the basal part of a young leaf.
**(J)** Twelve-day-old etiolated seedlings.

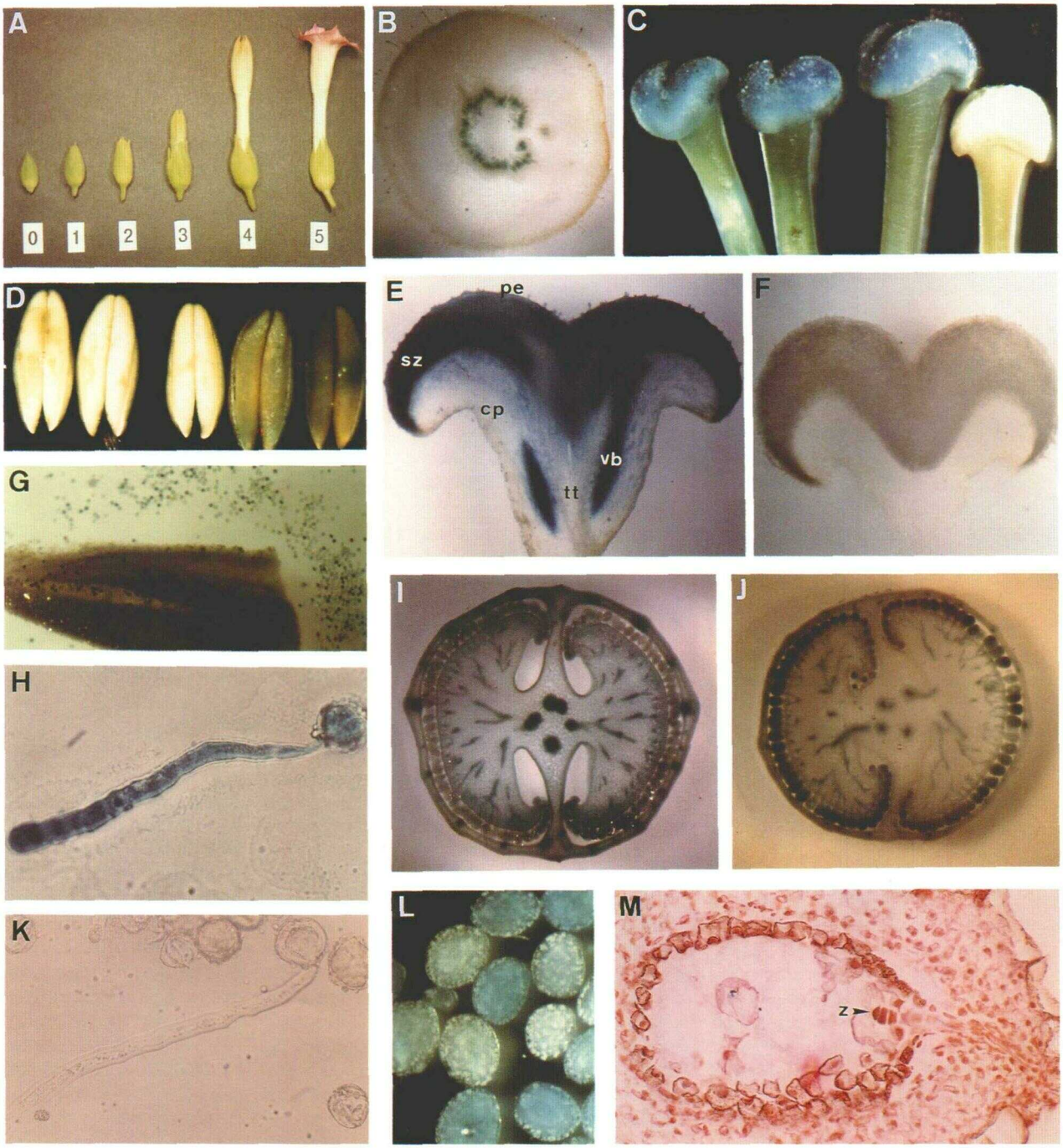


**Figure 5.** Histochemical Localization of GUS Activity in Floral Tissues of Transgenic Tobacco Plants Containing the (−857/+64)*HMG2–GUS* Construct.

**(A)** Different stages of tobacco flower development.
**(B)** Cross-section (80 μm thick) of a floral petiole.
**(C)** Stigmas at stages 3, 4, and 5 (left to right). Far right, stigma from a nontransgenic plant.
**(D)** Anthers corresponding to stages 1 to 5.
**(E)** and **(F)** Longitudinal sections (80 μm thick) of unfixed stigmas from transgenic and nontransgenic plants, respectively. pe, papillate epidermis; sz, secretory zone; tt, transmitting tissue; cp, cortical parenchyma; vb, vascular bundle.
**(G)** Anther and pollen grains at stage 5.
**(H)** Germinated pollen grain.
**(I)** and **(J)** Cross-sections of gynoecia before and after anthesis, respectively.
**(K)** Germinated pollen grain from a nontransgenic plant.
**(L)** Fecundated ovules.
**(M)** Cross-section (8 μm thick) of a fertilized ovule at the last stage when GUS activity was still detectable. z, zygote.

**Table 1.** Analysis of *GUS* Expression in Transgenic Tobacco Plants

| | Flowers | | | | Seedlings | |
|---|---|---|---|---|---|---|
| *GUS* Constructs | Pollen | Stigma | Ovary Vascular Tissue | Fecundated Ovules | Root Tip | Shoot Apex |
| (−857/+64)*HMG2–GUS* | + + + | + + + | + + + | + + + | + + + | + + + |
| (−502/+64)*HMG2–GUS* | + | +/− | − | − | − | +/− |
| (−217/+64)*HMG2–GUS* | + | − | − | − | − | − |
| (−56/+64)*HMG2–GUS* | + | − | − | − | − | − |
| (−857/+15)*HMG2–GUS* | + | − | − | − | − | − |
| CaMV 35S–*GUS*[a] | − | + + + | + + + | + + + | + + + | + + + |

+, presence of GUS activity; −, absence of GUS activity.

[a] Cauliflower mosaic virus 35S–*GUS* was used as a control of *GUS* expression.

plants revealed that GUS activity was no longer detected in meristematic or floral tissues, although low levels of GUS staining were still detected in mature pollen grains (Table 1).

Constructs containing sequential deletions of the 5′ flanking region were also generated and fused to the *GUS* reporter gene (Figure 3). The results obtained after histochemical analysis of GUS activity in the corresponding transgenic plants are summarized in Table 1. The dramatic loss of *GUS* expression in both meristematic and floral tissues in the transgenic plants containing constructs (−502/+64)*HMGR–GUS*, (−217/+64)*HMGR–GUS*, and (−56/+64)*HMGR–GUS* suggests that another *cis*-acting positive-regulatory element(s) is located between nucleotides −857 and −502.

The *HMG2–GUS* fusions analyzed in transgenic tobacco plants were also used in transient expression experiments with Arabidopsis protoplasts. The highest level of GUS activity was obtained with construct (−857/+64)*HMG2–GUS*. The expression driven by the deleted constructs (−857/+15)*HMG2–GUS*, (−502/+64)*HMG2–GUS*, (−217/+64)*HMG2–GUS*, and (−56/+64)*HMG2–GUS* was also dramatically affected in the Arabidopsis protoplast system (Figure 6).

## DISCUSSION

The great variety of essential cell functions involving isoprenoid products or derivatives suggests that plants must have elaborate control mechanisms to ensure their synthesis in the right place and at the right time. The synthesis of mevalonate, catalyzed by the enzyme HMGR, is considered the first major rate-limiting step of plant isoprenoid biosynthesis. However, the general occurrence in plants of different HMGR isozymes makes it difficult to assign a specific role to each HMGR isoform in the overall control of the isoprenoid pathway. Arabidopsis contains only two genes encoding HMGR, *HMG1* and *HMG2*, representing the smallest HMGR gene family reported in plants. The ubiquitous expression of *HMG1* suggests that HMGR1 probably corresponds to a housekeeping form of the enzyme; the restricted pattern of expression of *HMG2* points toward a more specific role for HMGR2 in the synthesis of isoprenoids in specific cell types or at specific developmental stages. To obtain more information about the significance of HMGR2 in isoprenoid biosynthesis, we used the *GUS* reporter system to characterize the temporal and spatial expression of the Arabidopsis *HMG2* gene in transgenic tobacco plants.

Our results showed that the 921-bp region comprising nucleotides −857 to +64 of the *HMG2* gene confers specific expression of the reporter gene in meristematic and in some floral tissues in transgenic plants. These results agree with our previous data showing that *HMG2* mRNA was detected only in young seedlings, roots, and inflorescences (Enjuto et al., 1994). The histochemical detection of GUS activity has revealed previously unknown patterns of *HMG2* expression.

Histochemical analyses showed that the Arabidopsis *HMG2* promoter directs *GUS* expression in meristematic tissues (root

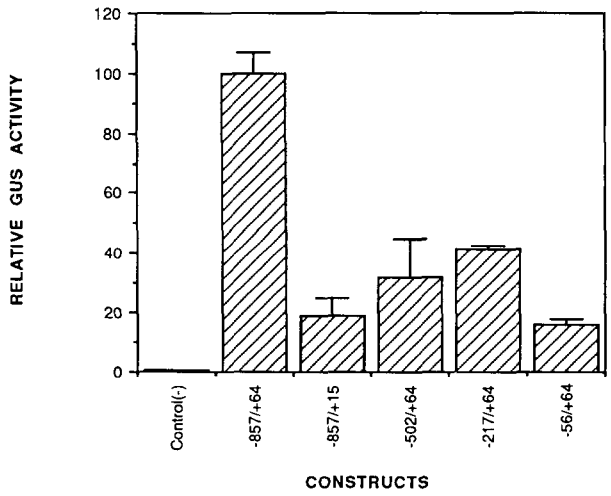


**Figure 6.** Transient Expression of the Promoter *HMG2–GUS* Constructs in Transfected Arabidopsis Protoplasts.

Relative GUS activities, normalized to the longest construct (−857/+64)*HMG2–GUS* (8 nmol of 4-methylumbelliferone per mg per min), are the average of at least three independent transfection experiments. A promoterless derivative of plasmid pBI221 was used as the control.

tips, shoot apices, and the sites initiating secondary roots). No expression was detected in old leaves, stems, or the elongation zone of roots. Expression of HMGR genes in rapidly dividing tissues has also been reported in other plants (Aoyagi et al., 1993; Cramer et al., 1993; Moore and Oishi, 1993). The expression of *HMG2* in meristematic tissues might be related primarily to the high rate of sterol biosynthesis required to sustain the active synthesis of membranes in actively dividing cells. However, *HMG2* gene expression in meristematic tissues might also be related to protein prenylation, a process that has been related to cell growth and division (Brown and Goldstein, 1980; Glomset et al., 1990; Goldstein and Brown, 1990; Maltese, 1990). Protein prenylation is a post-translational modification resulting in the covalent attachment of isoprenyl groups (either farnesyl or geranylgeranyl) to C-terminal cysteine residues of proteins (for a recent review, see Casey, 1992). Protein prenylation in plants has recently been reported (Randall et al., 1993; Zhu et al., 1993). A farnesyl transferase β subunit has been cloned from plants, and its expression pattern suggests that farnesyl transferase may have a critical role in regulating plant cell growth and division (Yang et al., 1993). The observation that the *HMG2* gene is expressed in the Arabidopsis T87 cell suspension culture (data not shown) further reinforces the proposed key role of HMGR2 in cell division.

The histochemical analysis of inflorescences revealed that the expression of the *HMG2–GUS* fusions was related not only to cell division. The expression of *HMG2–GUS* fusions was found in mature pollen grains, the secretory zone of the stigma, fertilized ovules, and the vascular tissue of the gynoecium. The high level of *GUS* expression in pollen driven by the *HMG2* promoter may be related to the need for the synthesis of specific isoprenoid compounds prior to the rapid growth of the pollen tube following germination. An abundant supply of sterols is obviously required for the synthesis of the enormous membrane surface synthesized during the growth of the pollen tube through the style. Given the extremely rapid growth of the pollen tube, it is likely that many of the transcripts and proteins that accumulate in the maturing pollen grain are synthesized in preparation for this effort (Bedinger, 1992; Mascarenhas, 1993). High levels of HMGR transcripts have also been reported in potato anthers, although the specific gene(s) responsible for this high level of expression has not been characterized (Choi et al., 1992).

GUS activity was also detected in the secretory cells of the stigma at stages preceding and following anthesis. In many plant species, the stigmatic surface is covered by a chemically complex exudate that provides a favorable environment for pollen adhesion and germination (Konar and Linskens, 1966; Knox, 1984). Although the chemical composition of these exudates has not been established, the detection of *GUS* expression driven by the *HMG2* promoter in the stigma secretory cells suggests that they may contain isoprenoid compounds or derivatives.

Our results also showed that the *HMG2* promoter directs *GUS* expression in fecundated ovules. The timing of *GUS* expression correlated with the stage in which rapid cell divisions occurred in the endosperm. However, GUS activity declined when the embryo started the cellular divisions leading to the globular stage. Analysis of HMGR activity during maize seed development has shown that levels are high in the endosperm and embryo during stages of rapid mitotic divisions (Moore and Oishi, 1993). Maize endosperm HMGR activity decreases in parallel to mitotic activity (Moore and Oishi, 1993). The restricted expression of the *HMG2–GUS* fusions in the dividing endosperm cells might reflect a role for HMGR in cell division similar to that previously discussed for the meristematic cells. However, the involvement of HMGR in the synthesis and accumulation of storage isoprenoids (or isoprenoid precursors) in seed cannot be ruled out. This latter possibility is supported by the observation that, in the presence of high concentrations of mevinolin (up to 50 μM), Arabidopsis seed germinated normally and the developing seedlings grew well for 2 to 3 days (A. Boronat and T.J. Bach, unpublished results). The presence of a pool of isoprenoid compounds or intermediates in quiescent maize seed has also been proposed (Moore and Oishi, 1993).

Major changes in plant isoprenoid metabolism have been reported in response to wounding and/or pathogen attack (Bostock and Stermer, 1989). Some of these metabolic changes have been correlated with changes in HMGR activity (Stermer and Bostock, 1987; Chapell et al., 1991) or differential expression of specific HMGR genes (Stermer et al., 1991; Yang et al., 1991; Choi et al., 1992; Cramer et al., 1993). We showed that the Arabidopsis *HMG2* gene promoter is not induced in wounded leaves of transgenic tobacco plants containing construct (−857/+64)*HMG2–GUS*. Under similar experimental conditions, the promoter of the *C. acuminata hmg1* gene was highly induced in transgenic tobacco plants (Burnett et al., 1993). Our results suggest that the Arabidopsis *HMG2* gene is not involved in the synthesis of specific isoprenoids in response to wounding. The lack of induction of the *HMG2* gene should not be surprising on the basis of the great diversity of metabolic responses induced by wounding or pathogen attack in different plant species. This is well illustrated by the synthesis of the antimicrobial phytoalexins induced by pathogen attack. Although some phytoalexins are widely distributed among many plant families, plants in the Leguminosae have not been reported to produce sesquiterpenoid phytoalexins, and those in the Solanaceae have not been reported to produce isoflavonoid phytoalexins (Kuc and Rush, 1985).

As a first approach to the identification of *cis* regulatory elements involved in the control of *HMG2* gene expression, we have analyzed truncated versions of the promoter in transgenic plants and transfected protoplasts. We showed that deletions removing sequences from either the 5′ or the 3′ end of the (−857/+64)*HMG2–GUS* fragment resulted in a dramatic loss of expression. Construct (−502/+64)*HMG2–GUS*, with a 5′ deletion of 355 bp, showed barely detectable *GUS* expression in meristematic tissues (Table 1). Expression in floral tissues was also dramatically reduced, although low levels of GUS activity were detected in mature pollen grains (Table 1). These results suggest that an important regulatory element(s) is

located within the deleted region. A computer search against the transcription factor data base (TFD) identified the sequence GGTGGATATA (located at position −825; Figure 2) as similar to a motif found not only in many viral enhancers (Weiher et al., 1983; Odell et al., 1985; Sassone-Corsi and Borrelli, 1986), but also upstream of several plant genes (Kaulen et al., 1986; Kuhlemeier et al., 1987; Maier et al., 1987; Hatzopoulos et al., 1990). In addition, deletion of 49 bp from the 3′ end, which removes sequences corresponding to the untranslated leader, also resulted in a lack of expression (Table 1).

No consensus sequences similar to previously characterized *cis* regulatory elements from other plant and sterol-regulated genes have been found within the deleted region. However, there are two short polypyrimidine stretches (TCTCCTCCTTCTCTCC and TTCTTCTTCTTCTCCTCTCTC) located at position +49 and −113, respectively (Figure 2). $d(CT)_n$ tracts in eukaryotic genomes may be involved in various processes, including replication (Baran et al., 1987; Caddle et al., 1990), recombination (Weinreb et al., 1990), transcription (Wells et al., 1988; Lu et al., 1993), and chromatin structure (Lu et al., 1993). Proteins that specifically bind $(CT)_n$·$(GA)_n$ promoter elements have been reported (Gilmour et al., 1989; Kolluri et al., 1992). Interestingly, a similar $(CT)_n$ element present in the leader region of the Arabidopsis *HMG1* gene is required for gene expression (V. Lumbreras and A. Boronat, unpublished results). The results shown in Figure 6 suggest that the *cis*-acting elements involved in the expression of the *HMG2* promoter in the transgenic tobacco plants are also involved in controlling *HMG2* gene expression in the homologous protoplast system. Experiments are currently in progress to determine the *cis* regulatory elements involved in the control of *HMG2* gene expression.

## METHODS

### *HMG2-GUS* Gene Fusions

DNA manipulations were performed according to the standard methods described by Sambrook et al. (1989) and Ausubel et al. (1987). The promoter region of the *HMG2* gene (encoding 3-hydroxy-3-methylglutaryl coenzyme A [CoA] reductase) was excised from the genomic clone λgAT1 (Enjuto et al., 1994) as a 921-bp EcoRI-HpaII fragment. This DNA fragment, which contains 857 bp of the 5′ flanking region and 64 bp of the untranslated leader sequence, was initially cloned into the pBluescript SK+ phagemid vector (Stratagene) and then fused to the β-glucuronidase (*GUS*) reporter gene in the plasmids pBI221 and pBI101.1 (Jefferson, 1987) to yield construct (−857/+64)*HMG2–GUS* (Figure 3). The deleted versions of the *HMG2* promoter shown in Figure 3 were constructed using unique restriction sites present in the sequence. Constructs in plasmid pBI221 were used for the transient expression analysis in protoplasts, and those in plasmid pBI101.1 were used to generate the transgenic tobacco plants. The pBI101.1 derivatives containing the *HMG2–GUS* fusions were transferred to *Agrobacterium tumefaciens* LBA4404 as described by An (1987).

### Plant Material

*Nicotiana tabacum* (cv Wisconsin 38) was transformed according to the Agrobacterium-mediated transformation methods described by Horsch et al. (1985). Transgenic tobacco plants were selected in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 100 mg/L kanamycin at 25°C under a 16-hr-light/8-hr-dark cycle. Seeds were obtained by self-fertilization of $R_0$ plants grown in culture soil. Self-fertilized progeny ($R_1$) were aseptically germinated on MS medium supplemented with 100 mg/L kanamycin. Histochemical analyses were performed with leaves, flowers, and seed of the primary transformants ($R_0$) and with seedlings, roots, leaves, and shoot apices of the $R_1$ progeny.

### Histochemical Staining

Stems, leaves, stigmas, ovaries, and fruits were embedded in PBS and 2% agar before sectioning using a vibratome (Lancet FTB, Bensheim). For GUS activity staining, plant material was immersed in X-gluc solution (1 mg/mL 5-bromo-4-chloro-3-indolyl β-D-glucuronide in 50 mM sodium phosphate, pH 7.0). Samples were placed under vacuum for 5 min and then incubated at 37°C for 2 to 18 hr (Jefferson, 1987). To improve the contrast of GUS staining in the reacting tissues, samples were briefly cleared with an aqueous solution of sodium hypochlorite before being mounted and observed by light microscopy (Boivin et al., 1990).

Ovarian material was fixed in a solution containing 10% formalin, 50% ethanol, and 5% acetic acid (Cox and Goldberg, 1988), embedded in paraffin (blocks), and sectioned by microtome. Tissue sections (8 μm thick) on gelatin-coated slides were treated with xylene to remove the paraffin and rehydrated through a progressive ethanol series before staining with Van Giesson picrofuchsin for 1 hr.

For GUS staining of germinated pollen grains, freshly collected pollen grains were rinsed with 50 mM phosphate, pH 7.5, placed on solidified germinating medium (10% sucrose, 0.01% boric acid, and 0.8% agar) on a microscope slide, and incubated in a moisture chamber at room temperature. After germination, a drop of X-gluc solution was applied over the agar layer, and the slides were incubated at 37°C for 2 to 5 hr.

### Protoplast Transfection

Protoplasts were prepared from an *Arabidopsis thaliana* (Columbia ecotype) cell suspension culture (line T87) according to Axelos et al. (1989). Cells were grown on MS medium supplemented with 1 μM naphthaleneacetic acid (Axelos et al., 1989) and collected at the mid-exponential growth phase. Transfections were performed according to the $Ca(NO_3)_2$–polyethylene glycol technique as described by Pröls et al. (1988) using 40 μg of cesium chloride–purified plasmid DNA and $10^7$ protoplasts. After incubation in the dark for 40 hr at 22°C, GUS activity was assayed by fluorometry (Jefferson, 1987). Protein concentration was determined by the method of Bradford (1976). All assays were performed with the constructs shown in Figure 3 subcloned into plasmid pBI221 (Jefferson et al., 1987). A promoterless derivative of plasmid pBI221 was used as a control.

### Wounding Treatment

Transgenic tobacco plants containing construct (−857/+64)*HMG2–GUS* were used. Plants were grown axenically for 3 to 4 weeks in MS agar

medium supplemented with kanamycin (100 mg/L). Leaves (2 to 3 cm) were excised and cut into small pieces (~0.5 cm) using a sterile razor blade. Leaf pieces were incubated in half-strength MS medium and analyzed for GUS activity (fluorometric assay) at 3, 6, 12, 24, 36, and 72 hr. Alternatively, the cut leaf pieces were incubated for 24 hr in X-gluc solution for histochemical staining.

### Computer Search Analysis

Motif searches have been done using the transcription factor data base (TFD, release 6.4) (Ghosh, 1993) and the Findpatterns program from the Genetics Computer Group (GCG) package (Devereux et al., 1984), allowing one mismatch between the sequences.

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