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Features of the *hmg* 1 subfamily of genes encoding HMG-CoA reductase in potato

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Abstract

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyzes a key step in isoprenoid metabolism leading to a range of compounds that are important for the growth, development and health of the plant. We have isolated 7 classes of genomic clones encoding HMGR from a potato genomic library. Comparison of nucleic acid sequences reveals a high degree of identity between all seven classes of clones and the potato *hmg* 1 gene described by Choi *et al.* (Plant Cell 4: 1333, 1992), indicating that all are members of the same subfamily in potato. A representative member (*hmg* 1.2) of the most abundant class of genomic clones was selected for further characterization. Transgenic tobacco and potato containing the β-glucuronidase (GUS) reporter gene under the control of the *hmg* 1.2 promoter expressed GUS activity constitutively at a low level in many plant tissues. High levels of GUS activity were observed only in the pollen. GUS assays of isolated pollen, correlations of GUS activity with the HMGR activity of anthers, *hmg* 1.2 promoter deletion studies, and segregation analysis of the expression of *hmg* 1.2::GUS among the R_2 pollen of R_1 progeny plants demonstrated that the *hmg* 1.2 promoter controls pollen expression.

Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGR; EC 1.1.1.34) catalyzes the conversion of HMG-CoA to mevalonic acid, the first committed step of the isoprenoid biosynthetic pathway. Isoprenoid compounds are a diverse class of molecules with important roles in the primary and secondary metabolism of plants. Mevalonate formed by HMGR is the major precursor of compounds such as abscisic acid, gibberellins, carotenoids, steroids, natural rubber, and the phytoalexins of solanaceous plants. In addition, isoprenoid groups derived from meva-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers L34823 (*hmg* 1.2), L34824 (*hmg* 1.3), L34825 (*hmg* 1.4), L34826 (*hmg* 1.5), L34827 (*hmg* 1.6), L34828 (*hmg* 1.7), L34829 (*hmg* 1.8) and L34830 (*hmg* 3.2).

lonic acid are incorporated into ubiquinone, dolichol, plastoquinone, chlorophyll and some tRNAs and proteins. The regulation of HMGR activity, often in coordination with other enzymes of isoprenoid biosynthesis, appears to be an important element in the production of many isoprenoid compounds [2, 8, 14, 30].

HMGR has been examined in a wide variety of plant species at the molecular and biochemical levels [3, 4, 24]. Potato, due to its synthesis of isoprenoid defense compounds such as sesquiterpenoid phytoalexins and steroid glycoalkaloids, has been the subject of several studies. Two separate HMGR activities which are differentially modulated by wounding, elicitor and light are discernible in potato tubers [30]. Differential activation of potato *HMGR* genes has been reported in response to pathogen, elicitor and wounding [9, 31, 38]. Three classes of genes have been described, *hmg* 1, *hmg* 2 and *hmg* 3. In tubers, *hmg* 1 mRNA accumulates to high levels after wounding but is strongly suppressed by additional treatment with elicitor or infection [9]. In contrast, the slight induction of *hmg* 2 and *hmg* 3 mRNA levels in wounded tuber tissues is greatly enhanced by treatment with elicitor or infection [9, 31, 38]. This change in the expression pattern of *HMGR* genes precedes a switch in wounded tubers from the synthesis of steroid glycoalkaloids to sesquiterpenoid phytoalexins upon elicitor treatment or infection [9].

Here we describe 7 classes of clones encoding HMGR from a potato genomic library. Partial sequencing demonstrates that all the *HMGR* clones share a high degree of identity to *hmg* 1 at the N-terminal region which is not conserved among *hmg* 1, *hmg* 2 and *hmg* 3. A representative gene (*hmg* 1.2) was selected for further characterization; its promoter was isolated, fused to the coding region of the GUS reporter gene and analyzed in transgenic tobacco and potato. Results show that the *hmg* 1.2 construct is expressed constitutively at a low level in many plant tissues, except for vascular tissue and pollen which had moderate and high levels of expression, respectively.

Materials and methods

Plant material

Certified tubers of potato cv. Kennebec were obtained commercially, and tubers of cv. Lemhi were provided by Dr J.J. Paved (USDA-ARS, Aberdeen, ID). The tubers were stored in the dark at 4 °C, and potato plants were grown under greenhouse conditions. Transgenic tobacco cv. Xanthi-nc was initially propagated aseptically in MS medium [25] and then transferred to the greenhouse. Seeds of self-pollinated transgenic plants were obtained by bagging the inflorescences of individual plants before flowering. Potato tuber disks were prepared and treated with the elicitor arachidonic acid or water droplets, or infected with a mycelial suspension of *Phytophthora infestans* as previously described [30]. One day after treatment, the treated surface layer of each tuber disk was harvested, frozen in liquid N_2 and stored at −70 °C until required. HMGR activity was assayed essentially as described by Russell [27].

Isolation of HMGR genomic clones

A genomic library from potato cv. Lemhi in the λFixII vector was obtained commercially (Stratagene, La Jolla, CA). The library (approximately 5×10^5 pfu) was screened using the HMGR cDNA pot 17 [31] as probe. Plaques were lifted onto nylon filters (NEN, Du Pont, colony/plaque screen) and hybridized to ^{32}P-labelled pot 17, by the method of Feinberg and Vogelstein [12], in hybridization buffer containing 1 M NaCl, 1% (w/v) SDS, 50% (v/v) formamide, 5× Denhardt's solution, 0.2 mg/ml denatured salmon sperm DNA at 42 °C overnight. Filters were washed twice in 2× SSC, 1% (w/v) SDS at 65 °C for 30 min followed by two washes in 0.2× SSC at room temperature for 30 min. Positive plaques were subjected to two further rounds of plaque purification.

Isolation of nucleic acids

Individual organs from either potato or tobacco plants grown in the greenhouse were harvested, frozen in liquid N_2 and stored at −70 °C until use. Total RNA was extracted according to the methods of Ward *et al.* [36]. The supernatant obtained after precipitation of total RNA was adjusted to a final concentration of 66% ethanol to precipitate the DNA; this was spooled out, dried briefly, resuspended in TE buffer and purified over $CsCl_2$ [22]. The poly(A)$^+$ RNA was purified with the PolyA Tract mRNA isolation kit (Promega, Madison, WI).

DNA sequencing

DNA fragments to be sequenced were cloned into pBluescript SK$^-$ and sequenced by the dideoxy chain termination method [28] on double-stranded DNA. Both strands were sequenced using either manual or automated (ABI model 373A, Applied Biosystems) methods.

DNA and RNA blot analysis

For DNA analysis, 10 ug of potato leaf genomic DNA were digested with *Hin*d III and the fragments separated on a 0.7% agarose gel and blotted onto a nitrocellulose filter according to Wahl *et al.* [35]. Blots were washed twice in 0.1 × SSC, 0.5% (w/v) SDS at 65 °C for 30 min prior to autoradiography. Total RNA was separated on agarose-formaldehyde gels then transferred to nitrocellulose membranes essentially as described by Maniatis *et al.* [22]. Radioactive probes were prepared according to Feinberg and Vogelstein [12].

Construction of HMGR promoter plasmids for plant transformation

The *hmg* 1.2 genomic clone (λ13) was cut with *Bam* HI and *Not* I, and the 7 kb *Bam* HI/*Not* I fragment was subcloned into pBluescript SK$^-$ to yield pHMGR1.2-7. The promoter sequence was then deleted from the 3′ end by exonuclease III using the Erase-a-Base kit of Promega (Promega Corporation, Madison, WI). Three clones, pHMGR1.2-7-1, HMGR1.2-7-2 and pHMGR1.2-7-3, were obtained, containing ca. 6.1, 5.8 and 5.5 kb HMGR fragments, respectively, derived from 3′ end deletion of the 7 kb HMGR fragment in pHMGR1.2-7. From these clones 3.5, 3.2 and 2.9 kb *Hin*d III (5′ end)/*Eco* RV (3′ end) fragments were obtained. These fragments were fused to pBI101 at the *Hin*d III and *Sma* I sites, to yield the corresponding HMGR promoter-GUS fusions pHMGR1.2-35, pHMGR1.2-32 and pHMGR1.2-29 in the binary vector. These plasmids were mobilized into *Agrobacterium tumefaciens* strain LBA4404 [15] by a direct DNA-transformation procedure [16].

Plant transformation and GUS assay

Tobacco (*N. tabacum* cv. Xanthi-nc) leaf disks were transformed with *A. tumefaciens* strain LBA4404 carrying the *hmg* 1.2-GUS fusions following the protocol of Horsch *et al.* [17]. The transformed plantlets were selected in MS medium [25] containing 100 μg/ml kanamycin sulfate and 250 μg/ml carbenicillin. Potato leaf tissue was transformed as described by Wenzler *et al.* [37]. Ten to 12 independent transgenic plants were isolated and transferred to the greenhouse. Fluorometric GUS assays and histochemical staining of calli and organs of transgenic plants were carried out according to Jefferson *et al.* [19]. Protein concentrations of the plant extracts used for fluorometric assay were obtained by the Bio-Rad (Hercules, CA) dye-binding assay according to the manufacturer's directions.

cDNA cloning

Poly(A)$^+$ RNAs isolated from anthers of cv. Kennebec were reverse transcribed using oligo-dT primers and cloned unidirectionally into the λ

phage vector Uni-ZAP (Stratagene, La Jolla, CA). A second library was constructed using primers complementary to sequences + 3 to + 41 nucleotides (relative to translation initiation codon) of *hmg* 1.2 (Fig. 2) in the λZAP vector (Stratagene).

Results

Isolation of clones encoding a subfamily of HMGR *genes*

Ca. 5×10^5 pfu of a potato genomic library in λFix II were screened with the potato *HMGR* cDNA clone pot 17, which hybridizes to the conserved region encoding the catalytic domain [31], and 18 positive clones were obtained after 3 rounds of plaque purification. The sizes of the inserts in these clones ranged from 12 to 17 kb. Fourteen randomly chosen clones were grouped into 7 classes (Table 1) on the basis of restriction analysis using pot 17 as a probe for Southern blots. The *hmg* 1-specific probe described by Choi *et al.* [9] hybridized with representatives of all 7 classes, but the gene specific probes for *hmg* 2 or *hmg* 3 did not hybridize (data not shown). The inserts from representatives of these groups were subcloned into the *Not* I site of plasmid vector LH1 [18] to yield pHMGR7, pHMGR9, pHMGR11, pHMGR13, pHMGR17, pHMGR19 and pHMGR22. A region (−78 to 247) spanning the ATG translation initiation codon of each clone was sequenced to confirm that they encoded HMGR (Fig. 1). The sequenced clones were very similar to each other in both their deduced N-terminal sequence and their 5′ non-coding region up to −339 (relative to the ATG initiation codon) where they diverged. Clone λ22 also contained a 19 bp deletion at −131.

The plasmid (pHMGR13) representing the most abundant class of *HMGR* genomic clones isolated from the library (*hmg* 1.2, Table 1) was selected for further study. Comparison of 88 deduced amino acids of the N-terminal sequence of *hmg* 1.2 with that of the potato *hmg* 1 gene (designated here as *hmg* 1.1) [9] revealed a high level of similarity (94% identity for *hmg* 1.2 as measured by the BESTFIT program of the GCG Wisconsin Package). The sequence up to 916 bp upstream of the ATG translation initiation codon contained multiple TATA and CCAAT motifs (Fig. 2). Primer extension analysis was carried out using two different oligonucleotides complementary to sequences upstream of the ATG translation initiation codon. The size of the primer-extended products indicated that the transcription initiation site for *hmg* 1.2 in potato tuber tissue is located 389 bp upstream of the ATG codon (data not shown). In addition to this major and common extended product from both primers, additional products were extended from either of the primers. This suggests the possibility of multiple transcript initiation sites or extended products from other *HMGR* genes. Because of this uncertainty the translation initiation site was used as a reference for nucleotides 5′ of the *hmg* 1.2 coding region. The putative transcription initiation site is upstream of three possible *cis*-acting elements that were present in all the *HMGR* sequences cloned, a G-box (TACACGTGTC) [13] located between −191 and −200 bp from the ATG codon, an imperfect H-box [ACT-ACC(N_7)CT] [39] between −163 and −178 bp upstream of the ATG codon and a TCAGTPy-box [1] between −282 and −288 (Fig. 2). Two 12 bp direct repeats (CCCATAACCCAA) 8 bp apart are located 11 bp downstream of the H-box. One of the direct repeats is absent from the *hmg* 1.8 promoter.

Genomic DNA from potato cvs. Kennebec and

Table 1. Classification of λ clones carrying *HMGR* gene sequences.

Class	λ clone	Corresponding plasmid
hmg 1.2	λ12, λ13, λ14, λ23, λ25, λ27	pHMGR13
hmg 1.3	λ7, λ20	pHMGR7
hmg 1.4	λ9, λ24	pHMGR9
hmg 1.5	λ11	pHMGR11
hmg 1.6	λ17	pHMGR17
hmg 1.7	λ19	pHMGR19
hmg 1.8	λ22	pHMGR22

Lemhi and pHMGR13 DNA was digested with *Hin*d III and electrophoresed in a genomic copy number reconstruction experiment. After blotting, the DNA was probed with a 2.9 kb fragment upstream of the ATG codon of *hmg* 1.2 (Fig. 3). The data indicate that the *hmg* 1.2 signal corresponded to the 4C amount of DNA in the potato genome. As potato is tetraploid, this suggests that *hmg* 1.2 is a single-copy gene. In addition to the specific band corresponding to *hmg* 1.2, a number of bands hybridized weakly to the probe, indicating the presence of multiple genes with promoter sequences related to the *hmg* 1.2 promoter.

Northern blot analysis of mRNAs from potato vegetative tissues was carried out using a probe that encompassed a sequence spanning the ATG codon of *hmg* 1.2 (Fig. 4). A high level of hybridizing mRNA accumulated only in wounded tuber tissue, but the mRNA levels were suppressed if the wounded tissue was treated with elicitor (arachidonic acid) or infected with *Phytophthora infestans*. A low level of expression was found in sprouts, stems and apical buds, and *hmg* 1.2-related transcripts were not detected in untreated leaves and tubers. Due to the possibility of cross-hybridization with sequences similar to *hmg* 1.2 (Fig. 1), we can only say that none of these related genes is elicitor- or infection-inducible, and that one or more are strongly wound-inducible.

Expression of hmg *1.2 promoter-GUS gene fusions in transgenic plants*

The *hmg* 1.2 promoter was isolated as a 3.5 kb fragment from −65 bp, relative to the translation start site, to the next upstream *Hin*d III site (Fig. 2) and was transcriptionally fused with the

A)

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hmg1.1      CTTTCTCTCATATCTCTGTTTTTTTTTTCTTCTTTTCAAAACTCCGGTGT   -28
hmg1.2      AAACAGAG  C AG ACTCAAAAA  ACAAAT  GG      A A        -28
hmg1.3      AAACAGAG  C AG ACTCAAAAA  ACAAAT  GG      A          -28
hmg1.4      AAACAGAG  C AG ACTCAAAAA  ACAAAT  GG      A          -28
hmg1.5      AAACAGAG  C AG ACTCAAAAA  ACAAAT  GG      A          -28
hmg1.6      AAACAGAG  C AG ACTCAAAA   ACAAAT  GG      A          -28
hmg1.7      AAACAGAG  C AG ACTCAAAAA  ACAAAT  GG   T  A          -28
hmg1.8      AAACAGAG  C AG ACTCAAAAA  ACAAAT  GG      A          -28
hmg3.2      *************************************** TTT   TC     -28

hmg1.1      TCCTACCGGAAAATCAACTAAATTTACAATGGACGTTCGCCGGCGACCTG    22
hmg1.2                                                            22
hmg1.3                                  G              T          22
hmg1.4                                                            22
hmg1.5                                                            22
hmg1.6                                                            22
hmg1.7                                           T                22
hmg1.8           T                                                22
hmg3.2      AAA T    CG CCGG AA GT  C  G                          22

hmg1.1      TTAAGCCTCTATACACATCTAATGATGCTTCCGCCGGCGAACCTCTGAAA    72
hmg1.2                            A                               72
hmg1.3                            A               A               72
hmg1.4                            A                               72
hmg1.5                            A        T                      72
hmg1.6                            A                               72
hmg1.7                            A       T                       72
hmg1.8                            A                               72
hmg3.2       A  A  C  T   C T  CG AC CAT    T******************   58

hmg1.1      CAACAAGAAGCTTCTCCTAAAGCATCTGATGCGCTTCCACTCCCATTGTA   122
hmg1.2         G   TTT                                           122
hmg1.3         G   TTT               T                           122
hmg1.4         G   TTT                                           122
hmg1.5         G   TTT                                           122
hmg1.6         G   TTT                                           122
hmg1.7         G   TTT                                           122
hmg1.8         G   TTT                 T                         122

hmg1.1      CCTAACCAATGGGTTGTTTTTCACCATGTTTTTCTCTGTTATGTATTTTC   172
hmg1.2              C                                            172
hmg1.3              C                   G                        172
hmg1.4              C                                            172
hmg1.5              C A                                          172
hmg1.6              C                                            172
hmg1.7              C                                            172
hmg1.8              C                       -                    171

hmg1.1      TTCTCGTAAGGTGGCGTGAGAAGATCCGTAATTCTATTCCTCTTCATGTG   222
hmg1.2           A                                          C    222
hmg1.3                                                           222
hmg1.4                                                           222
hmg1.5                                               T           222
hmg1.6                                                           222
hmg1.7                                                           222
hmg1.8                                                           221
hmg1.1      GTTACCCTTTCTGAATTGTTAGCTA                            247
hmg1.2                                                           247
hmg1.3                                                           247
hmg1.4                                                           247
hmg1.5                                                           247
hmg1.6                                                           247
hmg1.7                                                           247
hmg1.8                                                           246
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B)

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hmg1.1      MDVRRRPVKPLYTSKDASAGEPLKQQEVSSPKASDALPLPLYLTNGLFFT    50
hmg1.2                                                  T         50
hmg1.3      V   ----------------------------------------------     5
hmg1.4                                                  T         50
hmg1.5                     A                            T         50
hmg1.6                                                  T         50
hmg1.7         C             F                          T         50
hmg1.8                                        Y         T         50
hmg3.2                  P EHISS*******************************    14

hmg1.1      MFFSVMYFLLVRWREKIRNSIPLHVVTLSE                        80
hmg1.2                I            A                              80
hmg1.3       V                                                    30
hmg1.4                                                            80
hmg1.5                            F                               80
hmg1.6                                                            80
hmg1.7                                                            80
hmg1.8        SLLCI FS--------------------                        59
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Fig. 1. Partial sequences of different HMGR genes of potato. *hmg* 1.1 is the *hmg* 1 gene reported by Choi *et al.* [9]. *hmg* 1.2 through *hmg* 1.8 correspond to the genomic clones λ13, λ7, λ9, λ11, λ17, λ19 and λ22, respectively. *hmg* 3.2 is the sequence obtained from our potato anther cDNA library. Unknown sequences are noted by *. **A**. Nucleotide sequences; deletion of a nucleotide is indicated by –. **B**. Deduced amino acid sequences; sequence beyond a stop codon is indicated by –.

ACATCATACTTCTCGTACACATTTTTACACAATAGTCATTACTTATTCATACCAATATCA -857

TGCCAAATAGAATATCAAATTAATTTCAACCTATAAATATTAATTATAATTTCATACAAA -797

AAAAATAGTATATCAAATTATACTTTTAAACAGTATACCAAATTCATTTCATTATTATTA -737

TCAAATTACATTCAAATCAATATTCAGACCTTAAAAAACACATTTCATACATTTTTTGGG -677

2.9

CATACATTTCATAAATTTATAACGATAACATTGTTACTATTTCATACACTGTCATTATCA -617

AGAAAATAGTATACTCAAATCATTCAAACTAAATTCAAAATCGCAAACATCCATGGTATA -557

AACAGTATACAAATTACAAAAAAATAATATACAAAAATTTCATACCAACATTTATTCAAG -497

TTTCACAAAAATTATTATGTCAGATGAACAAAAGAATGTTCATATAAAAAACATCACAAT -437

3.2

TTAAATCTATTGTACACAAACAAAATCAACAAAATTTACTTTACAGTTTCATTGTTCTTA -377

TTTTTTTTTAACGTTTCATTAATATAAAAAAATAAAGGGAAAAGGGACAGATTTACCCCC -317

"TCAGTPy"-box

GAACTTTAATAAATGGTACGTATATGCCCTCCGTTATACTTTGCGTCCACATAAGCCCCT -257

G-box

TCCGTCCAATTATAGGTACACATATACCCCTCTCACTAACGGACCCCTAATTGCTTACAC -197

H-box I

GTGTCTTATTCCTATTGAACTACCCGTTTGACTCTTTTTTTTAACCCCATAACCCAACTAT -137

II

CCACCCCATAACCCAATATCCACCCAATTTCTTAGAACAAAGTTCAACAATCACCAGAAA -77

3.5

ACAGAGCACAAGTACTCAAAATGTACAAATTTGGCAAAACACCGGTGTTCCTACCGGAAA -17

ATCAACTAAATTTACA**ATG**GACGTTCGCCGGCGACCTGTTAAGCCTCTATACACATCTAA +44

AGATGCTTCCGCCGGCGAACCTCTGAAACAAGAAGTTTCTTCTCCTAAAGCATCTGATGC +104

GCTTCCACTCCCATTGTACCTAACCACTGGGTTGTTTTTCACCATGTTTTTCTCTGTTAT +164

GTATTTTCTTCTCGTAAGGTGGCGTGAGAAGATCCGTAATTCTATTCCTCTTCATGTGGT +224

TACCCTTTCTGAATTGTTAGCTATGGTGTCATTG

Fig. 2. Nucleotide sequence of *hmg* 1.2 including the N-terminal region and 916 bp upstream of the translation initiation codon. The putative *cis*-acting sequences are boxed; the direct repeats are underlined, and the translation initiation codon is in bold. The sites of the 3′-end promoter deletions are shown by a three-sided box marked with the resultant size (kb) of the promoter fragment used in GUS fusions.

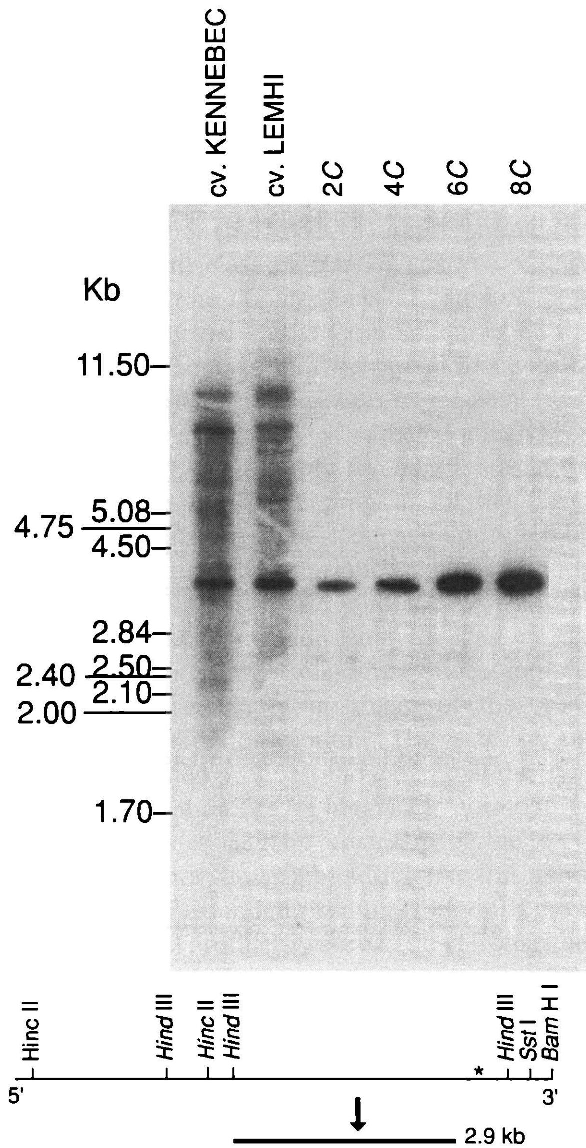

Fig. 3. Southern blot analysis of potato genomic DNA. DNA from potato cvs. Kennebec and Lemhi and pHMGR13 DNA (2, 4, 6, and 8 copy equivalents) was digested with *Hind* III, fractionated by electrophoresis and transferred to a nitrocellulose filter. The blot was probed with a 2.9 kb fragment 5′ of the ATG codon of *hmg* 1.2, washed and then subjected to autoradiography as described in Materials and methods. The restriction map indicates the origin of the hybridization probe. The star marks the transcription initiation site.

GUS reporter gene of the binary vector pBI101. The resultant plasmid (pHMGR1.2-35) was mobilized into *Agrobacterium tumefaciens* by the direct DNA transformation procedure [16]. Tobacco cv. Xanthi-nc was then transformed with *Agrobacterium* carrying the binary vector plasmid [17]. Ten transgenic plants derived from independent leaf disks and resistant to kanamycin sulfate were analyzed for expression of GUS activity. The highest levels of GUS activity were detected in anthers, roots and old petioles (Fig. 5). Lower GUS activities were detected in old leaves, young petioles, stems, ovaries and filaments. Assay of isolated pollen for GUS activity suggested that the activity in anthers was due to the pollen contained within the anthers (see inset, Fig. 5). Leaves of two independent transgenic plants, HMGR1.2-35-7 and HMGR1.2-35-8, were wounded, inoculated with tobacco mosaic virus (TMV) or treated with an emulsion of the elicitor arachidonic acid (0.5 mg/ml). Expression

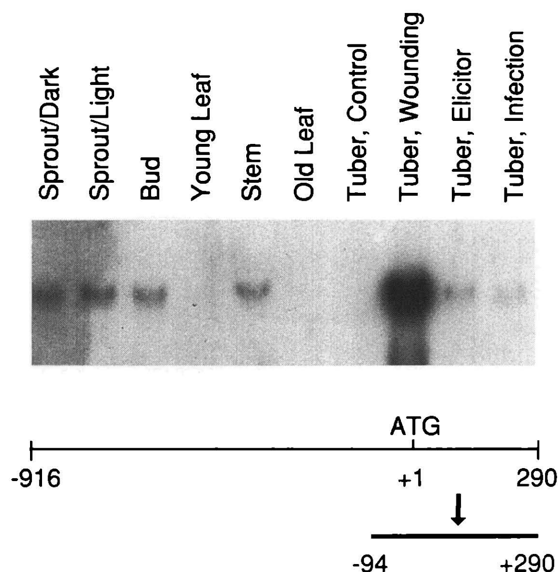

Fig. 4. Northern blot hybridization analysis of mRNAs from various vegative tissues of potato. Total RNA was extracted from tissues, fractionated by electrophoresis and transferred to nitrocellulose membranes as described in Materials and methods. The blot was probed with the fragment containing sequences around the ATG codon shown in the lower part of the figure, washed and then subjected to autoradiography.

of the *hmg* 1.2 promoter was not induced following any of these treatments (data not shown).

The *hmg* 1.2 promoter is comprised of a series of CCAAT and TATA boxes. To examine the roles of the pairs of CCAAT and TATA boxes in transcription initiation, two additional promoter-GUS fusion constructs were made. A 382 bp or 672 bp sequence was deleted from the 3′ end of the *hmg* 1.2 promoter yielding pHMGR1.2-32 and pHMGR1.2-29, respectively. For each of these two constructs, 10 tobacco plants derived from independent leaf disks and resistant to kanamycin sulfate were assayed for GUS expression in different organs as described for the pHMGR1.2-35 constructs. A much lower level of GUS expression was observed in all the organs of plants containing constructs with partially deleted promoters (Fig. 5).

Staining with the chromogen 5-bromo-4-chloro-3-indolyl glucuronic acid (X-gluc) is a much less sensitive indicator of GUS activity than the fluorescence assay, and failed to demonstrate activity in many of the tissues. Histochemical analyses of tobacco transformed with pHMGR1.2-35 constructs detected GUS activity in the mid-veins of old leaves (Fig. 6a) and in the vascular tissue of old petioles (Fig. 6b, c). Staining with X-gluc confirmed that the high level

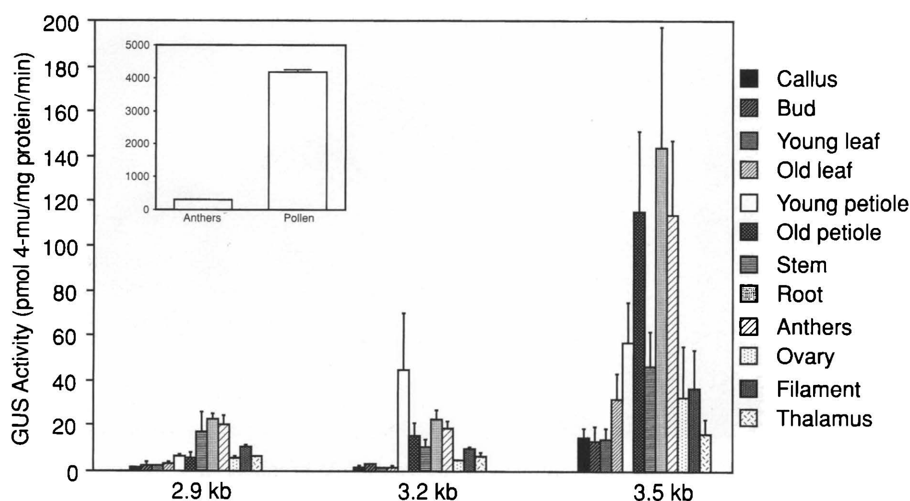

Fig. 5. Expression of *hmg* 1.2 promoter-GUS reporter gene fusions in transgenic tobacco. Fluorometric Assays for GUS activity were carried out on tissues from 10 to 12 independent transgenic plants as described in Materials and Methods. Plants containing *hmg* 1.2-35::GUS, *hmg* 1.2-32::GUS and *hmg* 1.2-29::GUS are designated by the 3.5 kb, 3.2 kb and 2.9 kb size of the *hmg* 1.2 5′ noncoding region they contain, respectively. The insert shows GUS activity for anthers and isolated pollen of *hmg* 1.2-35::GUS plants. The legend key is presented in the same order as the bars. Bars indicate the standard error.

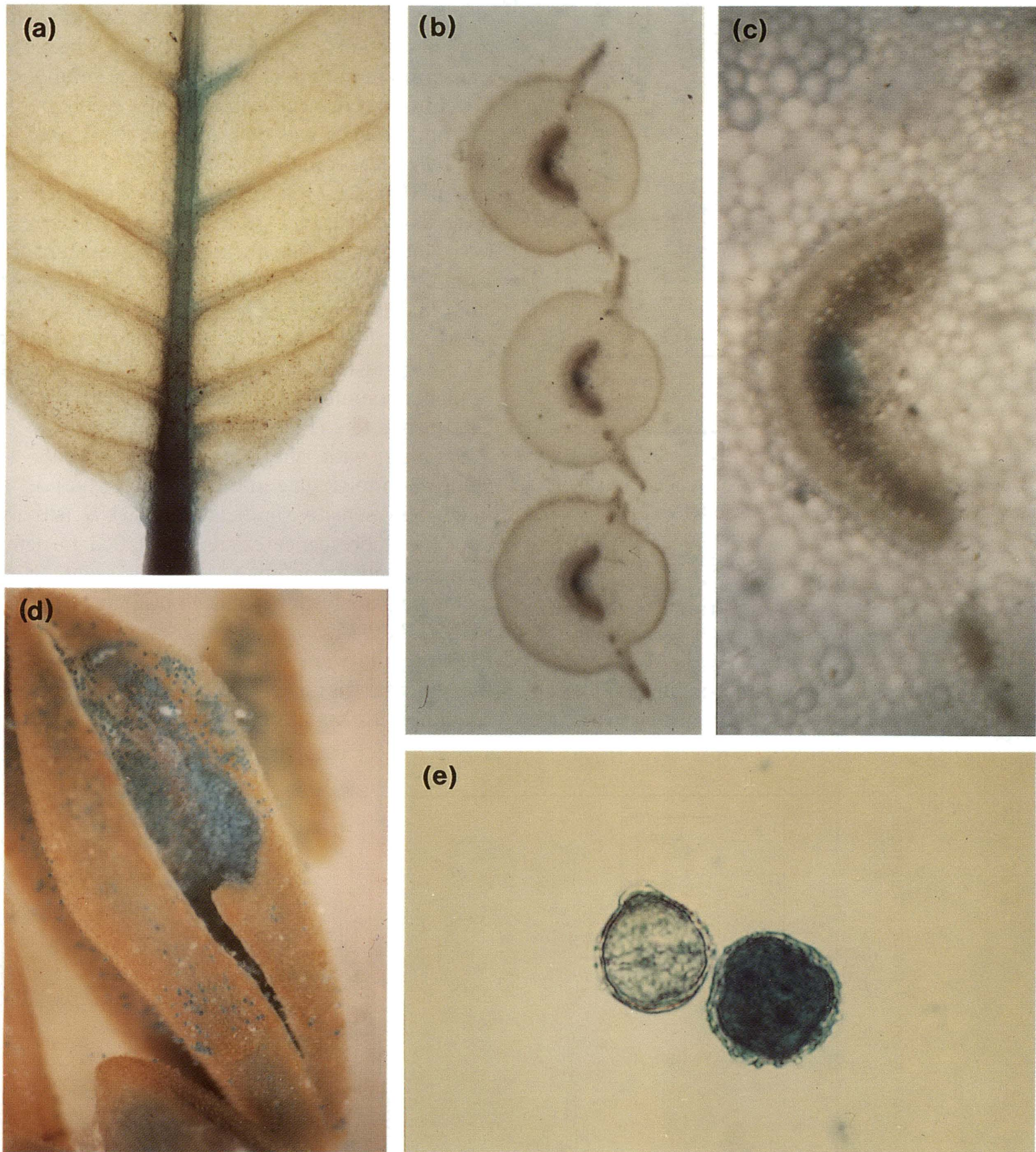

Fig. 6. Histochemical analysis of GUS expression controlled by the *hmg* 1.2-35 promoter in transgenic tobacco. Hand sectioned tissues were incubated in X-gluc from a few hours to overnight and then cleared in 70% ethanol. (**a**) young leaf; (**b**) cross section of petioles from mature leaf; (**c**) close-up of cross section of petiole from mature leaf; (**d**) anthers; (**e**) pollen.

of GUS activity in anthers was due to the activity in pollen (Fig. 6d, e). It has recently been suggested that apparent GUS expression in pollen can be an artifact of expression in the anther

tissue itself [23]. We tested this possibility by separating pollen from anther tissue, and separately stained each with X-gluc. Staining was only observed in pollen grains (Fig. 6e).

GUS activity was also measured in potato plants transformed with pHMGR1.2-35, pHMGR1.2-32 or pHMGR1.2-29. The overall results in potato paralleled those in tobacco, i.e., high levels of GUS activity were detected only in pollen. The levels of GUS activity in transgenic potato pollen were very high with the 3.5 kb fusion, as indicated by a strong blue staining with X-gluc; however, in contrast to tobacco, low levels of GUS activity were detected by X-gluc staining of potato pollen containing the 3.2 or 2.9 kb promoter fusions. GUS assays using either histochemical or fluorogenic substrates did not detect significant activity in leaves, petioles, stems, roots or tubers of transgenic potato. In addition, no induction of GUS activity was observed in any of the potato *hmg* 1.2-GUS transformants when tissues were wounded or treated with arachidonic acid, kinetin or methyl jasmonate (data not shown).

Table 2. Segregation of T-DNA in R_2 pollen carrying the *hmg* 1.2-35::GUS gene fusion.

R_1 progeny plant[1]	Color of pollen (R_2)[2]		Predicted ratio	T-DNA copy[3]	χ^2
	blue	white			
8-0-1-3	304	0	1:0	4	0[4]
8-0-1-7	329	0	1:0	4	0[4]
8-0-1-8	242	82	3:1	2	0.008[5]
8-0-1-12	288	0	1:0	3	0[4]

[1] R_1 progeny plants were derived by selfing the *hmg* 1.2-35-8 plant (R_0) which contained two functional T-DNA copies in separate loci.

[2] Pollen were stained for GUS activity with X-gluc as previously described [19].

[3] Number of *hmg* 1.2-35::GUS genes contained in R_1 progeny as determined by Southern blot analysis. The genomic DNA was digested with *Hin*d III and DNA blots were hybridized to a GUS coding sequence probe and washed at 65 °C in 2× SSC and then in 0.1× SSC. A non-specific 8 kb band which hybridized to the GUS probe under low-stringency washing conditions (2× SSC) was used as an internal control to estimate the copy number of the specific GUS bands representing the two T-DNA loci T_1 and T_2. Plants 8-0-1-3 and 8-0-1-7 contained two T-DNA copies in each of the two loci T_1 and T_2. Plant 8-0-1-8 contained only one T-DNA copy in each of the two loci (both loci were heterozygous). Plant 8-0-1-12 contained one copy in T_1 and two copies in the T_2 locus.

[4] Denotes significant fit at $P > 0.95$.

[5] Denotes significant fit at $P > 0.90$.

HMGR *expression in pollen grains*

Anthers from the R_1 tobacco plant 8-0-1-3 (see Table 2) containing the *hmg* 1.2-35::GUS fusion were dissected from flower buds and flowers at different stages of development. The different stages are termed 1 to 5, in which stage 1 represents the immature flower bud and stage 5 represents the oldest flower. The anthers were cut longitudinally and stained with X-gluc overnight at 37 °C in the dark. Pollen grains could be expressed from the anthers by mild pressure at all stages except stage 1. Pollen grains from immature buds (stage 1) were white or light yellowish in color after staining with X-gluc; a blue color first developed in some of the pollen grains from stage 2 flowers. The pollen of fully open flowers (stage 4), especially that of the oldest flowers (stage 5) stained dark blue suggesting a high level of *hmg* 1.2 gene expression. At each developmental stage there was a positive correlation of % blue pollen with the GUS activity and the endogenous HMGR activity in anthers of the transgenic plant studied (Fig. 7).

The pollen grains of the R_1 tobacco plants segregated for GUS activity indicating that the genome of the pollen (R_2 microspores after meiosis) controlled GUS expression. If the R_1 sporophytic tissue, i.e., surrounding anther tissue, controlled GUS expression in the pollen, then all the pollen should be GUS positive. Segregation analysis for *hmg* 1.2-35::GUS was carried out to confirm that the genome of the pollen was controlling expression of the fusion gene. Seeds from the transformed plants were germinated on MS medium containing kanamycin sulfate, and the selected resistant seedlings were grown to flowering in the greenhouse. The pollen of 4 R_1 plants de-

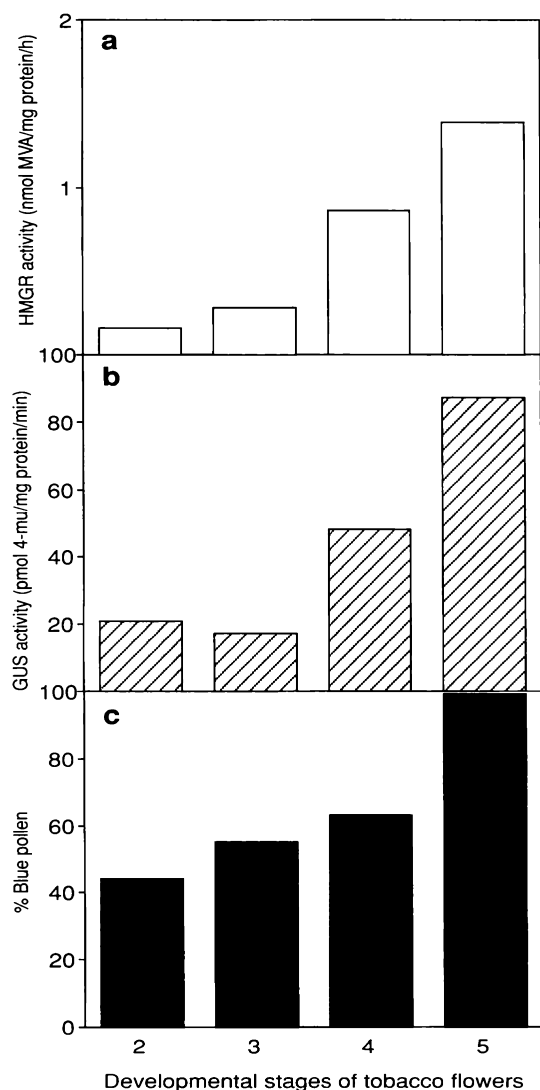

Fig. 7. Correlation of *hmg* 1.2-35::GUS expression with HMGR enzyme activity in tobacco anthers. Flowers, of a R_1 *hmg* 1.2-35-8 progeny (8-0-1-3, see Table 2) homozygous for the T-DNA insert containing the fusion gene, were collected at five developmental stages, from immature flower bud (1) to fully expanded flower (5), and the anthers dissected out for enzyme assays. The anther tissue, containing the pollen, was homogenized and assayed for HMGR activity (**a**) or GUS activity (**b**). Additional anthers were stained with X-gluc and the percentage of total pollen that were GUS-positive (% blue) was determined (**c**). Assay of GUS activity and histochemical staining were carried out as in Materials and methods.

rived from primary transformant HMGR1.2-35-8 (which carried two T-DNA inserts in different loci) were scored for segregation into blue and white pollen grains. The expected haploid segregation ratios for a single locus is 1:1 and for 2 loci is 3:1. Depending upon the genotypes of the R_1 plants the haploid pollen showed segregation in a 3:1 ratio (two heterozygous loci, each with one T-DNA copy segregrating independently), or there was no segregation for GUS activity (R_1 homozygous for T-DNA at one or both loci) (Table 2). The blue staining indicating GUS activity was not seen in pollen from transgenic tobacco transformed with promoter deletion constructs pHMGR1.2-32 or pHMGR1.2-29, or the promoterless pBI101. Thus, the expression of the *hmg* 1.2-35::GUS gene fusion is controlled by the genotype of the pollen grains (haploid microspores resulting from meiosis) rather than by the genotype of the parent R_1 plant (diploid anther tissue).

Analysis of the hmg *1.2-35::GUS transcript*

Two cDNA libraries were constructed from cv. Kennebec potato anther tissue which included pollen. The first library was constructed in Lambda Uni-ZAP. This library was screened using a probe representing sequences from −40 to + 250 of *hmg* 1.2 (Fig. 2). Of one hundred positive clones isolated from ca. 5×10^5 pfu, 15 representative clones were sequenced. Sequences of all 15 clones were identical to each other and *hmg* 3.2, a sequence having a high degree of identity to the *hmg* 3 subfamily (Fig. 1). The second cDNA library was constructed using a HMGR N-terminus specific primer in the λZAP vector and was screened using a probe representing −116 to + 5 nt of the *hmg* 1.2 sequence. Only one clone was obtained after screening ca. 3×10^5 pfu of this HMGR gene specific library, and it corresponded to *hmg* 1.1.

Poly(A)$^+$ RNA from the pollen-containing anther tissue of transgenic tobacco containing the *hmg* 1.2-35::GUS gene fusion was analyzed by northern blot hybridization with probes from the 5′ noncoding regions of *hmg* 1.2 (Fig. 8). No transcripts from *hmg* 1.2-35::GUS were detected

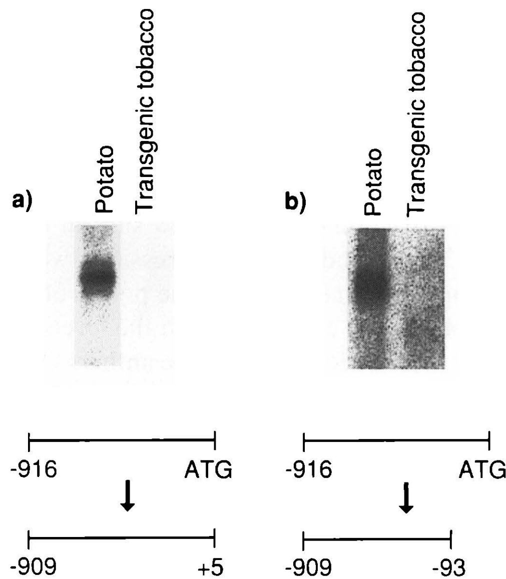

Fig. 8. Northern blot analysis of poly(A)$^+$ RNA from potato and *hmg* 1.2-35::GUS transgenic tobacco. Poly(A)$^+$ RNA from potato (cv. Kennebec) or transgenic tobacco anthers was separated on agarose-formaldehyde gels and then transferred to nitrocellulose membranes essentially as described by Maniatis *et al.* [22]. **a)** The probe used was the *hmg* 1.2 sequence from −909 to +5 relative to the ATG codon, which hybridizes to transcripts of *hmg* 1.1 through *hmg* 1.8 but not *hmg* 3.2. **b)** The probe used was as in the left figure but the coding and proximal 93 bp upstream were deleted, preventing hybridization to transcripts of both *hmg* 1.1 and *hmg* 3.2 but allowing detection of *hmg* 1.2 through *hmg* 1.8.

in tobacco with either of the two probes used or with GUS-coding sequences as a probe (data not shown). We were unable to detect the fusion transcript despite the expression of GUS activity in the pollen; apparently, this transcript is present at very low levels in the transgenic pollen, perhaps due to effects on the transcription, processing or stability of the fusion transcript, or presence of transcripts during a very short time window. The probe did not hybridize to the endogenous mRNAs of tobacco presumably because the non-coding potato sequence used for the probe was too divergent from the tobacco *hmg* genes. However, the probe in Fig. 8a did hybridize to mRNA from the anthers of potato representing *hmg* 1.1 (the strong band contained within a diffuse band). The diffuse band observed in both Fig. 8a and 8b may represent multiple transcription initiation sites for the *hmg* 1.2 gene or genes closely related to *hmg* 1.2.

Discussion

Presence of a complex hmg *1 subfamily of* HMGR-*genes in potato*

Screening a potato genomic library with a probe corresponding to a conserved region of the *HMGR* open reading frame [31] resulted in the isolation of 18 *HMGR* genomic clones of which 14 were selected for further study and placed into 7 classes (Table 1). Representatives of the 7 classes were very similar to each other at the nucleotide level, differing only in isolated point mutations, except for *hmg* 1.8 which contained a single deletion in the 5′-upstream region. It is not clear why our library screening appeared biased towards the isolation of a particular subfamily (*hmg* 1) of *HMGR* genes, and indeed a particular class (*hmg* 1.2, Table 1) within that subfamily. One possible explanation is the presence of introns in the region of the *HMGR* genes homologous to the 442 bp cDNA probe (Pot 17) used for screening. This region of the *Arabidopsis HMGR* gene contains two introns [21]. Interruption of the region of homology between probe and target, coupled with varying levels of mis-match between the probe and the various *HMGR* genes, could account for our failure to isolate all members of the potato *HMGR* family. The PCR-generated *HMGR* sequence Pot 17, which corresponds to arachidonic acid-induced transcripts, is only 70% identical to the *hmg* 1 clones isolated. Pot 17 corresponds to the *hmg* 2 subfamily, according to a recent classification of potato *HMGR* genes [9].

All 7 classes of genomic HMGR clones presented here are grouped with *hmg* 1, but not *hmg* 2 or *hmg* 3, of potato. The sequences of plant HMGRs are less well conserved in their N-terminal region compared to the C-terminal region, which contains the catalytic site [4]. Nevertheless, there is a high degree of identity between the N-terminal region encoded by the genomic clones described here and the *hmg* 1 (*hmg* 1.1)

gene described by Choi *et al.* [9], suggesting that these genes are members of a *hmg* 1 subfamily of *HMGR* genes in potato. In support of this notion, the *hmg* 1-specific probe described by Choi *et al.* [9] hybridized with members of all 7 classes of HMGR described in Table 1 (data not shown). Furthermore, northern blot hybridization experiments using a *hmg* 1.2 probe failed to demonstrate induction in response to either elicitor or infection, but did demonstrate wound induction of one or more members of this class (Fig. 4). This is consistent with the expression pattern of the *hmg* 1 subfamily. Southern blot hybridization analysis with the same *hmg* 1-specific probe suggested the presence of 7 or 8 different *hmg* 1 genes [29], in contrast to the 1 to 3 reported by Choi *et al.* [9]. Our sequence analysis of genomic and cDNA clones confirms the presence of at least 8 different members of the *hmg* 1 subfamily.

The diverse functions of isoprenoid compounds in potato are consistent with the existence of an *HMGR* multigene family, members of which could potentially encode different isoforms of the enzyme with different tissue-specific expression patterns and possibly different biochemical regulation. Multigene families for HMGR have been observed in other plant species [7, 10, 24]. Because potato cv. Lemhi is a tetraploid species and propagated asexually, some of the multigenes may represent allelic variants. Also, one or more of the *HMGR* genes describe here may not be expressed. *hmg* 1.3 and *hmg* 1.8 contain stop codons in their coding regions, indicating that they are not translated into functional proteins. Furthermore, we have been unable to isolate cDNAs corresponding to the genomic clones reported here.

Pollen-specific expression of hmg *1.2::GUS*

The haploid pollen grains (male gametophytes) develop from pollen mother cells through meiosis. Although only a single cell, around 20 000–24 000 genes are transcribed in the haploid pollen grain. Of these, 10–20% are expressed specifically only in pollen [32]. *Hmg* 1.2-35::GUS is expressed to a very high level in pollen grains. Several lines of evidence indicate that this high level of GUS expression is a true representation of the pollen-specific expression of *hmg* 1.2 rather than an artifact. First, isolated pollen grains stain blue with X-gluc, indicating that the GUS activity is contained in the pollen and does not result from diffusion of reaction products from sites in the anther [23]. Second, GUS expression, which is developmentally regulated in the pollen of transgenic tobacco, is correlated with the levels of endogenous HMGR activity in the anthers (Fig. 6). Third, deletions of the *hmg* 1.2 promoter dramatically reduced expression of the promoter-GUS gene fusion in pollen, indicating the unlikelyhood that a cryptic promoter in the coding region of the GUS gene is controlling expression, as has been recently suggested [33, 34]. Last, segregation analysis of the expression of *hmg* 1.2-35::GUS among the R_2 pollen of R_1 progeny plants (Table 2) demonstrates that GUS expression is controlled by the genotype of the pollen, not the genotype of the parental plant tissues.

Transcript analyses by cDNA cloning and northern blots indicate that *hmg* transcripts from at least two genes are present in the pollen-containing anthers. Of these two, *hmg* 3.2 is more abundant than *hmg* 1.1. A putative third gene or genes similar or identical to *hmg* 1.2 may also express in anther tissue to a very low level (faint diffuse band in Fig. 8a and 8b). The high level of pollen-specific expression of *hmg* indicates an important role for mevalonate in pollen, perhaps in carotenoid or sterol biosynthesis.

Putative cis-*acting elements and* hmg *1.2 expression*

The 5′ sequence flanking the coding region of *hmg* 1.2 contains a consensus G-box (CACGTG) [13], a TCAGTPy box [1] (identical to one present in a *Hevea* HMGR promoter [10]) and a variant of the H-box (consensus CCTA-CC(N7)CT) [39]. Sequences upstream of these three boxes include multiple TATA motifs, which are believed necessary to initiate transcription in most eukaryotic genes [5], but no TATA

sequences are found downstream of the three putative *cis*-acting sequences. This downstream region also lacks GC-rich (CCGCCC or GGGCGG) hexanucleotide sequences which are important positive elements in the promoters of animal HMGRs [26]. In addition to the H, G and TCAGTPy boxes, two 12 bp direct repeats (CCCATAACCCAA) 20 bp apart are located downstream from the H box. Deletion of this region from the untranslated leader sequence of the *hmg* 1.2-GUS fusion strongly reduced the expression of GUS activity in all organs tested, including pollen grains. Interestingly, the recent study of a *Camptotheca acuminata* HMGR promoter in GUS gene fusions expressed in transgenic tobacco showed that a fragment containing the −165 bp 5′ of the transcriptional start site was sufficient to confer developmental and environmental regulation [6]. This promoter fragment did not contain a H box, G box or the 12 bp direct repeats; however, two consensus TCAGTPy boxes were present on the complementary DNA strand. Recent work has shown that mutations in the untranslated leader sequence of the maize Adh1 gene can change its pollen-specific expression by altering mRNA levels, suggesting elements in the untranslated leader sequence can be important for expression [11]. Another possibility is that unknown *cis* elements in the leader sequence could increase translation; thus, deletion of these putative elements would also reduce GUS expression. Further deletional and mutational analyses will be necessary to evaluate the role, if any, of the putative *cis*-acting motifs or repeat element in *hmg* 1.2 expression, particullarly since the first two C residues of the H box were shown to be necessary for binding [39], not AC as here.

In summary, the results presented here indicate that HMGR in potato is encoded by a complex family of genes with several interesting features. *hmg* 1 represents a subfamily of genes containing at least 9 members, 7 corresponding to genomic clones and 2 identified as cDNA clones. This appears to be considerably larger than either the *hmg* 2 or *hmg* 3 subfamilies. The presence of a large *HMGR* gene family has not been reported for other plants; however potato may have evolved multiple copies of certain genes for some yet to be discovered advantage. For example, phenylalanine ammonia-lyase (PAL), which catalyzes the first committed step of phenylpropanoid synthesis, is encoded by 40–50 genes per haploid genome in potato [20]. Not only do our results extend the previously reported number of *HMGR* genes in potato, but the promoter fusion studies demonstrate that the promoter of a member of the *hmg* 1 family (*hmg* 1.2) is able to drive high levels of GUS expression in the pollen of transgenic plants.

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