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Isolation and Characterization of cDNAs Encoding Wheat 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase

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The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC 1.1.1.34) is a key enzyme in the isoprenoid biosynthetic pathway. We have isolated partial cDNAs from wheat (*Triticum aestivum*) using the polymerase chain reaction. Comparison of deduced amino acid sequences of these cDNAs shows that they represent a small family of genes that share a high degree of sequence homology among themselves as well as among genes from other organisms including tomato, *Arabidopsis*, hamster, human, *Drosophila*, and yeast. Southern blot analysis reveals the presence of at least four genes. Our results concerning the tissue-specific expression as well as developmental regulation of these HMGR cDNAs highlight the important role of this enzyme in the growth and development of wheat.

The enzyme HMGR (EC 1.1.1.34) catalyzes the synthesis of mevalonate, the precursor of isoprenoid compounds that give rise to a number of products, including growth regulators, photosynthetic pigments, phytoalexins, steroid glycoalkaloids, and sterols (Garg and Douglas, 1983).

HMGR has been extensively studied in mammals, in which it controls cholesterol biosynthesis (for review, see Sabin, 1983). cDNAs and genomic HMGR clones have been isolated from *Drosophila* (Gertler et al., 1988), yeast (Basson et al., 1988), sea urchin (Woodward et al., 1988), hamster (Chin et al., 1984), and human (Luskey and Stevens, 1985). Recently, with the use of hamster and yeast probes plant HMGR cDNAs and genomic clones have been isolated from *Arabidopsis* (Caelles et al., 1989; Learned and Fink, 1989), tomato (Narita and Gruissem, 1989; Narita et al., 1991), and *Hevea* (Chye et al., 1991). Sequence similarities among HMGR genes from different organisms are evident in the catalytic site regions.

We are interested in examining the regulation and the level of expression of wheat (*Triticum aestivum*) HMGR genes because of the potential uses of their molecular components. For example, the *cis*-regulatory element of their promoters can be utilized in future genetic engineering applications that require similar tissue-specific or developmental regulation. As a first step in characterizing the spatial and temporal regulation of this enzyme in wheat, we have isolated wheat HMGR cDNAs using PCR with primers designed specifically for the conserved gene sequences. In the studies described here, we examined the levels of HMGR mRNA in different tissues, as well as in different sections of seedlings, to determine the extent to which its gene expression is regulated. We demonstrate here that HMGR genes show not only tissue-specific gene expression but also developmental gene regulation.

MATERIALS AND METHODS

Plant Materials

Triticum aestivum L. cv Yecora rojo was grown in a growth chamber under a 12-h light regimen unless otherwise mentioned. Total RNA was prepared from 7-d-old wheat seedlings by the method of Chomczynski and Sacchi (1987) or that of Chirgwin et al. (1979). Poly(A)$^+$ RNA was isolated from the total RNA using oligo(dT)-cellulose columns (Pharmacia). First-strand cDNA was synthesized from total RNA (5 μg) using oligo(dT)-primer and Moloney murine leukemia virus reverse transcriptase (Maniatis et al., 1989). Dissection of apical domes was carried out under a dissecting microscope to ensure removal of leaf and root primordia, leaving apical domes intact.

PCR Amplification of HMGR cDNA

Three sense-strand primers (Nos. 73, 116, and 117) and one antisense-strand primer (No. 118) were constructed to the conserved active site regions of tomato, yeast, hamster, and *Drosophila* (Narita and Gruissem, 1989). Primer number 73 is a degenerate primer coding for NVLDYL (20 mer), number 116 coding for DKKPAAVNW (27 mer), number 117 coding for GDAMGMNM (24 mer), and number 118 coding for TMPSIEVGT (26 mer), respectively. One-tenth of the cDNA was mixed with the above primers and PCR reagents (Perkin-Elmer Cetus) following the manufacturer's instruc-

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Abbreviations: HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; PCR, polymerase chain reaction; SSU, Rubisco small subunit.

tions. Thirty cycles of PCR were done under the following conditions: annealing at 45°C, 2 min; extension at 72°C, 2 min; denaturation at 94°C, 1 min. For amplification of HMGR cDNAs using the specific primers, the annealing temperature was increased to 55°C. DNA fragments were analyzed by agarose gel electrophoresis.

Cloning and Characterization of cDNA

The PCR products were blunt-end ligated into the *Sma*I site of Bluescript plasmid (Stratagene) and sequenced directly in the double-stranded plasmid DNA form by the standard dideoxy chain-termination method using Sequenase (United States Biochemical). Subsequently, selected clones were subcloned into the pGEM.4Z vector (Promega) to generate riboprobes. Hybridization and washes were done by the standard method (Maniatis et al., 1989). Sequence analysis was done using the PC/GENE program (IntelliGenetics).

RNA Blot Analysis

For a northern blot, 10 μg of total RNA were denatured at 50°C in the presence of formaldehyde, separated by electrophoresis in a 1.2% agarose gel, and blotted onto Nytran filters (Schleicher & Schuell). For a dot blot, 1 to 10 μg of total RNA were blotted onto a Nytran filter using a manifold (Schleicher & Schuell). The inserts from HMGR 18 cDNA or wheat SSU were gel purified and nick translated in the presence of ^{32}P-labeled deoxynucleotide triphosphates. Riboprobes were made according to the manufacturer's recommendation (Promega). Hybridization and washes for the nick-translated probe were done by the standard method (Maniatis et al., 1989); however, for riboprobes, the hybridization conditions were 5× SSPE, 50% formamide, 5× Denhardt's solution, 1% SDS, 100 μg mL^{-1} tRNA at 55°C for 18 h, and washes were 2× SSPE, 0.1% SDS twice at 50°C for 30 min each and then 0.1× SSPE, 0.1% SDS twice at 65°C for 30 min each.

Southern Blot Analysis

Genomic DNA was isolated from wheat seedling using the cetyltrimethylammonium bromide method of Metler (1979). Southern blots were probed with ^{32}P-labeled, randomly primed insert DNA prepared from HMGR 10, 18, and 23 cDNA clones following the manufacturer's suggested protocol (Boehringer Mannheim Biochemicals).

RESULTS

Isolation and Characterization of HMGR cDNA by PCR

PCR amplification of cDNA prepared from wheat seedlings by the use of degenerate primers produced distinct DNA bands on a 1% agarose gel (Fig. 1). The primer number 116 (lane 3) produced a major PCR product of about 0.35 kb. In contrast, the other two primers, numbers 117 and 73, produced multiple nonspecific fragments in addition to the major PCR products (lanes 4 and 5). Sequencing of these putative cDNA clones and the comparison of their nucleotide sequences to HMGR genes from other organisms indicated that they all share a high degree of homology. Sequenced wheat cDNAs were grouped into four sets (HMGR 6, 10, 18, and 23; however, HMGR 6 was lost during cloning) based on restriction endonuclease analysis and sequence homology (Fig. 2). At the amino acid level, the similarity of each cDNA relative to HMGR 18 is 85% for HMGR 6, 82% for HMGR 10, and 91% for HMGR 23. The arrows show the position of PCR primers used for cDNA synthesis. The degree of sequence homology among the three cDNAs is very high, as indicated by the shading. Figure 3 shows sequence homology comparison among HMGR genes by computer alignment of the translated wheat HMGR 18 partial cDNA sequence with that from *Arabidopsis* (Caelles et al., 1989; Learned and Fink, 1989), tomato (Narita and Gruissem, 1989; Narita et al., 1991), human (Luskey and Stevens, 1985), yeast (Basson et al., 1988), and *Drosophila* (Gertler et al., 1988).

Genomic Southern analysis showed that there are four genes encoding HMGR in wheat. The pattern of DNA blot hybridization is presented in Figure 4. The restriction enzymes used in this analysis cleave genomic DNA outside of the sequence recognized by the probes. Therefore, the probes should only recognize one unique DNA fragment per gene, unless by chance the genomic sequence contains an intron with one of the restriction enzyme sites. We know this is not the case for HMGR 10. Our results suggest that there are at least four copies of HMGR in the wheat genome, the same number of genes found in tomato (Narita et al., 1991). The result is also consistent with the four sets of cDNAs we obtained by the PCR method as well as with hybridization patterns of the cDNAs to RNA blots under very stringent conditions (data not shown).

Tissue-Specific HMGR Expression

To distinguish three HMGR genes that share sequences of homology, we designed gene-specific primers to regions of

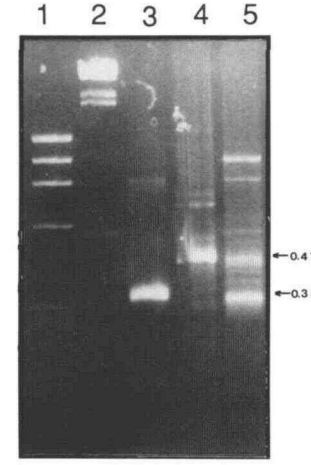

Figure 1. HMGR PCR products. cDNA templates were derived from wheat seedling RNA and the degenerate PCR primer sets. Reaction A used primers 116/118, reaction B used 117/118, and reaction C used 73/118. Expected lengths (kb) are about 0.35, 0.47, and 0.33, respectively. Lane 1, ϕX174 RF DNA *Hae*III digest; lane 2, λDNA *Hind*III digest; lane 3, PCR reaction A; lane 4, PCR reaction B; lane 5, PCR reaction C. The relative positions of the PCR primers are shown in Figure 3.

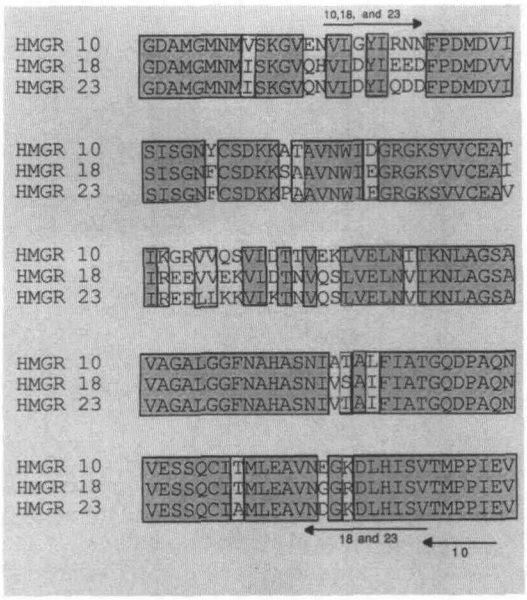

Figure 2. Homology among wheat HMGR cDNAs. Computer alignment of the amino acid sequences derived from the wheat HMGR partial sequences. Residues that are identical or conserved are shown in gray, and the residues that are neither identical nor conserved are shown in white. The numbered arrows indicate the position of gene-specific HMGR primers used for differential PCR analysis.

least homology (Fig. 2). When we tested the amplification by these primers of plasmids containing cDNA inserts at a relatively high-stringency annealing temperature (55°C), only the specific gene of interest was amplified and no others. In addition, diagnostic restriction endonuclease analysis was carried out to confirm the PCR results (data not shown). Figure 5 shows gene-specific differential accumulation of HMGR mRNA in wheat anther, seedling, apical dome, root, and leaf as reflected by PCR-amplified products. HMGR 10 and 18 are more amplified in apical dome, whereas HMGR 23 is undetectable. On the other hand, HMGR 23 is the predominant, amplified product in the root. Even though it is difficult if not impossible to quantitate RNA abundance adequately from PCR results, the data suggest that, in general, HMGR is relatively abundant in many rapidly growing tissues such as immature anther and apical dome. This abundance was also reflected in the relative number of cDNA clones isolated from our anther cDNA library (K. Aoyagi, unpublished observation). We have found at least one member of the HMGR family expressed in all the tissues we examined thus far.

Because template amplification suggested that HMGR mRNA is relatively abundant in rapidly growing tissues, we compared the level of mRNA in callus to that in leaf. Figure 6 shows a significantly higher level (50-fold for HMGR 10, 20-fold for HMGR 18 and 23) of HMGR mRNA in callus than in leaf. To confirm the PCR results suggesting higher abundance of HMGR 18 mRNA in meristematic tissues, we have carried out northern blot analysis using a wheat SSU probe as a control. Total RNA isolated from microdissected apical dome sections (see "Materials and Methods") or the whole leaf were compared for the accumulation of HMGR 18 and SSU in the light and the dark (Fig. 7). Our result shows that HMGR mRNA is abundant in meristem-enriched apical dome section grown in the light. In contrast, the level of SSU mRNA is the highest in whole green leaf, and it is reduced in etiolated leaf. Because meristem sections do not contain chloroplasts, it was not unexpected that SSU mRNA was not detectable.

Developmental Gene Expression

To further investigate whether higher levels of HMGR gene expression are associated with rapidly growing cells, the wheat leaf was chosen as a model system. Because all cell division in wheat occurs in the basal meristem, the region above the meristem provides a gradient of cellular development. We have studied the levels of HMGR mRNA in leaf sections. The first section at the base of the leaf is the youngest, and the tip section is the oldest (Fig. 8). Our results indicate that HMGR 18 and 23 are not only developmentally regulated but also regulated by light.

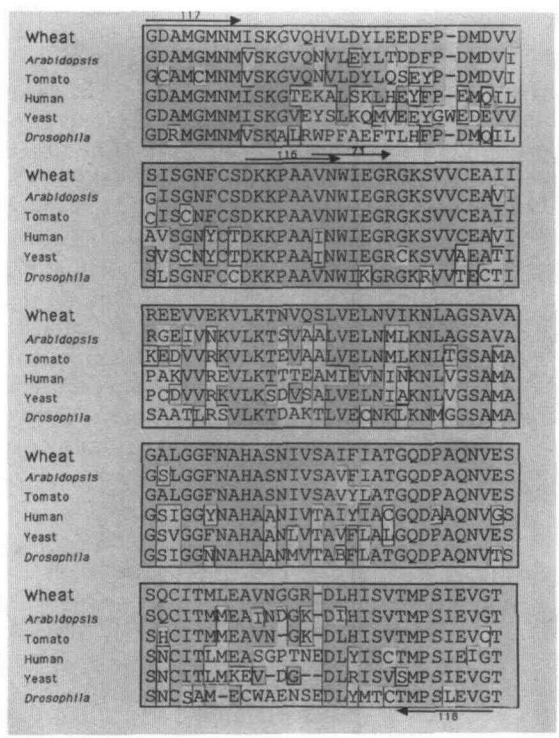

Figure 3. Sequence homology among HMGR genes. Computer alignment of the translated wheat HMGR 18 partial cDNA sequence with HMGR amino acid sequences from *Arabidopsis* (Caelles et al., 1989; Learned and Fink, 1989), tomato (Narita and Gruissem, 1989), human (Luskey and Stevens, 1985), yeast (Basson et al., 1988), and *Drosophila* (Gertler et al., 1988). Relative to the wheat sequence, identical or conserved residues are shown in gray and residues that are neither identical nor conserved are shown in white. The numbered arrows indicate the highly conserved regions used to design degenerate HMGR PCR primers.

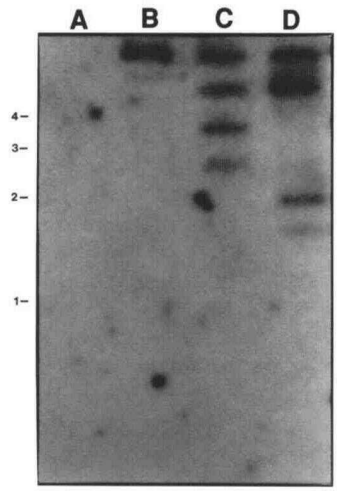

Figure 4. Southern blot analysis of wheat genomic DNA. Wheat (cv Mustang) genomic DNA (5 μg per lane) was digested with restriction enzymes, and the Southern blot was hybridized to a mixture of random-primed HMGR PCR fragments. Lane A, Uncut DNA; lane B, *Afl*II; lane C, *Eco*RV; lane D, *Afl*II and *Eco*RV. Length standards are given on the side: 4, 3, 2, and 1 kb, respectively, from the top to the bottom.

DISCUSSION

We have isolated partial HMGR cDNAs from wheat by using three 5′-PCR primers, numbers 73, 116, and 117 (see "Materials and Methods"). All three primers in combination with the 3′ primer number 118 gave rise to HMGR cDNA products, but the greatest degree of specificity was observed with primer number 116 (Fig. 1). There are probably two reasons for this. One possible reason for this is that primer number 116 was designed to match the region of the highest homology among all the organisms previously studied and especially among plants (Fig. 3). Another reason is that we incorporated inosine in place of using all possible nucleotides when there were multiple choices. Narita and Gruissem (1989) used an oligonucleotide probe that consisted of 50

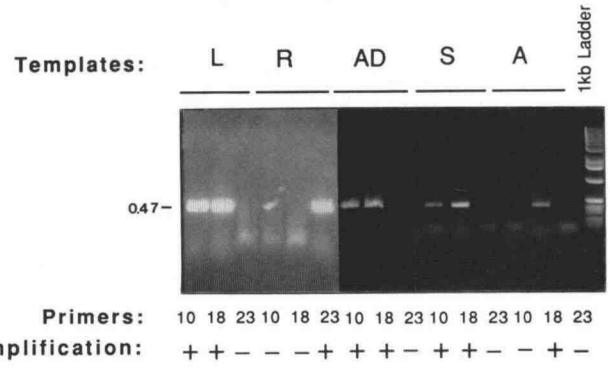

Figure 5. Tissue-specific gene expression in wheat. RNA from anther (A), apical dome (AD), leaf (L), root (R), and seedlings (S) was used to make cDNA templates for differential PCR amplification. See Figure 2 for positions of the gene-specific PCR primers. The arrows indicate the amplified HMGR cDNA (0.47 kb).

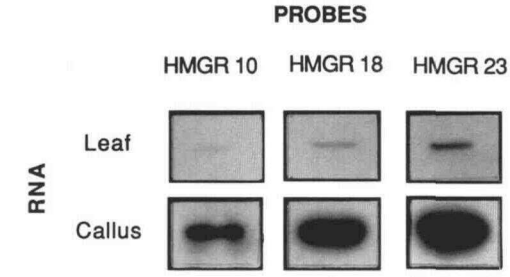

Figure 6. HMGR gene expression in callus. Total RNA (1 μg) was immobilized on Nytran filters and hybridized to HMGR 10, 18, and 23 ^{32}P-labeled riboprobes. Exposure times for the autoradiography were 1 d for HMGR 18 and 23 and 3 d for HMGR 10.

residues within the same region as our primer number 116. Perhaps the same primer set will be helpful in the cloning of HMGR genes from other plants in the future.

We have shown that the cDNAs that we isolated share a high degree of homology among themselves as well as with HMGR sequences from other organisms (Figs. 2 and 3). This high degree of similarity among these cDNAs is probably due to the constraints dictated by the nucleotides coding for the conserved catalytic site region. This similarity extends to genomic clones in which all three introns interrupt the coding sequence at the same position (data not shown). Based on the genomic Southern blot analysis (Fig. 4), there seem to be at least four genes in wheat. Hybridization with each individual HMGR cDNA would have been interesting in providing information regarding specific gene fragments on the Southern blot, but in our judgment these cDNA probes were too closely related to distinguish one gene from another.

We have examined tissue-specific gene expression of

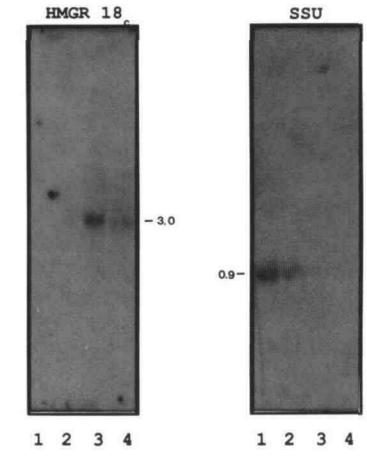

Figure 7. HMGR and SSU in meristem-enriched tissue versus whole leaf. A northern blot was prepared in duplicate and probed with nick-translated HMGR 18 or SSU. Total RNA (10 μg) from the following tissues was loaded per lane: lane 1, green leaf; lane 2, etiolated leaf; lane 3, green meristem enriched; lane 4, etiolated meristem enriched. The exposure time of autoradiography was 24 h for the SSU probe and 3 d for the HMGR 18 probe. The sizes of HMGR and SSU mRNA are about 3 and 0.9 kb, respectively.

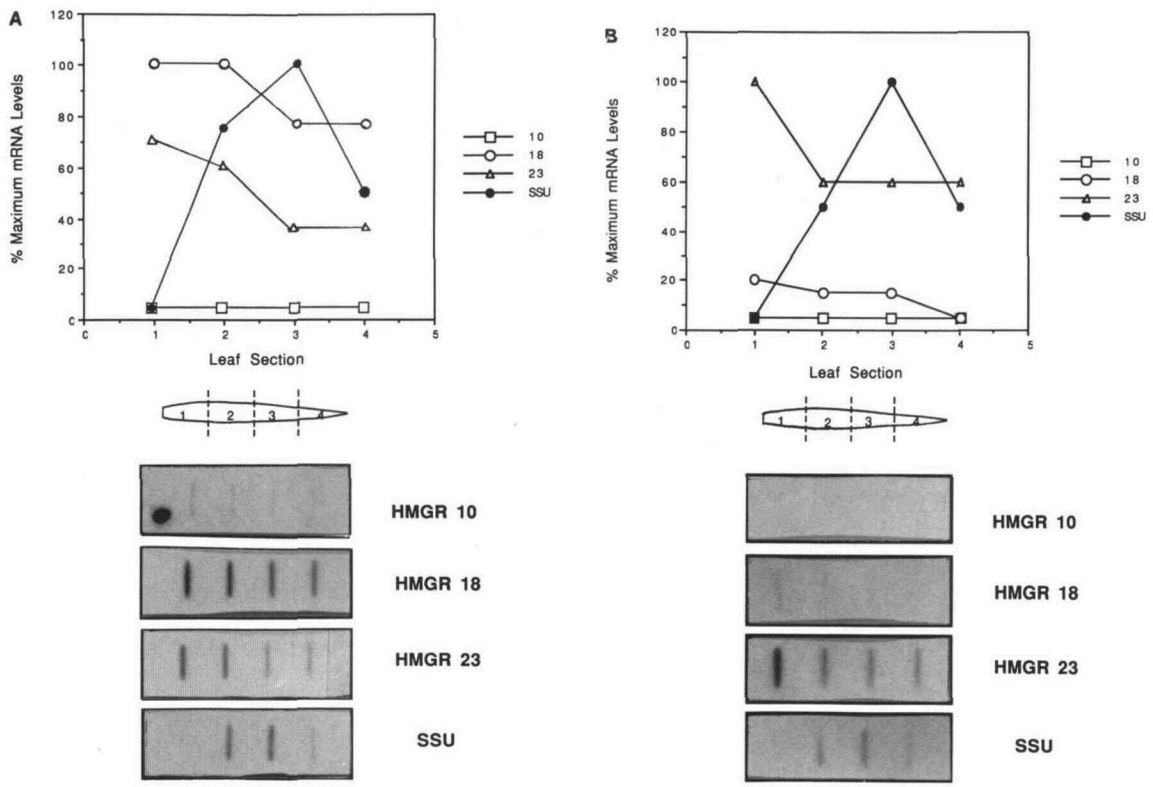

Figure 8. Developmental gene expression of HMGR in seedlings. Leaf sections were made from 8-d-old seedlings grown in light (A) or in dark (B) from the base to the tip along the age gradient (section 1, 0–2.5 cm; section 2, 2.5–5.0 cm; section 3, 5.0–7.5 cm; section 4, 7.5–10.0 cm). HMGR mRNA levels were estimated by slot blot analysis of total leaf RNA (1, 3, and 10 μg per well) using ^{32}P-labeled HMGR 10, 18, and 23 cDNA as probes. As a control, a wheat SSU probe (Broglie et al., 1983) was used on the blots. The highest level of HMGR mRNA detected was assigned a value of 100%, and the relative mRNA levels among the three were estimated based on this maximum value. Similarly, the highest level of SSU accumulation was taken as 100%, and the relative amounts of SSU mRNA were based on this value.

HMGR 10, 18, and 23 and found an HMGR gene to be expressed in every tissue (Fig. 5). This ubiquitous occurrence of HMGR mRNA is consistent with HMGR's important role as a key enzyme in the isoprenoid biopathway and with its reported location in plastids (Brooker and Russell, 1975), mitochondria (Suzuki and Uritani, 1976), and microsomes (Wong et al., 1982). HMGR mRNAs appear to be relatively abundant in rapidly growing tissues such as apical dome (Fig. 7, and by in situ mRNA hybridization, data not shown) and immature anther (Fig. 5; K. Aoyagi, unpublished observation). The reason for the elevated level of HMGR mRNA in callus (Fig. 6) is unknown; however, it may be partially attributed to the presence of auxin (2,4-D) in the medium, which generally is required for sustained cell growth and division of plant tissue in culture. It was reported that removal of 2,4-D from the medium of a carrot cell culture resulted in decreased incorporation of [^{14}C] mevalonic acid into sterols, accompanied by a decrease in microsomal HMGR activity (Nishi and Tsuritani, 1983), pointing to a coordinated control of enzymes involved in sterol biosynthesis. However, the effect of auxin on HMGR gene expression in wheat callus needs further investigation, as do other factors in addition to those influencing growth rate.

The size of the transcript based on northern blots is approximately 3.0 kb (Fig. 6). Occasionally, we observed a smaller transcript of about 2.4 kb in leaf tissues that is less abundant (about one-tenth) than the 3.0-kb mRNA. The sizes of the mRNA are similar to those reported in tomato (Narita and Gruissem, 1989) and *Arabidopsis* (Caelles et al., 1989; Learned and Fink, 1989). These transcripts may represent different forms of the enzyme present in different subcellular compartments. This contrasts with the mammalian and yeast enzymes, which appear to be membrane bound (Brown and Simoni, 1984; Wright et al., 1988).

The level of HMGR 18 is relatively high in rapidly growing tissues such as meristem (Fig. 7). In meristem-enriched apical dome tissue the level of HMGR 18 mRNA is about 1% of total mRNA (estimated from a comparison of the exposure time needed for SSU and HMGR autoradiography).

Using wheat leaf as a model system, we examined the levels of HMGR 10, 18, and 23 to study developmental regulation. HMGR 18 and 23 are developmentally regulated, whereas HMGR 10 mRNA is constitutive in the leaf (Fig. 8). Because the level of HMGR 10 mRNA is always very low, it is likely that HMGR 10 plays a different role from that of HMGR 18 and 23, possibly in defense against pathogens or in response to wounding, as reported for a potato HMGR isogene (Yang et al., 1991). However, our preliminary wound-

ing experiment did not show a substantial increase in the HMGR 10 mRNA level. Failure to observe differences in expression levels may be due to the methods we used. It will be interesting to repeat the experiment using a control system such as the potato plant to compare the response. Our results also suggest light regulation of HMGR 18 and 23 genes. It seems that HMGR 18 mRNA accumulates to a higher level than HMGR 23 mRNA in the light, whereas HMGR 23 mRNA accumulates to a higher level in the dark. It will be very interesting to compare the different gene-specific RNAs that accumulate under different light conditions. Previous studies show high sterol levels associated with young and developing tissue (Kemp et al., 1967). The higher level of HMGR mRNA accumulation in young tissue may be a consequence of a greater structural requirement for higher sterol levels. In plants, the mechanism of HMGR gene regulation seems more complex than in mammals. Our data are consistent with the important role this enzyme plays in plant cells. Further studies are necessary to elucidate the role of specific HMGR genes in wheat.

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