Molecular Cloning, Characterization, and Expression of

3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Gene from Tomato

by

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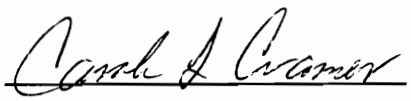

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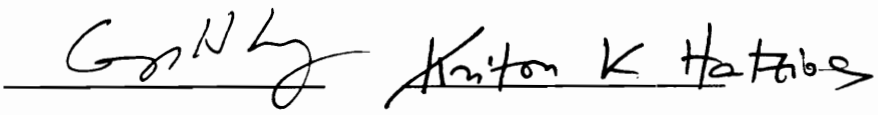

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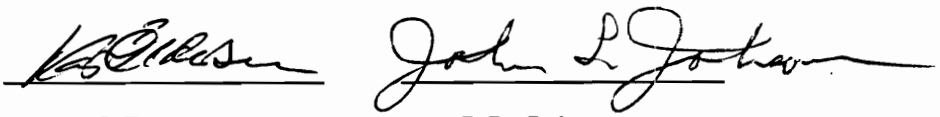

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Plant Pathology, Physiology, and Weed Science

(ABSTRACT)

In plants, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC 1.1.1.34) is a key enzyme regulating biosynthesis of phytosterols, plant growth regulators, carotenoids, antimicrobial defense compounds, and numerous other isoprenoids. To initiate molecular studies of HMGR in relation to defense responses in plants, we utilized yeast HMGR cDNA sequences to isolate tomato genomic sequences encoding HMGR. The nucleic acid sequence and gene structure was determined. The tomato HMGR gene (HMG2) contains four exons seperated by three introns and encodes a polypeptide of 602 amino acid residues (about 64,714 Da). Two membrane-spanning regions are contained in the NH_2-terminus of the HMGR polypeptide. The COOH-terminus shares significant homology with HMGR sequences from different species. Genomic Southern hybridization analyses reveals that tomato contains 3 to 4 HMGR genes. The HMG2 gene cross-hybridizes to mRNA of about 2.7 kb which is highly induced in tomato cells treated with fungal elicitors and in stems, leaves, or roots stressed by wounding suggesting that the HMG2 is a defense-related gene in tomato. Hybridization with a gene specific probe indicates that the HMG2 gene

is induced specifically during defense responses and is distinct from the gene(s) expressed during fruit development and ripening.

To Whom I Love

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CHAPTER 1

Literature Review

1. 3-Hydroxy-3-methylglutaryl coenzyme A Reductase (HMGR)

1.1. What Is HMGR?

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [HMGR; EC 1.1.1.34 - mevalonate:$NADP^+$ oxidoreductase (CoA acylating)] catalyzes the irreversible reaction (Durr and Rudney, 1960):

HMG-CoA + 2NADPH + $2H^+$ ⇒ mevalonate + CoASH + $2NADP^+$.

HMGR is a key regulatory enzyme of the isoprenoid biosynthetic pathway that catalyzes the rate-limiting step diverting acetyl CoA derivatives from general cell metabolism into mevalonic acid, the major precursor of the isoprene unit. In higher plants, isopentenyl pyrophosphate (IPP) functions as the active isoprene unit and is used as an essential five-carbon skeleton of all isoprenoid compounds as illustrated in Fig. 1. The isoprenoid compounds play indispensable roles in metabolism, cell growth, differentiation and development, and disease resistance. Thus, the mode of regulation by HMGR in the synthesis of isoprenoid com-

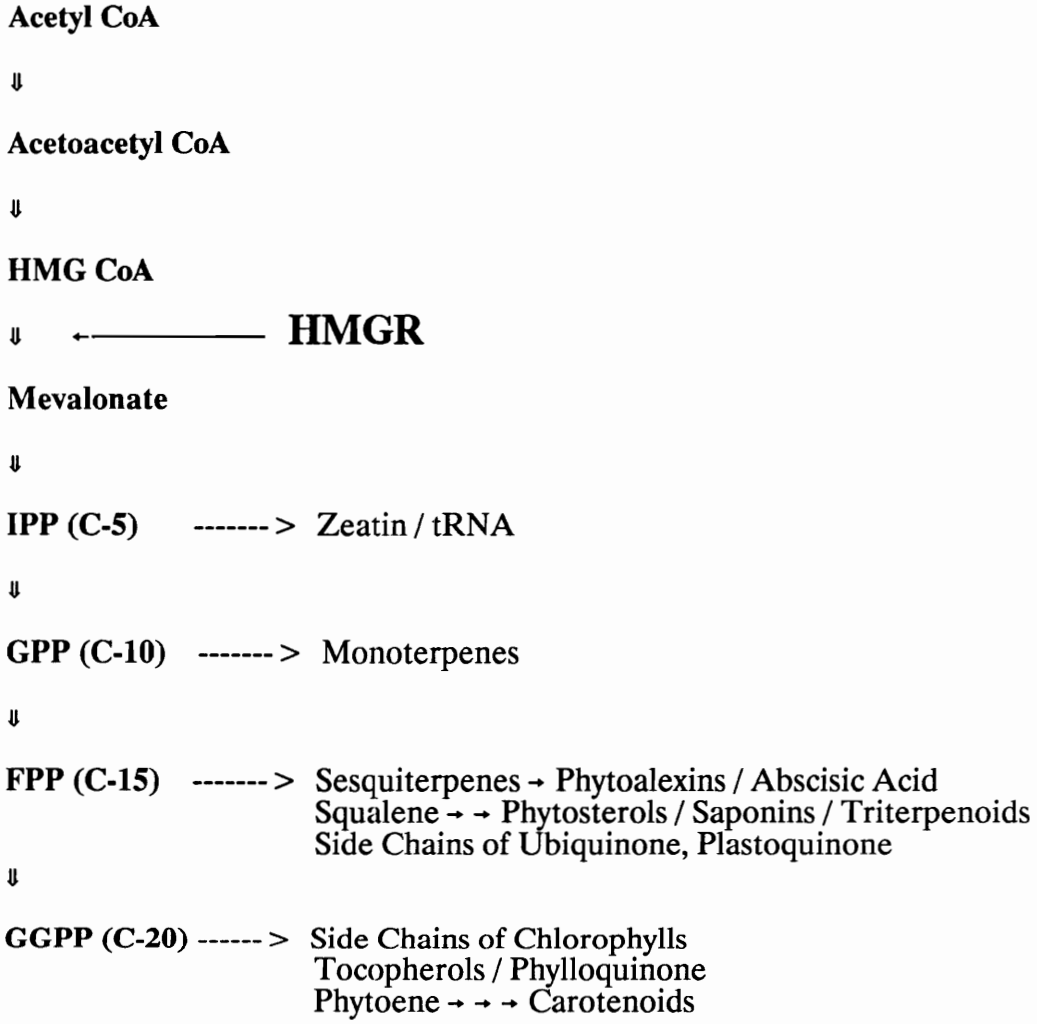

Fig. 1. Synthesis of mevalonate and its flow into isoprenoid compounds. IPP, isopentenyl pyrophosphate (PP); GPP, geranyl-PP; FPP, farnesyl-PP; GGPP, geranylgeranyl-PP.

pounds is believed to impose considerable effect on plant growth and survival (Bach, 1986; 1987; Bach and Lichtenthaler, 1983).

1.2. Isoprenoid Pathway

In mammalian cells, HMGR catalyzes a reaction which is common to the synthetic pathway of the three major isoprenoid compounds; cholesterol, dolichol, and the side chain of ubiquinone. Each of these compounds contains a polyisoprene structure derived from repeated polymerization of isopentenyl pyrophosphate (IPP). Initially, IPP is coupled with its isomer, 3,3-dimethylallyl pyrophosphate (DMAPP), in a head-to-tail condensation to form geranyl pyrophosphate (C-10). This compounds then reacts with another DMAPP to form farnesylpyrophosphate (C-15). This compound, as the major branch-point in polyisoprene biosynthesis, can participate in three different enzymatic reactions to form (i) squalene (C-30) that is the first committed intermediate in the sterol synthetic pathway, (ii) long-chain polyprenols, of which, for example, C-50 polyprenyl group is transferred to an aromatic ring derived from tyrosine to initiate the synthesis of ubiquinone, and (iii) the long-chain polyisoprenyl alcohol, dolichol, through repeated additions of up to 16 additional isopentenyl residues (Brown and Goldstein, 1980; Gray, 1987).

Compared to mammalian systems, the isoprenoid pathway in plants is much more complex in its branch pathways (see Fig. 1). Isoprenoid biosynthesis in plants is subdivided into a main pathway from acetyl-CoA through mevalonate and IPP to long-chain prenyl diphosphates, with a large number of branch points leading to individual isoprenoid compounds (Bach, 1986; 1987). As many as 10,000 different isoprenoid compounds have been estimated in plants and are

associated not only with key functions in growth and differentiation but also many of the pigments, flavor, and aroma components important in many crops. Phytosterols (campesterol, sitosterol, and stigmasterol) are components of cell membranes and are important in cell division and plant growth and development (Geuns, 1973; Grunwald, 1975). Gibberellic acids and abscisic acid function as plant growth regulators. Diterpene and sesquiterpenoid phytoalexins are used during defense responses against pathogenic attack (Kuc and Rush, 1985; Stermer and Bostock, 1987). Carotenoids have roles as accessary pigments in photosynthesis, coloring pigments in fruit and flowers, and protectants against photooxidative damage (Spurgeon and Porter, 1980). Quinones (plastoquinone and ubiquinone) containing the prenyl moiety are required for electron transport during photosynthesis and respiration (Threlfall, 1980).

1.3. Properties and Regulation of HMGR

Due to the importance of regulation of cholesterol levels in mammalian systems, biochemical and molecular studies have been focused on the properties and regulation of HMGR, the major rate-limiting step in cholesterol biosynthesis. In mammalian cells, HMGR is a trans-membrane glycoprotein with its COOH-terminal site facing the cytosol and a carbohydrate-containing NH_2-terminal site oriented toward the lumen of the endoplasmic reticulum (ER) (Brown and Simoni, 1984; Chin *et al.*, 1984; Liscum *et al.*, 1985). The molecular weight of hamster HMGR, deduced from the amino acid sequence is 97,092 daltons. HMGR is a protein of 887 amino acids (888 in human; Luskey and Stevens, 1985) containing three potential sites for asparagine-linked glycosylation (Chin *et al.*, 1984). The NH_2-terminal half of the polypeptide is anchored to the membrane

and contains seven hydrophobic regions, each of which encompasses 20 amino acids. The COOH-terminal half of HMGR is hydrophilic and contains the catalytic site of the enzyme (Gil *et al.*, 1985). HMGR lacks a prepeptide or signal peptide sequence (Brown and Simoni, 1984; Chin *et al.*, 1984).

Studies suggest multiple control mechanisms for HMGR activity and mevalonate formation: (i) transcriptional regulation, (ii) post-transcriptional modifications (Clarke *et al.*, 1983; Liscum *et al.*, 1983), (iii) enzyme degradation (Skalnik *et al.*, 1988), (iv) changes in the membrane composition and membrane fluidity (Sipat and Sabine, 1981), and (v) phosphorylation and dephosphorylation of HMGR. The phosphorylation/dephosphorylation may be controlled by different kinases including reductase kinase, phospholipid-dependent protein kinase (protein kinase C), and a Ca^{2+}, calmodulin-dependent protein kinase (Beg *et al.*, 1980; 1985; 1987; Kennelly and Rodwell, 1985).

In higher plants, the properties of HMGR have been determined mostly from microsomal preparation of different sources of plants (Bach *et al.*, 1986; Bach and Lichtenthaler, 1983; Brooker and Russell, 1975b; Narita and Gruissem, 1989; Sipat, 1982; Suzuki *et al.*, 1974). The major location of the reductase activity appears to be the endoplasmic reticulum (ER), but enzyme activity has also been reported to be associated with mitochondria and plastids (Bach and Lichtenthaler, 1983; Brooker and Russell, 1975b; Wong *et al.*, 1982). The HMGR enzyme from microsomal preparations of pea seedlings uses NADPH as the source of reducing power. The enzyme activity appears to require the presence of free thiol groups. HMGR exhibits optimal activity near neutral pH. The microsomal HMGR from pea seedlings has an optimum activity at pH 6.8 (Brooker and Russell, 1975b). The molecular properties of HMGR enzyme were determined

using a microsomal fraction isolated from radish seedlings (Bach *et al.*, 1986). HMGR was purified and an apparent molecular mass of 180 kDa with a subunit of 45 kDa was determined by electrophoresis. However, molecular study of HMGR from *Arabidopsis thaliana* revealed a polypeptide of 592 residues with a molecular mass of 63,605 Da deduced from the HMGR cDNA (Caelles *et al.*, 1989). A sequence of 407 amino acids lying within the COOH-terminal site shows a high level of amino-acid sequence homology to the catalytic site of enzymes from different organisms. The NH_2-terminal domain contains one (Learned and Fink, 1989) to two (Caelles *et al.*, 1989) putative membrane-spanning regions, in contrast to the enzymes from *Saccharomyces cerevisiae* and mammalian systems (Basson *et al.*, 1988) which have seven trans-membrane regions.

Plant HMGR appears to be closely regulated by phytochrome, phytohormones, and feedback mechanisms. In pea seedlings, mevalonic acid did not appear to inhibit HMGR activity at concentrations up to 1 mM. In contrast, the presence of free CoA was inhibitory (Brooker and Russell, 1975a). Cholesterol and abscisic acid (ABA) applied to etiolated seedlings reduced activity of HMGR but had no effect *in vitro* (Brooker and Russell, 1979). Gibberellic acid enhanced HMGR activity and this stimulation was blocked by ABA (Russell and Davidson, 1982). It seems likely that these responses are due to the hormonal role of these compounds rather than to feedback inhibition as end products. Light plays a crucial role in the growth and development of plants, and light-induced changes in the levels of plant isoprenoids have been reported (Brooker and Russell, 1975b; 1979). Studies with etiolated seedlings have indicated that brief red light irradiation (5 min) of etiolated pea seedlings caused up to 50% decline of the specific

activity of the microsomal enzyme. When the red-light pulse is followed immediately with a far-red pulse there is a partial reversal of the red-light inhibition. This indicates that the light effect is phytochrome-mediated (Brooker and Russell, 1979).

2.0. Host Defense And HMGR

Plants have evolved a number of inducible defense responses following pathogenic attack, or wounding. These responses include (i) synthesis of phytoalexins, which have been defined as antimicrobial compounds of low-molecular weight that are synthesized by and accumulate in plants after the exposure of the plant to microorganisms; (ii) fortification of cell walls by deposition of callose, lignin and related wall-bound phenolics, and accumulation of hydroxyproline-rich glycoproteins (HRGPs); (iii) production of proteinase inhibitors and lytic enzymes such as chitinase and glucanase; and (iv) accumulation of pathogenesis-related (PR) proteins, some of which have recently been identified as proteinase inhibitors, chitinases, and glucanases (Collinge and Slusarenko, 1987; Cramer and Radin, 1990; Ebel, 1986). These host defense responses can also be triggered in intact plant tissues or in cultured plant cells by a variety of compounds termed "elicitors". Elicitors are defined as substances which can elicit phytoalexin production in the absence of a live organism. Generally, induction of defense responses involves transcriptional activation of the corresponding defense genes as part of massive switch in the pattern of host gene expression (Cramer *et al.*, 1985). For example, in suspension-cultured bean cells treated with fungal elicitor,

defense genes encoding chitinase (Hedrick *et al.*, 1988) and enzymes of phenylpropanoid biosynthesis involved in isoflavonoid phytoalexin and lignin induction (phenylalanine-ammonia lyase, chalcone synthase, chalcone isomerase, and cinnamyl-alcohol dehydrogenase) are known to be activated within 2 to 3 min of elicitor treatment (Bell *et al.*, 1984; Bolwell *et al.*, 1985; Lawton and Lamb, 1987; Mehdy and Lamb, 1987; Walter *et al.,* 1988). This response implies few steps between elicitor binding to a probable receptor and specific transcriptional activation of these genes (Lamb *et al.*, 1989). In contrast, transcriptional activation of cell wall HRGP gene by elicitor is observed only after 1 hr, showing slow kinetics for transcript accumulation (Corbin *et al.*, 1987). These notably different induction kinetics for the activation of defense-related genes suggest the possibility of complex signalling and gene activation events in response to these stresses.

Mechanical damage or pathogenic infection often alters the pattern of phytoalexin biosynthesis in the plant. Phytoalexins constitute a variety of compounds belonging to several classes including isoflavonoids, sesquiterpenes, diterpenes, polyacetylenes, dihydrophenanthrenes, stilbenes, and others (Ebel, 1986; Kuc and Rush, 1985). They are not present as constitutive metabolites in the healthy plant, but their production can be monitored in living cells of the plant in contact with a fungal pathogen. It is commonly observed that phytoalexins are built up from non-detectable levels to substantial levels within 12 to 48 hr after fungal inoculation (West, 1981). For examples, glyceollin can increase to greater than 10% of the dry weight of soy bean tissues within 24 to 48 hr after infection with *Phytophthora meganosperma* var. *sojae*, which implies a set of regulatory controls for phytoalexin accumulation (Keen and Horsch, 1972). *Cladosporium fulvum* excretes potent inducers (high-molecular-weight glycoproteins) of the accumula-

tion of rishitin, a sesquiterpenoid phytoalexin, in tomato fruits. These inducers appeared to be neither race- nor cultivar-specific with respect to the accumulation of phytoalexins in tomato leaves and fruits (De Wit and Kodde, 1981). In contrast, intercellular fluids of compatible race-cultivar interactions of *Cladosporium fulvum* and tomato were shown to contain specific elicitors of necrosis (De Wit and Spikman, 1982). These fungal elicitors induced chlorosis and necrosis in resistant but not in susceptible plants. Specificity of the elicitors was not determined by the gene(s) for resistance present in the cultivar but by the virulence gene(s) present in the specific fungal race (De Wit *et al.*, 1985). Model systems using plant tissue and cell cultures treated with elicitors have been widely used for providing insight into defense-related mechanism associated with phytoalexin synthesis in plants, although these systems often fail to demonstrate the host cultivar:pathogen race specificity seen in intact plant:pathogen interactions (Templeton and Lamb, 1988). Phytoalexins have been detected in suspension cell cultures derived from members of the Leguminosae and the Solanaceae. The phytoalexins studied include pisatin from *Pisum sativum* (Bailey, 1970), glyceollin from *Glycine max* (Ebel *et al.*, 1976), phaseollin from *Phaseolus vulgaris* (Dixon and Fuller, 1978), medicarpin from *Canavalia ensiformis* (Gustine *et al.*, 1978), and capsidiol from *Nicotiana tabacum* (Helgeson *et al.*, 1978). These data cannot be translated directly to the intact plant systems because elicitors prepared from a particular race of a pathogen are generally active on plant cultivars regardless of whether that race is virulent or avirulent on that cultivar (Templeton and Lamb, 1988).

Three basic pathways are responsible for the biosynthesis of phytoalexins -- the acetate-malonate, acetate-mevalonate, and shikimate/phenylpropanoids

pathway. Sesquiterpenoid phytoalexins are produced from the acetate-mevalonate or isoprenoid pathway and, as one of the major classes of phytoalexins, participate in disease resistance in higher plants (Kuc and Rush, 1985). Increases in HMGR activity leading to the induction of sesquiterpenoid phytoalexin synthesis have been reported in different plant species. Sweet potato, inoculated with *Ceratocystis fimbriata*, the fungal pathogen causing black rot in roots and stems, showed induction of HMGR activity preceding the increased synthesis of the furanoterpenoid phytoalexin ipomeamarone (Suzuki *et al.*, 1974). Large amounts of the sesquiterpenoid phytoalexin capsidiol accumulated in tobacco cell suspension culture medium upon addition of fungal elicitor. The accumulation of capsidiol was preceded by a transient increase in HMGR activity, which paralleled the changes in ^{14}C-acetate incorporation into capsidiol (Chappell and Nable, 1987). Wounding of potato tubers produced a large temporary increase in HMGR activity of the microsomal and organellar fraction. Treatment of wounded tuber tissue with the elicitor, arachidonic acid, further increased and prolonged the HMGR activity in the microsomal but not the organelle fraction (Stermer and Bostock, 1987). Intact tubers of potato contain a very low activity of HMGR. The activity increased first in response to slicing, and again in response to additional treatments such as inoculation with an incompatible race of *Phytophthora infestans* (Stermer and Bostock, 1987). With respect to phytoalexin accumulation in the Solanaceae, potato has been thoroughly investigated, and a number of sesquiterpenoid phytoalexins such as rishitin, rubimin, phytuberin, phytuberol, hydroxyrubimin, rishitinol, anhydro β-rotunol, and solavetivone have been isolated following challenge with fungi or bacteria (Coxon *et al.*, 1974; Katsui *et al.*, 1971; Tomiyama *et al.*, 1968; Varns *et al.*, 1971). Tomato has a number

of advantages for analyses of host-pathogen interactions due to the excellent development as a genetic system, identification of specific genes associated with resistance to a variety of pathogens, and the availability of isogenic line differing only in these resistant alleles. In tomato, only rishitin has been isolated as the major sesquiterpenoid phytoalexin. Tomato plants resistant to *Verticillium albo-atrum* (Tjamos and Smith, 1974) were reported to accumulate as much of four-fold higher rishitin levels as susceptible tomato varieties (McCance and Drysdale, 1975). Unidentified antifungal compounds were also detected as phytoalexins. Recently several polyacetylene phytoalexins have been identified in tomato and associated with resistance to the leaf spot pathogen, *Cladosporium fulvum* (De Wit *et al.*, 1985).

3.0. Molecular Studies of HMGR

In animals, cholesterol can be obtained from one of two sources — the receptor-mediated uptake of plasma lipoproteins or *de novo* synthesis. The rate-limiting enzyme of cholestrol synthesis is HMGR and its activity is regulated by a negative feedback mechanism in which cholesterol and other end products of the metabolic pathway suppress the enzyme in a multivalent fashion (Gil *et al.*, 1985; Nakanishi *et al.*, 1988). Since HMGR cDNA sequences were initially isolated from a hamster cell line (UT-1) resistant to compactin, a competitive inhibitor of HMGR (Endo *et al.*, 1976), molecular studies have been accelerated toward understanding mechanisms of HMGR regulation (Chin *et al.*, 1982; Gertler *et al.*, 1988; Hardeman *et al.*, 1983; Luskey *et al.*, 1983). These studies (Luskey, 1987;

Luskey and Stevens, 1985; Reynolds *et al.*, 1984 and 1985; Skalnik *et al.*, 1988) have determined that (i) the mammalian genome contains a single HMGR gene, (ii) the 25-kb hamster HMGR gene contains 20 exons, up to eight ATG codons upstream of translation initiation codon, multiple transcription initiation sites, and multiple (hamster) or single (human) donor sites for an intron in the 5' untranslated region, (iii) the HMGR promoter lacks a classic TATA box or CCAAT box, but contains repeats of the sequence 5'-GGGCGG-3' or its complement within 300 nucleotides of the transcription initiation sites --part of the recognition sequence for SP1, a cellular transcriptional factor, (iv) human and hamster show a highly conserved region from 42 to 220 nucleotides upstream of the transcription sites -- the interspecies conservation of the regulatory element, (v) human HMGR (888 amino acids) and hamster HMGR (887 amino acids) share a high degree of homology in NH_2-terminal membrane-bound domain (seven substitutions out of 339 amino acids) and COOH-terminal catalytic domain (22 substitutions out of 439 amino acids). Cholesterol balance in mammalian cells is maintained in part by sterol-mediated repression of gene transcription for the low-density lipoprotein (LDL) receptor and enzymes in the cholesterol biosynthetic pathway. A sequence essential for sterol-mediated repression of transcription has been defined through HMGR and LDL receptor gene promoters (Dawson *et al.*, 1988; Rajavashisth *et al.*, 1989; Sudhof *et al.*, 1987). The sensitivity to end product repression depends upon an element in the 5'-flanking region, designated the sterol regulatory element (SRE). This sequence contains two 16-bp direct repeats that exhibit positive and negative transcriptional regulation activities. Recently, a zinc finger repressor protein that binds to the conserved SRE octanucleotide in both a sequence specific and single-strand-specific

manner has been identified (Rajavashisth *et al.*, 1989).

HMGR genes have been also studied in other organisms including *S. cerevisiae* and *Drosophila melanogaster.* Although *D. melanogaster* does not contain sterol-repressed HMGR, the HMGR enzyme, a polypeptide of 916 amino acids (M_r, 98,165), shares sequence homology with hamster HMGR (56% identical residues in the COOH-terminal region) and contains seven potential transmembrane domains within the NH_2-terminus as in hamster. Two HMGR mRNA transcripts of 3.2- and 4-kb from the single HMGR gene are differentially expressed throughout *D. melanogaster* development (Gertler *et al.*, 1988). In contrast to mammals and *D. melanogaster*, *S. cerevisiae* contains two HMGR genes, HMG1 and HMG2 (Basson *et al.*, 1986 and 1988). Cells containing a mutant allele of HMG1 or HMG2 have only subtle growth defects. However, cells containing mutant alleles of both HMG1 and HMG2 are inviable (Basson *et al.*, 1988). HMG1 is responsible for most of the enzyme activity. Based on deduced amino acid sequences, HMG1 and HMG2 contain 1054 and 1045 amino acids, respectively. The COOH-terminal regions of the two isozymes are highly conserved with respect to both mammalian HMGR and each other. Amino acids 667 to 1025 of the HMG1 protein are 65% identical and 25% conserved with amino acids 512 to 871 of human HMGR (Basson *et al.*, 1988). No primary sequence similarity is observed in the NH_2-terminal region among the *S. cerevisiae* proteins and the human protein. However, the predicted membrane-spanning domains of the yeast and human proteins are similar in that (i) they contain seven membrane-spanning domains, amino acids of which are usually charged and (ii) the spacer regions between the membrane-spanning domains are predicted to contain amphipathic helices which can associate with the lipid bilayer

so that the hydrophobic face would be embedded in the interior of the bilayer and the hydrophilic face would interact with the charged phospholipid head groups of the bilayer (Barnes *et al.*, 1984; Basson *et al.*, 1986; 1988). When I initiated studies of tomato HMGR in 1986, no analogous information on plant HMGR was known.

4.0. Objectives of Tomato HMGR Gene Study

Despite the important properties of the HMGR enzyme in higher plants, its regulation at the molecular level has been little studied compared to mammalian systems. This is due primarily to two major reasons; difficulty in purifying the membrane-integrated HMGR enzyme and its occurrence in organelles including mitochondria and chloroplasts as well as endoplasmic reticulum (ER). Because of the problems associated with studying this enzyme at protein level, I focused on developing alternative molecular tools for analyzing the regulation of this enzyme in plants. My overall strategy was to utilize heterologous probes from yeast HMGR to identify and clone HMGR sequences from tomato for analysis of HMGR at the gene and mRNA levels especially as it relates to host defense and disease resistance. Ultimately, our goal in studying tomato HMGR study lies in providing a fundamental understanding of the molecular events between plant pathogens and their hosts as well as determining the potential for engineering enhanced resistance in crops.

I am addressing several major questions about HMGR and its regulation at the gene and protein level. Does HMGR function as a major regulatory enzyme

in plants as in animal systems? Are there multiple forms of HMGR enzyme? Are there multiple HMGR isogenes, directing synthesis of distinct HMGR isozymes? Is HMGR activated at the gene level in response to pathogens as are other enzymes involved in defense? Therefore, I studied HMGR gene number, HMGR gene structure, and defense-related HMGR regulation, using molecular techniques. My objectives were:

(A) Isolation and characterization of HMGR genes from tomato: (i) develop a plant cell culture system for studying the induction of HMGR by elicitors, (ii) isolate tomato genomic clones, and (iii) identify gene number and characterize the gene structure.

(B) Regulation of HMGR gene expression: (i) analyze defense-related expression of HMGR genes in response to elicitors or mechanical wounding, (ii) determine the gene-specific expression of one HMGR gene during development versus defense responses.

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CHAPTER 2

Defense-Related Expression of A Tomato 3-Hydroxy-3-methylglutaryl coenzyme A Reductase Isogene*

(* To be submitted to Proceedings of National Academy Science USA with Park, H., Denbow, C. J., Yang, Z., and Cramer, C. L. as authors.)

ABSTRACT

In plants, 3-hydroxy-3-methylglutaryl coenzyme A reductase [HMGR; hydroxymethylglutaryl-coenzyme A reductase (NADPH); EC 1.1.1.34] regulates biosynthesis of phytosterols, plant growth regulators, carotenoids, antimicrobial defense compounds, and numerous other isoprenoids. We utilized *Saccharomyces cerevisiae* HMGR cDNA sequences to isolate tomato (*Lycopersicon esculentum*) genomic DNA sequences encoding HMGR. Partial derived amino acid sequences of a region in the tomato gene show greater than 90%, 82%, and 80% sequence similarity with *Arabidopsis thaliana*, *S. cerevisiae*, and human proteins, respectively. Genomic Southern hybridization analyses reveals that tomato contains 3 to 4 HMGR genes in contrast to the single copy found in animal systems. In tomato, host defense responses are associated with accumulation of sesquiterpenoid phytoalexins. HMGR DNA sequences cross-hybridize to mRNA of about 2.7 kb which is highly induced in tomato cells treated with fungal elicitors and in stems, leaves, or roots stressed by wounding suggesting that HMGR gene is a "defense-related gene" in tomato. Elicitor induction of HMGR mRNA levels is transient with maximum levels 9 to 12 hr after treatment. Hybridizations with a gene specific probe indicate that the cloned gene is specifically induced during defense responses and is distinct from the gene(s) expressed during fruit development and ripening. Our results indicate that HMGR is encoded by a gene family whose members are differentially expressed in response to environmental and developmental cues. This is consistant with reports of plant HMGR activity which suggest multiple enzymatic forms showing distinct kinetic properties, regulation, and subcellular localization.

INTRODUCTION

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [HMGR; hydroxymethylglutaryl coenzyme A reductase (NADPH); EC 1.1.1.34] catalyzes the conversion of HMG-CoA to mevalonic acid, a key step in the biosynthesis of isopentenyl pyrophosphate, the building block of all isoprenoid compounds. This enzyme, and the genes that encode it, have been extensively studied in animal systems due to its rate-limiting role in regulating cholesterol biosynthesis (Nakanishi *et al.*, 1988, Sabine, 1983). In animals, HMGR mediates the production of a discrete number of isoprenoids: sterols, ubiquinone, dolichol, isopentenylated adenosine, and isoprenoid moieties involved in post-translation modification of a number of cellular proteins (Farnsworth *et al.*, 1990; Rilling *et al.*, 1990; Schafer *et al.,* 1989). In contrast, plants synthesize a significantly greater array of unique isoprenoid compounds estimated in the thousands. These include phytosterols, plant growth regulators (gibberellins, abscisic acid, and cytokinins), antimicrobial phytoalexins, carotenoid pigments, components of chlorophyll and plastoquinone, rubber, and a variety of specialized isoprenoids associated with insect attraction, fragrance, flavor, feeding deterrents, and allelopathy. HMGR is also considered to be a major control point in plant isoprenoid biosynthesis and thus has been studied in a number of plant species (reviewed in Bach, 1987). Consistent with the increased complexity of the isoprenoid pathway in plants, multiple isozymes of HMGR appear to differ in both kinetic properties and regulation by light, growth regulators, or pathway end-products (Brooker and Russell, 1979, Wong *et al.*, 1982). HMGR enzyme activity has been associated with membranes of subcellular compartments including endoplasmic reticulum (site of HMGR in

animals and *Saccharomyces cerevisiae*), plastids, and mitochondria (reviewed in Bach, 1987; Russell, 1985) suggesting that compartmentalization of HMGR isozymes and other enzymes in the isoprenoid pathway may facilitate localized production of specific products. Definitive localization and evidence that HMGR isoforms direct production of specific isoprenoids have been difficult at the protein level due to its multiple forms and membrane association.

In several of plant species, increases in HMGR enzyme activity in response to pathogens, wounding, or elicitors are associated with accumulation of diterpene (Dueber *et al.*, 1978) or sesquiterpene phytoalexins (Shih and Kuc, 1973; Stermer and Bostock, 1987; reviewed in Kuc and Rush, 1985; Stoessl *et al.*, 1976; Suzuki *et al.*, 1974) and, therefore, suggest involvement in host defense responses. Rishitin, the major sesquiterpene phytoalexin in tomato, is elevated in response of inoculation with the wilt-inducing fungi, *Verticillium albo-atrum* and *Fusarium oxysporum*, and the leaf spot pathogen, *Cladosporium fulvum* (De Wit and Flach, 1979; McCance and Drysdale, 1975; Tjamos and Smith, 1974). To initiate studies of HMGR gene regulation, protein localization, and regulation during defense responses in tomato, we cloned a gene encoding tomato HMGR and studied its regulation at the mRNA level in tomato cells treated with fungal elicitors of host defense responses.

MATERIALS AND METHODS

Plant and Fungal Material. Tomato *(Lycopersicon esculentum* cv. EP7) plants were grown under greenhouse conditions. Suspension cultures of EP7, provided

by Dr. David N. Radin (Virginia Polytechnic Institute and State University, Blacksburg, VA), were maintained in the dark in a modified MS medium (Fosket and Radin, 1983). *Verticillium albo-atrum* (race 1) and *Fusarium oxysporum* (race 1), provided by Dr. Martha Mutschler (Cornell University, Ithaca, NY), were maintained on 2.4% potato dextrose agar and grown in 2.4% liquid medium for cell wall isolation. Fungal elicitor, the high molecular weight material heat-released from isolated mycelial walls was obtained and quantitated as described (Ayers *et al.*, 1976).

Library Screening. Recombinant clones (500,000) of a tomato genomic DNA library (*L. esculentum* cv. VFNT Cherry) constructed in lambda Charon 35 [provided by Dr. Robert Fischer (University of California, Berkeley, CA)] were screened by plaque hybridization (Maniatis *et al.*, 1982). Hybridization probe was the 1.75 kb *Eco*RI fragment of pJR326, provided by Dr. Jasper Rine (University of California, Berkeley, CA), which contains the region of *S. cerevisiae* HMG1 most highly conserved with hamster HMGR (Basson *et al.*, 1986). Initial screening was at relatively low stringency conditions [30% formamide, 6X SSC, 5X Denhardt solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA (Sigma)]. Plaques giving positive hybridization signals were carried through at least three rounds of purification prior to further characterization. From six positive clones, two were chosen for further characterization based on strong cross-hybridization with *S. cerevisiae* HMG1 sequences on Southern blots of plaque-purified DNA. One of these clones was a false positive. A 7 kb *Hin*dIII fragment of the second clone (designated TH29) was subcloned into the *Hin*dIII site of transcription vector pSP6/T7 [Melton *et al.*, 1984; from Bethesda Research Laboratories

(BRL), Gaithersburg, MD] and designated pTH295.

Sequence Analyses. Dideoxy chain-termination sequencing (Sanger *et al.,* 1977) was utilized with pTH295 and its subclones generated by restriction endonucleases, exonuclease III digestion in Bluescript (SK^-) vectors (Stratagene, San Diego, CA), or T4 polymerase in M13 mp19 [International Biotechnologies, Inc. (IBI), New Haven, CT]. Sequence analyses used the University of Wisconsin Genetics Computer Group Sequence Analysis Package (Devereux *et al.*, 1984).

Generation of cDNA Sequences. RNA (15 μg) from tomato cells harvested 9 hr after treatment with *V. albo-atrum* elicitor (50 μg glucose equivalents/ml) was used for first strand cDNA synthesis utilizing oligo(dT)-18mer (Pharmacia, Piscataway, NJ) and Moloney Murine Leukemia Virus reverse transcriptase (BRL) following the manufacturers protocols. Oligonucleotide primers, based on sequences of pTH295 were synthesized by Dr. Tom Reynolds (Institute of Biotechnology, Medical College of Virginia, Richmond, VA). Primer #1 (5'-CGCAAGCT-TGGTGATGCAATGGGAATGAACATGGT) contained 26 bases of coding strand plus a 5' *Hin*dIII site. Primer #2 (5'-TGAGATG-CAAGCTGAGTTCCACCTCC) is complementary strand sequence beginning 202 bases upstream from the presumptive stop codon. Polymerase chain reactions (PCR) were carried out with 0.5 μM of each primer and *Taq* polymerase (Promega, Madison WI) with temperature cycles as described by Frohman *et al.* (1988). PCR reactions were performed in 100 μl reaction mixtures for 35 cycles (95°C, 1 min; 65°C, 2 min; 72°C, 3 min) with final polymerization step for 15 min using an automated thermocycler (model TCX15A, Ericomp, Inc., San Diego,

CA). PCR product was processed by phenol extractions, ethanol precipitation, *Hin*dIII digestion, and electrophoresis in a 2% NuSieve GTG (FMB, Rockland, ME) gel. DNA from the excised band was inserted into Bluescript SK$^-$ (Stratagene) digested with *Hin*dIII.

Nucleic Acid Isolation. Total DNA was isolated from tomato leaves according to the method of Draper and Scott (1988). For RNA isolation, suspension cultured tomato cells treated with elicitors, healthy roots, stems, and leaves treated by wounding (cut into 1 mm slices with a razor blade and compressed with a pestle), or intact fruit at various stages of development were stored at -70^{o}C. Total RNA was isolated from 1 to 3 g fresh weight of tissue ground in liquid nitrogen and homogenized directly in a phenol:0.1 M Tris (pH 9.0) emulsion as described previously (Haffner *et al.*, 1978).

Hybridization Analyses. For genomic Southern analyses, 10 μg/lane total DNA was digested with restriction endonucleases, separated on 0.8% agarose gels, and transferred to Nytran membranes (Schleicher and Schuell, Keene, NH) as described (Maniatis *et al.*, 1982). For Northern analyses, total RNA (5 to 20 μg/lane) was denatured by treatment with glyoxal prior to electrophoresis in 1.2% agarose for gel analyses or application directly to Nytran filters utilizing a slot blotting apparatus. For hybridizations aimed at revealing all members of the HMGR gene family, either the 1.5 kb *Eco*RI fragment of pTH295 (see Fig. 1) or the 486 bp pCD1 insert [probes derived from the 3' end of the gene which is most highly conserved between species (Basson *et al.*, 1988; Learned and Fink, 1989)] were ^{32}P-labeled by random-primer methods (Multi-prime Labeling System,

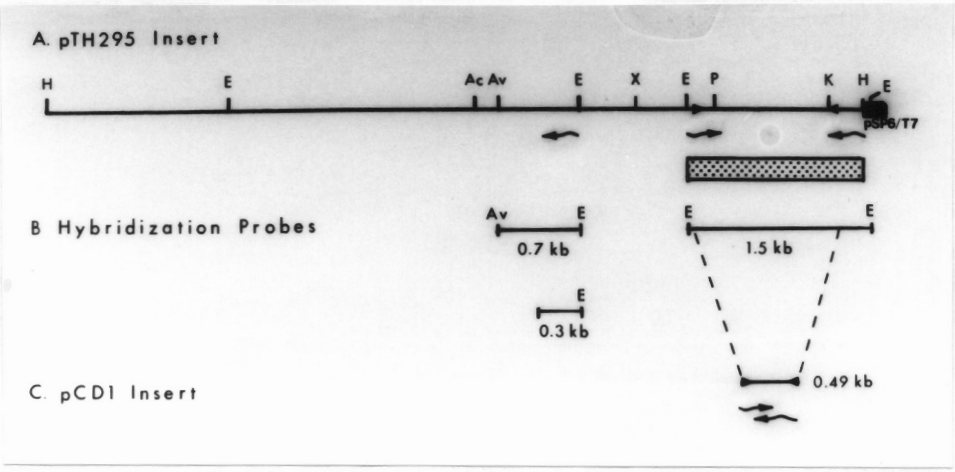

FIG. 1. Characterization of tomato genomic clone pTH295. (A) Partial restriction map of the 7 kb *Hin*dIII fragment of VNFT Cherry tomato DNA in pTH295 (pSP6/T7 plasmid vector is marked at the right end.). (B) Location of HMGR sequences utilized as hybridization probes containing divergent (0.7 kb *Ava*I-*Eco*RI or deleted 0.3 kb probe) or conserved (1.5 kb *Eco*RI) regions of the gene. The downstream *Eco*RI site of the 1.5 kb fragment is derived from the multiple cloning region of the vector. (C) Insert of PCR-generated cDNA probe in pCD1. Arrows represent location of sequence used for construction of oligonucleotides for PCR. Stippled box indicates region of pTH295 cross-hybridizing to yeast HMG1 sequences. Wavy arrows indicate regions sequenced. Restriction endonucleases are Ac = *Acc*I, Av = *Ava*I, E = *Eco*RI, H = *Hin*dIII, K = *Kpn*I, P = *Pst*I, and X = *Xba*I.

Amersham, UK). Membranes were prehybridized overnight without labeled probe and hybridized in the presence of ^{32}P-labeled probe for 24 to 48 hr at 42^{o}C in solution containing 40% formamide, 6X SSC, 5X Denhardt solution, 5 mM EDTA, 0.1% SDS, 100 μg/ml salmon sperm DNA (Sigma). For analyses aimed at monitoring hybridization of sequences specific only for the HMGR gene encoded by pTH295, the 0.7 kb *Ava*I-*Eco*RI fragment encoding 5-untranslated regions and the 5' end of the gene or a smaller 340 bp subclone derived from this fragment and lacking the upstream region (Fig. 1) was utilized. Hybridization conditions were as described above except that 50% formamide and 5X SSC were used. Following hybridization, membranes were washed (final wash was 0.1X SSC at 50^{o}C for 1 hr) to remove unbound label prior to X-ray film exposure.

RESULTS

Isolation and Sequence Confirmation of A Tomato HMGR Gene. Our strategy for isolating tomato HMGR gene sequences was based on cross-hybridization with *S. cerevisiae* HMG1 sequences shown to contain regions highly conserved with hamster HMGR cDNA sequences (Basson *et al.*, 1986). A VFNT cherry tomato genomic library was screened by plaque hybridization using 1.75 kb *Eco*RI fragment of *S. cerevisiae* HMG1 as probe. This fragment encodes the COOH-terminal half of *S. cerevisiae* HMGR, the region showing greatest sequence identity with human and hamster HMGR (Basson *et al.*, 1986; 1988). Approximately 500,000 recombinant plaques were screened by low-stringency hybridization. DNA from plaque-purified cross-hybridizing phage was analyzed by

restriction digestion and Southern hybridizations using the *S. cerevisiae* HMG1 sequences as probe. A 7 kb *Hin*dIII insert of the lambda TH29 clone, which showed strong hybridization to the *S. cerevisiae* HMG1 sequences at higher stringencies, was subcloned into plasmid vector pSP6/T7, and the recombinant plasmid designated pTH295. Further analysis of this plasmid indicated that *S. cerevisiae* HMG1 sequences cross-hybridized to a 1.5 kb *Eco*RI-*Hin*dIII fragment of pTH295 as indicated in Fig. 1A. Sequence analysis of this fragment revealed regions exceeding 65% sequence identity with *S. cerevisiae* HMG1. Based on this sequence, oligonucleotide primers were synthesized and used for PCR amplification of tomato HMGR cDNA sequences as described in Materials and Methods (Fig. 1). Sequence analysis of the resulting clone, pCD1, revealed the expected 486 bp insert identical to 3'-end coding regions of pTH295.

The derived amino acid sequence of CD1 was compared to sequences predicted from *A. thaliana*, *S. cerevisiae*, and human HMGR genes (Fig. 2) and shows 88%, 72%, and 64% sequence identity, respectively, within this region. Sequence similarity, based on sequence analysis programs utilizing the algorithm of Smith and Waterman (Devereux *et al.*, 1984) was 90% with *A. thaliana*, 82% with *S. cerevisiae*, and 80% with human. The sequence of CD1 (designated HMG2, Fig. 2) is closely related to but distinct from an independently isolated tomato HMGR cDNA (designated HMG1) derived from mRNA of immature VFNT cherry tomato fruit (Narita and Gruissem, 1989). Comparison of pCD1 sequences with genomic sequences of pTH295 revealed the location of two introns (arrows, Fig. 2) within this region.

Tomato HMGR Is Encoded by A Multigene Family. Total genomic DNA, isolated

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        *********                          ⇓
Tom2    NMVSKGVQNVLDYLQNEYP..DMDVIGISGNFCSDKKPAA
Tom1    ::::::::::::::S:::..::::::::::::::::::::
Ara     ::::S::::::E::TDDF:..::::::::::::::::::::
Yst     ::I::::EYS:KQMVE::GWE::E:VSV:::Y:T::::::
Hum     ::I:::TEKA:SK:HEYF:..E:KILAV:::Y:T::::::

Tom2    VNWIEGRGKSVVCEAIITEEVVKKVLKTEVAALVELNMLK
Tom1    ::::::::::::::::::K:D::::::::::::::::::::
Ara     ::::::::::::::I:V:RG:I:N:::::S::::::::::::
Yst     I::::::::::::A::T:PGD::R::::SD:S::::::IA:
Hum     I::::::::::::::V:PAK::RE::::TTE:MI:V:IN:

Tom2    NLTGSAMAGALGGFNAHASNIVSAVFIATGQDPAQNVESS
Tom1    ::::::::::::::::::::::::::TL::::::::::::::
Ara     ::A:::V::S:::::::::::::::::FIA::::::::::::
Yst     ::V::::::SV:::::::::L:T:::L:L::::::::::::
Hum     ::V::::::SI::Y::::A:::T:IY::C:::A::::G::

                                          ⇓          *****
Tom2    HCITMMEAVN.DGKDLHISVTMPSIEVGTVGGGTQLASQS
Tom1    ::::::::::.:::::::::::::::::::::::::::::::
Ara     Q:::::::I:.::::I:::::::::::::::::::::::::
Yst     N:::L:KE:D.G..::R:::S::::::::I::::V:EP:G
Hum     N:::L:::SGPTNE::Y::C::::::I:::::::N:LP:Q

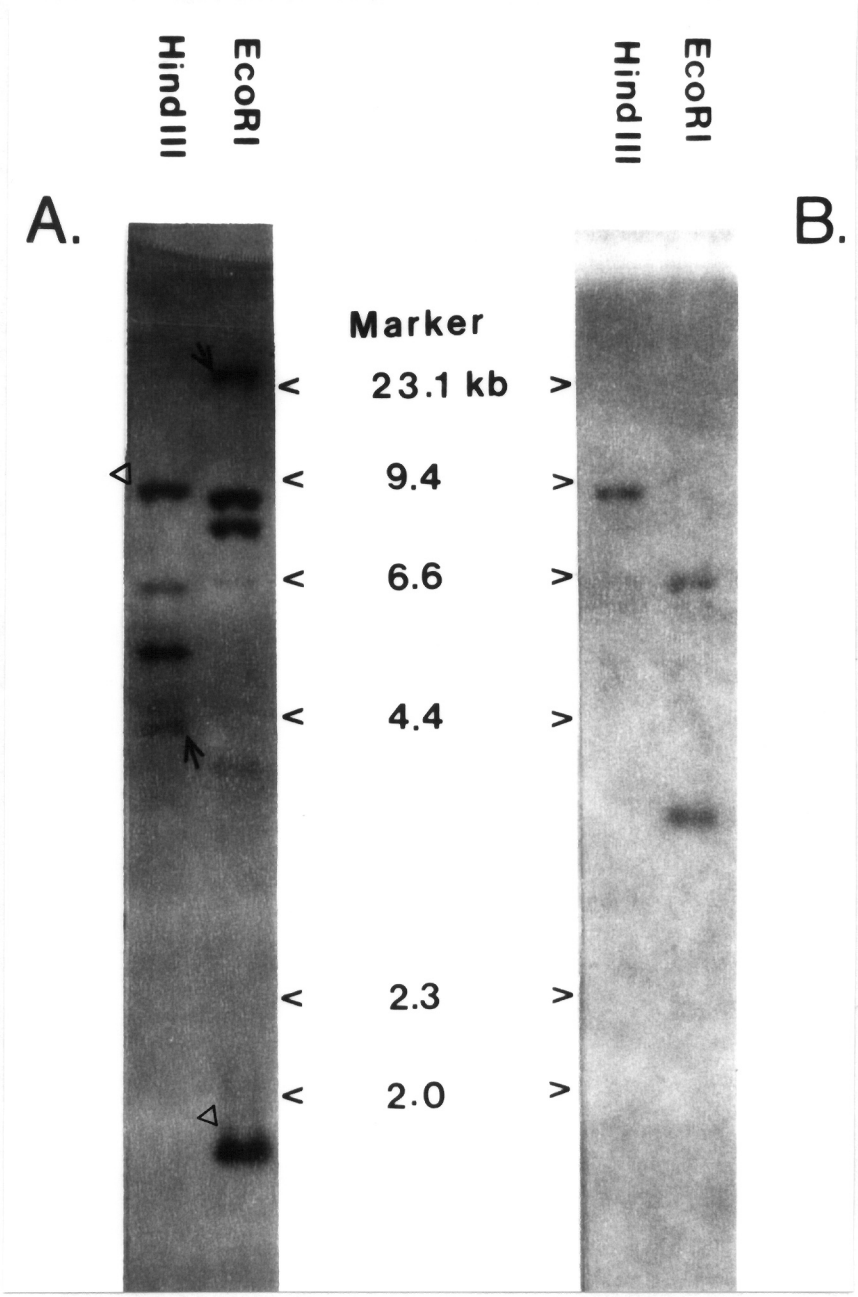
        ***
Tom2    ACL
Tom1    :::
Ara     :::
Yst     :M:
Hum     :::
```

Fig. 2. Comparison of derived amino acid sequences of partial tomato cDNA CD1 (Tom2) with that of tomato HMG1 (Tom1, Narita and Gruissem, 1989), *A. thaliana* HMG1 (Ara; Learned and Fink; 1989), *S. cerevisiae* HMG1 (Yst; Basson *et al.*, 1988) and human HMGR (Hum; Luskey and Stevens, 1985). Sequence identity to tomato clone pTH295 (Tom2) is indicated by a colon; differences are as marked. Gaps are represented as a single dot. Asterisks indicate region used for synthesis of PCR primers to generate pCD1. Arrows represent location of introns within this region of tomato HMGR.

from leaves of inbred tomato line, EP7, was digested with restriction endonucleases and used for Southern hybridization analyses (Fig. 3). Tomato DNA, hybridized with the conserved-region cDNA probe CD1 (Fig. 3A) or the 1.5 kb EcoRI fragment of pTH295 (data not shown), showed at least four cross-hybridizing bands in all digestions tested. The 1.5 kb *Eco*RI fragment of pTH295 hybridized to all fragments indicated by CD1 but hybridized most strongly to the largest *Hin*dIII fragment (~9.0 kb) and the 1.6 kb *Eco*RI fragment and gave identical hybridization pattern on EcoRI or HindIII-digested DNA from cultivars EP7 and VFNT cherry (data not shown). The additional bands may represent distinct HMGR genes suggesting that HMGR is encoded by at least three and possibly more HMGR genes. Comparison of these results with Southern hybridizations published by Narita and Gruissem (1989) suggest that their cDNA probe hybridizes almost exclusively to the 4.2 kb *Hin*dIII fragment and the EcoRI fragment >23 kb (arrows, Fig. 3A).

Comparisons of cloned HMGR sequences from a number of species indicate that the region encoding the NH_2-terminus is much less conserved between species than that encoding the COOH-half of the enzyme which contains the catalytic site (Basson *et al.*, 1988; Learned and Fink, 1989). For this reason, we undertook identification of the 5'-region of the HMGR gene encoded by pTH295. Sequence analysis of the ends of the 3.0 kb *Eco*RI fragment of pTH295 (Fig. 1A) indicated that the 3'-*Eco*RI site encoded a region showing extensive sequence identity to the *A. thaliana* HMG1 (Learned and Fink, 1989) from nucleotide 239 in the open reading frame toward the ATG start codon. We used the 0.3 kb fragment of pTH295 (Fig. 1B) containing this region and, presumably, about 100 bp of 5'-untranslated sequence to probe the tomato genomic DNA. As

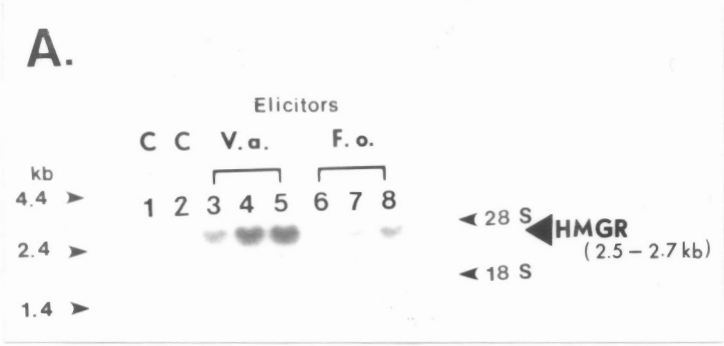
Fig. 3. DNA gel blots of tomato HMGR gene sequences to tomato cultivar EP7 genomic DNA. Total genomic DNA (10 μg/lane) was digested with restriction endonucleases, fractionated by agarose gel electrophoresis, denatured, transferred to Nytran membranes, and hybridized to ^{32}P-labeled probes. Hybridization probes were (A) CD1, a 486 bp cDNA containing the region of HMGR most highly conserved between species, and (B) a 300 bp genomic sequence derived from pTH295 and containing 127 bp of ExonI and about 180 bp of 5'-upstream sequences (Fig. 1). Nucleotide length of DNAs, indicated to the left of autogradiogram, was based on *Hin*dIII-digested lambda molecular size markers (BRL). Triangles in (A) indicate bands hybridizing most strongly to pTH295 sequences. Arrows represent major bands hybridizing to the independently cloned tomato HMGR cDNA sequences based on Southern analyses published by Narita and Gruissem (1989).

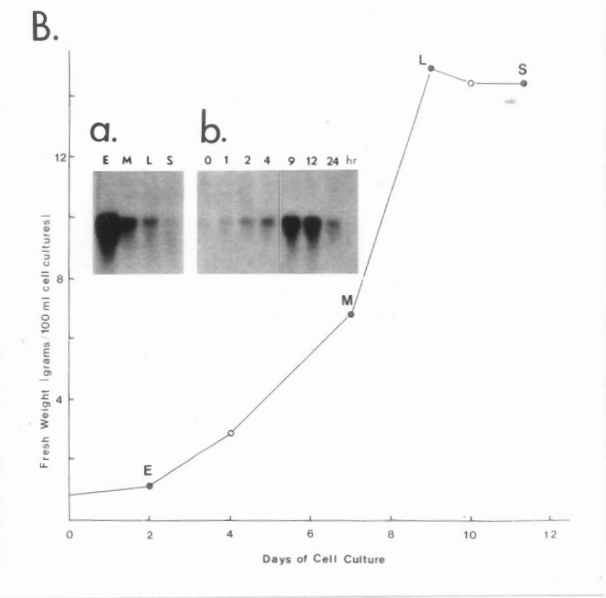

A.
B.
Hind III
EcoR I
Hind III
EcoR I
Marker
23.1 kb
9.4
6.6
4.4
2.3
2.0

shown in Fig. 3B, this "divergent region" probe hybridized to a single fragment (e.g., ∿9.0 kb *Hin*dIII fragment, 3.4 kb *Eco*RI fragment) in each digest. The more faintly hybridizing band at around 6.8 kb in the EcoRI and *Hin*dIII digests was not detected in other Southerns and varied in intensity depending on probe preparation. Thus it probably does not represent HMGR sequences but is due to probe contamination which cross-hybridizes with a repetitive sequences within the genome. This 0.3 kb or 0.7 kb AvaI-EcoRI fragment (Fig. 1B) is used as a gene-specific probe in subsequent analyses of tomato mRNA.

Fungal Elicitors and Wounding Induce HMG-CoA Reductase mRNA Levels. Suspension-cultured cells of tomato cultivar EP7 were treated with elicitors isolated from cell wall fractions of *V. albo-atrum* or *F. oxysporum.* Total RNA, isolated 9 hr after elicitor treatment, was used for RNA blot hybridization analyses using the 1.5 kb *Eco*RI pTH295 fragment (conserved region) as probe. A transcript of about 2.7 kb was induced by both elicitors in a dose-dependent manner (Fig. 4A). Elicitor from *V. albo-atrum* was a more active inducer of HMGR mRNA levels than *F. oxysporum* elicitor. The competency of tomato cells to respond to elicitors was highly dependent on their growth phase; elicitor induction was most effective in early-log cells (Fig. 4B.a). A more detailed time course of elicitor-induced responses indicates that HMGR mRNA induction is transient with maximal mRNA levels at 9 to 12 hr (Fig. 4B.b). The kinetics of HMGR mRNA induction is similar for both *F. oxysporum* and *V. albo-atrum* elicitors but is distinct from that of another defense-related gene, phenylalanine ammonia-lyase (PAL) which shows maximal mRNA levels at 3 to 4 hr (Denbow and Cramer, unpublished results).

Fig. 4. Induction of HMGR mRNA levels in fungal elicitor-treated tomato cells. Northern hybridization analysis were performed on total RNA isolated from suspension-cultured cells of tomato cultivar EP7 following treatment with water (control) or elicitors heat-released from fungal cell wall preparation of *V. albo-atrum* or *F. oxysporum.* The 1.5 kb EcoRI fragment of pTH295 (Fig. 1) containing regions highly conserved among species, was used as probe under condition of moderate stringency [see Materials and Methods]. (A) Elicitor dose response. RNA samples (20 μg/ml) were from O hr control cells (lane 1); 9 hr mock (H_2O-treated) control cells (lane 2); elicitor-treated cells 9 hr after addition of 20 (lane 3), 50 (lane 4), or 100 (lane 5) μg glucose equivalents/ml elicitor isolated from *V. albo-atrum*; elicitor-treated cells 9 hr after addition of 20 (lane 6), 50 (lane 7), or 100 (lane 8) μg glucose equivalents/ml elicitor isolated from *F. oxysporum.* (B.a) Growth phase-dependence of elicitor induction. Cells at various stages of growth following transfer (E: early-, M: middle-, L: late-exponential; S: stationary; 15 μg RNA/lane) were treated with *V. albo-atrum* elicitor (50 μg glucose equivalents/ml) for 9 hr. Growth, as grams fresh weight, and times of harvest (solid circles) are shown. (B.b) Time course of elicitor induction. RNA (15 μg/lane) was isolated from early exponentially growing tomato cell treated with *V. albo-atrum* elicitor (50 μg glucose equivalents/ml) at 0 hr and harvested at the times indicated.

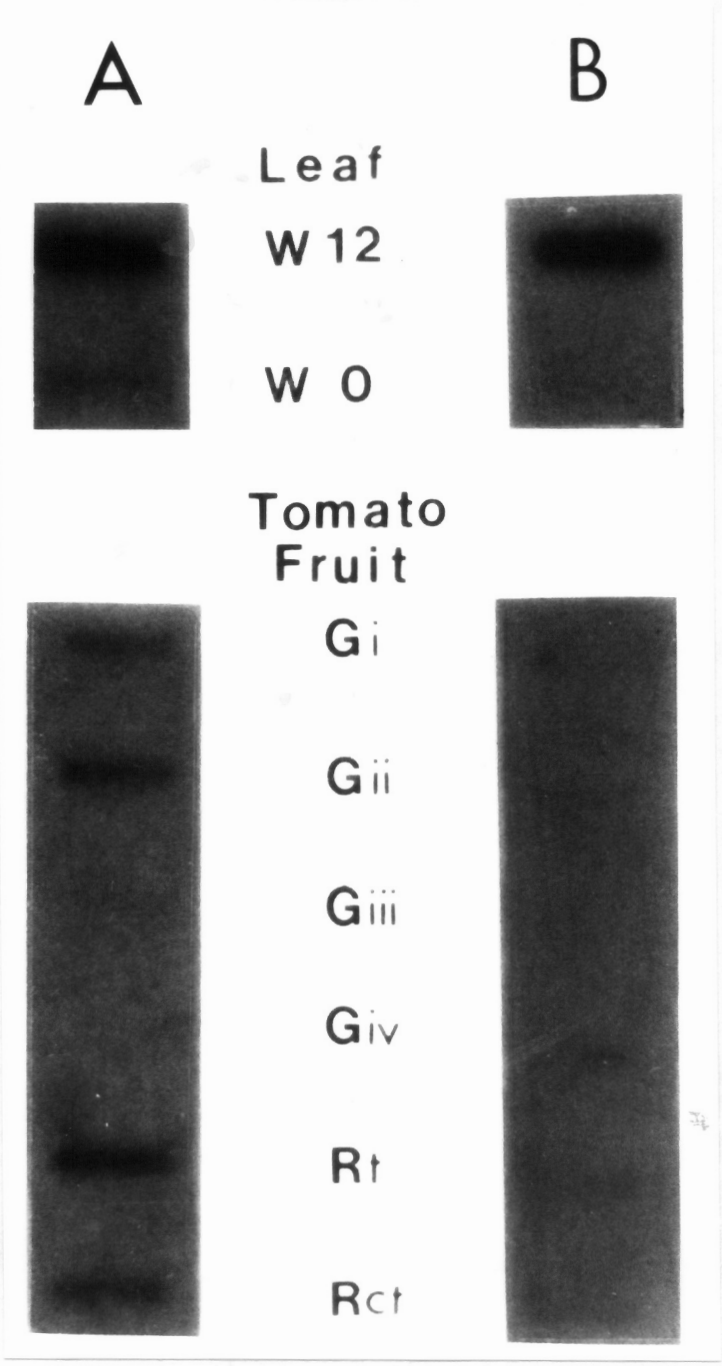

A.
Elicitors
C C V.a. F.o.
kb
4.4
2.4
1.4
1 2 3 4 5 6 7 8
28 S
HMGR
(2.5 – 2.7 kb)
18 S


B.
a.
E M L S
b.
0 1 2 4 9 12 24 hr
Fresh Weight (grams/100 ml cell cultures)
Days of Cell Culture
E
M
L
S

HMGR enzyme activity is elevated in potato tubers or sweet potato following wounding (Stermer and Bostock, 1987; Oba *et al.*, 1985; Yang *et al.*, 1990). To test if wounding triggered an increase in HMGR mRNA levels in tomato, RNA was isolated from roots, stems, and leaves of tomato following wounding. HMGR mRNA levels were elevated in all tissues in response to wounding; the response in leaves is shown in Fig. 5. In all tissues, the induction of HMGR mRNA was transient, with maximal levels at around 12 hr. Wound-induced HMGR mRNA cross-hybridized to the 700 bp *Ava*I-*Eco*RI fragment identified as a gene-specific probe as well as to the CD1 conserved probe for the HMGR gene encoded by pTH295 (Fig. 5A and 5B).

Hybridization of pTH295 Sequences with Fruit HMGR mRNA. Narita and Gruissem (1989) reported that HMGR mRNA and enzyme levels were elevated in young post-anthesis tomato fruit but not in mature fruit. To test if the HMGR gene encoded by pTH295 is expressed during fruit development, we isolated mRNA from fruit of cultivar EP7 at various stages in the maturation and ripening process. These mRNAs were analyzed for cross-hybridization to both conserved region probes and pTH295-gene-specific probes. As shown in Fig. 5A, HMGR mRNA is present in young fruit (2 to 3 mm) but not in larger green fruit. Mature red fruit of both EP7 and VFNT cherry also contained detectable levels of HMGR mRNA. The mRNA on the same blot, stripped and reprobed with the "gene-specific" 0.7 kb *Ava*I-*Eco*RI fragment of pTH295, showed virtually no cross hybridization in contrast to the strong signal seen with mRNA isolated from wounded leaves (Fig. 5B).

Fig. 5. HMGR mRNA levels in wounded leaf tissue and developing fruit. Total RNA (9 μg/sample) was isolated from young leaves 12 hr after wounding (W 12), and untreated leaves (W 0), and tomato fruit at each stages of development (Gi, 2 to 3 mm; Gii, 10 to 20 mm; Giii, 40 to 50 mm; Giv, mature green; Rt and Rct, mature red tomato and cherry tomato, respectively) and utilized for slot blot analyses. (A) Conserved region probe: Immobilized RNA is hybridized with the 1.5 kb *Eco*RI fragment of pTH295. (B) Gene-specific probe: The same blot is stripped and reprobed with the 700 bp *Ava*I-*Eco*RI fragment of pTH295. Fruit and leaf RNAs are from different regions of the same filter. All samples are from tomato cultivar EP7 except Rct which is from tomato cultivar VNFT cherry tomato.


A
B
Leaf
W 12
W 0
Tomato
Fruit
Gi
Gii
Giii
Giv
Rt
Rct

DISCUSSION

We have isolated a tomato genomic clone, pTH295, encoding HMGR utilizing a *Saccharomyces cerevisiae* HMG1 probe. Initial sequence analyses has identified regions showing extensive identity with *S. cerevisiae*, *A. thaliana*, and human HMGR proteins. Although the sequence presented here is limited, it clearly indicates that the gene encoded by pTH295 is distinct from the HMGR cDNA independently isolated by Narita and Gruissem (1989); the derived amino acid sequence differed by 5 residues in 161 (Fig. 2). Besides these two tomato HMGR isogenes, Southern analyses of tomato inbred line EP7, probed at relatively low stringency with cDNA sequences containing regions most highly conserved between species, revealed the presence of at least two addition bands with the restriction endonucleases tested. Thus, the HMGR gene family contains at least three and probably four genes in tomato. It is possible that other more divergent members were not detected by this analysis.

The presence of multiple copies of HMGR is not surprising based on protein level studies which indicate multiple isozymes and distinct subcellular localizations (Bach and Lichtenthaler, 1983; Oba *et al.*, 1985; Russell, 1985; Stermer and Bostock, 1987). However, initial reports on cloned plant HMGR sequences from *A. thaliana* and tomato concluded that the reductase was probably encoded by a single gene (Learned and Fink, 1989; Narita and Gruissem, 1989). Caelles *et al.* (1989) independently cloned an *A. thaliana* HMGR cDNA and used this sequence to identify genomic sequences revealing two reductase genes, HMG1 and HMG2, in *A. thaliana*. These two genes, however, did not cross-hybridize with each other on genomic Southerns of *A. thaliana* DNA

(Caelles *et al.*, 1989). In our hands, even cDNA probes encoding the region most highly conserved among species would function as a gene-specific probe at high stringencies. Thus it appears that distinct HMGR isogenes have diverged significantly. By comparison, mammalian systems and *D. melanogaster* contain only a single HMGR gene (Gertler *et al.*, 1988; Lusky and Stevens, 1985) and *S. cerevisiae* contain two genes (Basson *et al.*, 1986; 1988). It will be of interest to determine whether this divergence has been associated with regulatory and functional specialization of specific isogenes and isogene products.

In responses to pathogenic challenge, plants synthesize phytoalexins and defense-related proteins. This defense response is also observed in plant cell suspension cultures treated with fungal elicitors (reviewed in Templeton and Lamb, 1988) . Increases in HMGR activity leading to terpenoid phytoalexin accumulation have been associated with expression of disease resistance and wound responses in a number of plants including tomato and potato (Oba *et al.*, 1985; Stermer and Bostock, 1987; Tjamos and Smith, 1974). Fungal elicitors have been shown to induce HMGR enzyme activity and sesquiterpene phytoalexin accumulation in suspension-cultured cells of tobacco (Chappell and Nable, 1987). We, therefore, tested if wounding or fungal elicitor derived from tomato pathogens induce HMGR mRNA levels. Elicitor-treated tomato cells gave the most dramatic increase in mRNA levels. This increase was transient with maximal level occurring about 9 hr after elicitor treatment. The kinetics of induction are similar to other defense-related genes (reviewed in Collinge and Slusarenko, 1987; Corbin *et al.*, 1987; Cramer and Radin, 1990; Dixon *et al.*, 1986; Lamb *et al.*, 1989) but were slower than the induction pattern for PAL mRNA, another defense-related gene product, (mRNA maximum at 4 hr, data not shown), in the

same cells. While tomato cell suspension cultures respond to fungal elicitors by increasing the level of HMGR mRNA, further experimentation is required to correlate the induction of HMGR mRNA levels with host responses associated with expression of disease resistance in intact plants.

HMGR mRNA levels were also elevated in response to wounding in leaves, roots, and stem. Both the elicitor-and wound-induced transcripts cross-hybridized strongly to the gene-specific region of pTH295 indicating that this isogene is involved in these stress responses. Interestingly, wounded tomato tissues did not display the very rapid wound response (mRNA maximum at 30 min after wounding) seen in potato tubers (Yang *et al.*, 1990). The 0.7 kb *Ava*I-*Eco*RI fragment of pTH295 also serves as a gene-specific probe in potato and these studies indicated that the HMGR mRNA rapidly induced by wounding is distinct from the potato gene analogous to pTH295 in tomato. However, the pTH295-like gene is induced in potato tubers inoculated by the soft rot bacterium, *Erwinia carotovora* subsp. *carotovora* (Yang *et al.*, 1990). Further studies are required to determine if the rapid wound response is limited to potato or is a tuber-specific response.

Phytoalexin accumulation is one of a number of host responses induced during defense responses against pathogens, wounding, or elicitor. Other responses include cell wall deposition of lignins, glycine-rich proteins, and hydroxyproline-rich glycoproteins, and production of degradative enzymes such as chitinases and glucanases. A number of the genes encoding these defense compounds or the enzymes involved in their biosynthesis have been cloned and characterized (reviewed in Collinge and Slusarenko, 1987; Cramer and Radin, 1990; Dixon *et al.*, 1986; Lamb *et al.*, 1989). Increases in these stress-related

compounds or enzymes involve *de novo* protein synthesis resulting ultimately from activation of the respective defense-related genes (Lawton and Lamb, 1987; Lamb *et al.*, 1989). Significant effort is now being focused on understanding both signal perception and signal transduction in mediating the activation of these genes. Although the rate of gene transcription was not tested directly, it is likely that elicitor-and wound-induced increases in HMGR mRNA are due to activation at the gene level. We are interested in analyzing the promoter region of pTH295 to determine regions responsible for directing the elicitor and wound activation and to compare these sequences with defense-response elements defined for other defense-related genes in plants.

Several of the defense-related genes that have been characterized are encoded by gene families, specific members of which are differentially induced in response to environmental and developmental signals (Corbin *et al.*, 1987; Liang *et al.*, 1989; Wingender *et al.*, 1989). For example, PAL from bean is encoded by 3 to 4 genes, one of which is involved primarily in developmental expression of anthocyanin pigments, another in responses to pathogens associated with phytoalexin production (Liang *et al.*, 1989). Our data suggest that an analogous situation exists in the HMGR gene family. Utilizing DNA probes specific for sequences of the HMGR gene encoded by pTH295, we determined that wound- and elicitor-induced transcripts were derived from this gene but not transcripts associated with tomato fruit development. Apparently, our gene is different from the cDNA cloned by Narita and Gruissem (1989). Their HMGR cDNA was derived from young fruit and isolated using cross-hybridization with a HMGR-specific synthetic oligonucleotide. Using this cDNA probe, they detected elevated HMGR mRNA levels in young fruit similar to results shown with our "conserved-region" probes.

This expression is presumably associated with sterol synthesis during this rapid period of growth and not with carotenogenesis which occurs later during ripening (Narita and Gruissem, 1989). Unlike their results, we detected HMGR mRNA in mature red fruit. These transcripts were detected using a probe containing the regions most highly conserved between species and hybridization conditions of relatively low stringency which may account for this difference. It may be that HMGR transcripts expressed in the later stages of ripening are encoded by a gene distinct from either "HMG1" or "HMG2" possibly associated with carotenogenesis. Mevinolin, a specific HMGR inhibitor, blocked growth and ripening when injected into young fruit but did not block carotenogenesis when injected into mature green fruit (Narita and Gruissem, 1989) suggesting that HMGR enzyme activity is not required for synthesis of fruit pigments. However, earlier studies have suggested that mevinolin may have differential access to HMGR isozymes localized in various organelles (Schindler *et al.*, 1984) and thus lack of mevinolin inhibition may not be definitive in determining the role of HMGR in plastid-localized isoprenoid biosynthesis. Clarification of the role of HMGR in ripening-associated carotenogenesis may require cloning the sequences encoding the presumptive plastid-localized HMGR isozyme(s) and characterization of its expression during fruit development and ripening.

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CHAPTER 3

Tomato 3-Hydroxy-3-methylglutaryl coenzyme A Reductase Gene: Sequence and Structure*

*To be submitted to Plant Molecular Biology with Park, H., Denbow, C. J., and Cramer, C. L. as authors.

ABSTRACT

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [(HMGR) EC 1.1.1.34], catalyzing the formation of mevalonate, controls the synthesis of isoprenoid compounds in plants. The sequence and gene structure of a full length tomato HMGR gene (HMG2) has been determined. Intron splicing sites were identified by comparison with cDNA sequences generated by polymerase chain reaction (PCR)-assisted amplification. The HMG2 gene contains four exons seperated by three introns. The combined HMG2 exons encode a polypeptide of 602 amino acid residues with a molecular mass of 64,714 Da. The overall properties of tomato HMG2 closely resemble *Arabidopsis thaliana* HMG1. In the COOH-terminus, which is most highly conserved between species, amino acid residues 188 to 602 of the tomato HMG2 share as high as 91% similarity and 81% identity with the corresponding region of *A. thaliana* HMG1 (172 to 586). Nucleic acid sequence identity is 72% within this region and introns are placed identically. Tomato HMGR is similar to *A. thaliana* in that it is substantially truncated with respect to *Saccharomyces cerevisae*, *Drosophila melanogaster*, and mammalian HMGR and contains two putative membrane-spanning regions as opposed to seven regions in these other species.

INTRODUCTION

Plants produce a broad array of isoprenoid compounds with diverse functions. They play important roles in defense responses (sesquiterpenoid phytoalexins), membrane biogenesis (sterols), growth and development (gibberellic acid and abscisic acid), pigmentation (carotenoids), photosynthesis (chlorophyll and plastoquinone), and respiration (ubiquinone). A number of industrially important compounds are also produced including rubber, and a variety of compounds associated with flavor and aroma compounds (Bach, 1986; 1987). Mevalonic acid, as a precursor to all these compounds, is produced under the control of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; EC 1.1.1.34). HMGR is considered a key regulatory enzyme in isoprenoid biosynthesis, and its activity has been observed to change in response to light (Brooker and Russell, 1979), stresses including fungal infection, elicitors, or wounding (Chappell and Nable, 1987; Oba *et al.*, 1985; Stermer and Bostock, 1987; Suzuki *et al.*, 1974), pathway end products, and growth regulators (Brooker and Russell, 1975). Currently, little is known about the molecular mechanism controlling plant HMGR regulation and its compartmentation during isoprenoid biosynthesis.

The mammalian HMGR gene/enzyme system and multiple control mechanisms have been well characterized due to its critical role in cholesterol synthesis (Beg *et al.*, 1987; Brown and Goldstein, 1980; Nakanishi *et al.*, 1988). Molecular studies have been also carried out on HMGR from other organisms including *Saccharomyces cerevisiae* (Basson *et al.*, 1986; 1988), *Drosophila melanogater* (Gertler *et al.*, 1988), and *Arabidopsis thaliana* (Caelles *et al.*, 1989; Learned and Fink, 1989). One of the striking features shared between different organisms is

the highly conserved HMGR sequence in the COOH-terminal half which contains the active site (Chin *et al.*, 1984) but variability in NH_2-terminus.

Sequences encoding HMGR have been isolated recently from plants by utilizing heterologous probes containing this highly conserved region (Narita and Gruissem; 1989; Caelles *et al.*, 1989; Learned and Fink, 1989). *A. thaliana* contains two HMGR genes, and the *A. thaliana* cDNA HMG1 gene has been characterized to have 1776 nucleotide bases encoding 592 amino acid residues with a estimated molecular mass of 63,605 Da. This HMG1 gene transcript was detected in leaves and seedlings (Caelles *et al.*, 1989). The *A. thaliana* HMG1 gene was shown to confer viability to *S. cerevisiae* mutants lacking HMGR (Learned and Fink, 1989). In tomato, HMGR mRNA was detected at the early developmental stages of fruit maturation presumably associated with membrane biogenesis (Narita and Gruissem, 1989). However, in our studies, tomato was found to contain at least three HMGR genes, and the tomato HMG2 gene was strongly expressed in response to fungal elicitor (Park *et al.*, 1990). To further characterize HMG2, we present the sequence and structure of tomato HMG2 gene.

MATERIALS AND METHODS

Materials. Primers were purchased from several sources; T3 and T7 primers from Promega (Madison, WI), M13 primer from Bethesda Research Laboratory (BRL, Indianapolis, IN), and 22-mer from International Biotechnologies, Inc. (IBI, New Haven, CT). For sequencing, Sequenase sequencing kit from United

States Biochemical Corporation (USB, Cleveland, OH) was used. For polymerase chain reaction (PCR), synthetic oligonucleotide primers were obtained from the Biology Department, Virginia Polytechnic Institute and State University (Blacksburg, VA) or the Institute of Biotechnology, Virginia Medical Institute (Richmond, VA). ^{35}S-dATP was from NEN (Boston, MA).

Elicitor Preparation. *Verticillium albo-atrum* (race 1), a tomato pathogenic fungus (Tjamos and Smith, 1974), was maintained in 2.4% potato dextrose broth (Difco, Detroit, MI) agar (PDA) and grown in 2.4 % liquid (PDL) medium as described (De Wit and Kodde, 1981). From purified fungal cell walls prepared by the method of Ayers *et al.* (1976), heat-released elicitor was isolated following the procedure by Bostock *et al.* (1982) and quantitated as the amount of anthrone carbohydrate (Ayers *et al.*, 1976).

RNA Isolation. Tomato suspension cells (*Lycopersicon esculentum* cv. EP7) were grown in modified MS medium (Fosket and Radin, 1983). Cells at early-growth phase are most responsive to fungal elicitor (Park *et al.*, 1990) and were treated with elicitor (50 μg glucose equivalents/ml of culture) for 9 hr. Samples were frozen in liquid nitrogen and stored at -70°C. Total RNA was isolated from frozen samples (1.5 g) by the method of Haffner *et al.* (1978).

Nucleotide Sequencing. Tomato HMGR genomic clone, pTH295, previously identified as described (Park *et al.*, 1990), was further cut with *Eco*RI or *Ava*I, and the resulting fragments (*Eco*RI, 1.5 kb and 0.8 kb; *Eco*RI-*Ava*I, 0.7 kb) were introduced into appropriate restriction sites of M13mp18 or mp19 phage vectors

(BRL), or pSK$^-$ or pKS$^-$ plasmids (Stratagene; La Jolla, CA)). A sequential series of overlapping clones were generated by single-stranded M13 deletions (Dale *et al.*, 1985) or Stratagene's pBluescriptII exonucleaseIII/mungbean nuclease system for double-stranded DNA deletion. The extent of deletion was measured on agarose gel following NaOH/SDS- (Maniatis *et al.*, 1982) or phenol-cracking of the deleted transformants. In the phenol-cracking method, the colony was suspended in 30 μl of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA), extracted with the same volume of TE-saturated phenol by vortexing for 10 sec, and centrifuged 5 min at 13,000 rpm. DNA from the aqueous layer was electrophoresed in 1% agarose. DNA sequences were determined by the dideoxy chain termination method of Sanger *et al.* (1977) using Sequenase and protococls from USB. DNA samples were loaded on an 8 M urea-8% polyacrylamide gel and electrophoresed for appropriate amounts of time. Analysis of sequence data was aided by University of Wisconsin Genetics Computer Group Sequence Analysis Package (Devereux *et al.*, 1984).

Generation of cDNA Clones. Partial cDNA clones were generated from mRNA by the synthesis of the first strand cDNA followed by PCR amplification (Mullis and Faloona, 1987) and transformation of the ligate (pKS- or pSK$^-$/PCR product) into *E. coli* DH5α (Maniatis *et al.*, 1982). The first strand cDNA was synthesized in 20 μl mixture containing cDNA synthesis buffer (BRL), RNA template (20 μg), 0.5 mM of each dNTP, 100 *p*moles of random primer or oligo dT (BRL), and 200 units of MoMuLV reverse transcriptase. The reaction continued for 1 hr at 37°C and 1000 units of T_1 RNase was added for 1 hr additional incubation at 37°C. For PCR amplification, the oligonucleotide primer sets 5'-CGCAAGCTTGGT-

GATGCAATGGGAATGAACATGGT-3' (primer 1), 5'-TGAGATG-CAAGCTGAGTTCC-ACCTCC-3' (primer 2) and 5'-GATAAGCTTGGG-TACGTGCAGATACCAG-3' (primer 3), 5'-AACAAGCTTCCGATGACATC-CATGTCGG-3' (primer 4) were used. To 20 μl of cDNA reaction, 80 μl containing 10 μl of 10X GeneAmp buffer (Perkin Elmer Cetus; Norwalk, CT), 50 *p*moles each of primer 1 and 2 (or primer 3 or 4), 2 μl each of dNTP (2.5 mM), and 1 unit of *Taq* polymerase was added. Temperature cycling was established by programming a TwinBlock thermocycler (model TCX15A, Ericomp, Inc., San Diego, CA). Cycle 1 was 95°C, 5 min/ 72°C, 5 min/ 65°C, 2 min. Cycle 2, 95°C, 1 min/ 65°C, 2 min/ 72°C, 3 min/ was repeated 34 times. Cycle 3 was 72°C for 15 min. After completion, top-layered mineral oil used during the reaction was extracted with TE-saturated chloroform. Amplified cDNAs were cloned into the *Hin*dIII or *Sma*I site of the vector and designated pCD1 (primer1---primer2 / pSK^-) or pCD3 (primer3---primer4 / pKS^-), respectively.

RESULTS

Characterization of Tomato HMG2. We described previously the isolation of tomato HMGR genomic DNA clone (pTH295) (Park et al., 1990). It was designated tomato HMG2 to follow the first tomato HMGR cDNA clone (HMG1) isolated by Narita and Gruissem (1989). Its identity as HMGR gene was confirmed by the presence of sequences homologous to the most conserved HMGR sequences among different organisms. These studies indicated that HMG2 is activated in response to fungal elicitors and wounding (Park *et al.*, 1990). A 3.2

kb *Acc*I-*Hin*dIII fragment of the 7 kb insert of pTH295 or derivatives of that fragment subcloned into the M13 or plasmid vector systems as described in Methods. The restriction map of tomato HMG2 gene and the sequencing strategy are shown in Fig. 1. The nucleotide sequence and deduced amino acid sequence of tomato HMG2 is shown in Fig. 2. To determine the HMG2 gene structure, we compared genomic sequences to those of HMG2 cDNA sequences generated by PCR. Oligonucleotide primers (primer #1, #2, #3, and #4, see Fig.1) were designed to flank presumptive introns suggested by the genomic sequence and comparison with introns located in *A. thaliana* HMG1 (Learned and Fink, 1989). RNA from tomato cells induced for HMGR expression by treatment with fungal elicitor was used for cDNA synthesis. Based on comparison of genomic sequences with the cDNAs, pCD1, and pCD3, we established that tomato HMG2 contains four exons (I, 1037 bp; II, 182 bp; III, 347 bp; IV, 240 bp) interupted by three introns (I, 82 bp; II, 282 bp; III, 423 bp). The intron-exon junctions defined in the HMG2 gene (Fig. 2) generally follow the GT/AG rule of intron splicing sites in higher plants (Brown, 1986). The polypeptide deduced from tomato HMG2 is predicted to have an open reading frame of 602 amino acids corresponding to a protein of 64,714 Da.

Transcription and Translation Start Site. An initial primer extension experiment (Wang *et al.*, 1988) to determine the transcription start site of the pTH295 transcript was not definitive. However, an alternative approach involving RNase-protection analysis (Liang *et al.*, 1989) of labeled *in vitro*-synthesized RNA from the 5' end of the gene encoded by pTH295 hybridized with RNA from elicitor-treated tomato cells (C. Cramer, unpublished results) has identified the transcrip-

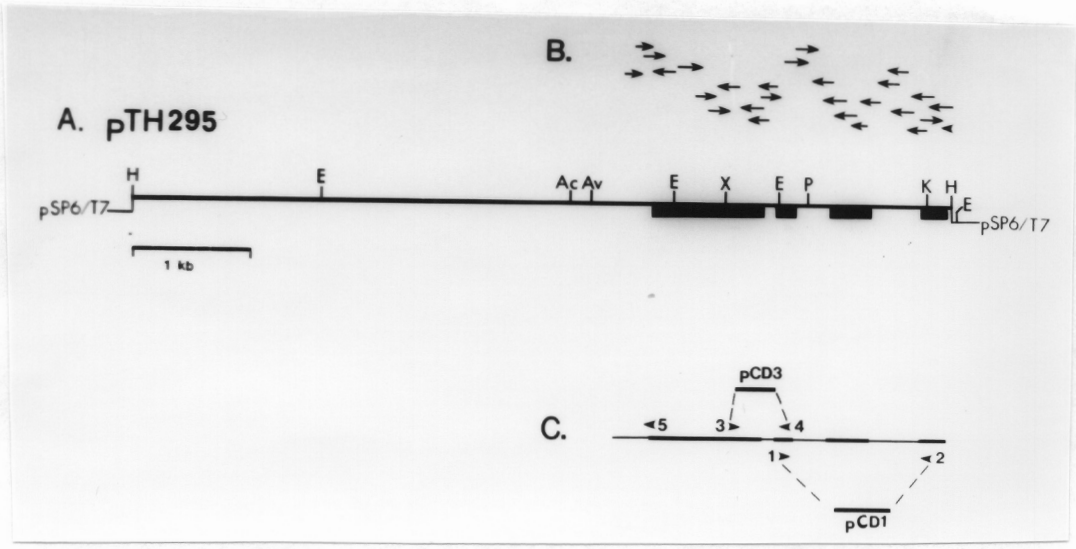

Fig. 1. Structural determination of the tomato HMG2 gene. (A). Restriction map of the pTH295 insert and the tomato HMG2 gene structure. Thickened closed boxes represent exons. Introns are horizontal lines between exons. pSP6/T7 is positioned at both ends of the map as the vector. Restriction sites: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; P, *Pst*I; X, *Xba*I. (B). Strategy used to determine the sequence. Arrows indicate sequencing direction. (C). Primers used for PCR-assisted cDNA cloning or the primer extension experiment (primer5). Primers used for their synthesis.

Fig. 2. Nucleotide and deduced amino acid sequence of the tomato HMG2 gene. Exons and deduced amino acids are written in capital letters. Numbering starts at the putative ATG initiation codon. Introns and other regions are written in small letters and not considered in the numbering. Intron sequences are positioned below exon-intron junction (underlined). Putative transcriptional initiation site (+-->) and TATA box (= = =) are marked at 5' end.

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                                aaaaaattgaaaaaaaaaagaaagtatattattctaaaa

          gataatactccattcaaaatataaaatgaaaaaagtccagcgcggcaaccg
                                =======
          ggggcctctataaatacatttcctacatcttctcttctcctcacatcccat
                              +--->
          cactgcttctttaacaattatacttgtcaatcatcaatcccacaaacaac

          acttttctctcctctttttcctcaccggcggcagacttaccggtgaaaaa

1         ATGGACGTTCGCCGGAGATCTGAAGAGCCTGTTTATCCATCTAAGGTCTTT
          M  D  V  R  R  R  S  E  E  P  V  Y  P  S  K  V  F

52        GCCGCCGATGAAAAACCTCTCAAACCCCACAAGAAACAACAACAACAACAA
          A  A  D  E  K  P  L  K  P  H  K  K  Q  Q  Q  Q  Q

103       GAGGACAAGAATACCCTTCTCATTGATGCTTCCGATGCTCTCCCACTTCCT
          E  D  K  N  T  L  L  I  D  A  S  D  A  L  P  L  P

154       TTGTATCTCACGACGAATGGCTTGTTTTTCACCATGTTTTTCTCTGTTATG
          L  Y  L  T  T  N  G  L  F  F  T  M  F  F  S  V  M

205       TATTTTCTTCTATCAAGGTGGCGTGAGAAAATCAGGAATTCCACTCCTTTA
          Y  F  L  L  S  R  W  R  E  K  I  R  N  S  T  P  L

256       CATGTCGTTACGCTTTCTGAATTGGGTGCTATTGTTTCGTTAATTGCTTCT
          H  V  V  T  L  S  E  L  G  A  I  V  S  L  I  A  S

307       GTCATTTATCTTCTTGGTTTCTTTGGGATTGGGTTTGTTCAGACGTTTGTG
          V  I  Y  L  L  G  F  F  G  I  G  F  V  Q  T  F  V

358       TCAAGGGGAAATAATGATTCATGGGATGAAAATGATGAGGAATTTCTATTG
          S  R  G  N  N  D  S  W  D  E  N  D  E  E  F  L  L

409       AAGGAAGATAGTCGTTGTGGGCCTGCAACTACTCTTGGTTGTGCTGTTCCT
          K  E  D  S  R  C  G  P  A  T  T  L  G  C  A  V  P

460       GCACCACCTGCTCGACAAATTGCCCCAATGGCACCACCTCAACCTTCTATG
          A  P  P  A  R  Q  I  A  P  M  A  P  P  Q  P  S  M

511       TCTATGGTAGAGAAACCTGCACCGTTGATAACATCAGCTTCGTCTGGGGAA
          S  M  V  E  K  P  A  P  L  I  T  S  A  S  S  G  E
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562   GACGAAGAGATAATTAAATCCGTGGTGCAGGGGAAAATACCATCATACTCA
        D  E  E  I  I  K  S  V  V  Q  G  K  I  P  S  Y  S

613   TTGGAATCCAAGCTCGGTGATTGTAAGCGCGCTGCTTCGATAAGGAAAGAG
        L  E  S  K  L  G  D  C  K  R  A  A  S  I  R  K  E

664   GTGATGCAGAGGATTACAGGGAAGTCTCTAGAAGGGCTACCATTGGAAGGA
        V  M  Q  R  I  T  G  K  S  L  E  G  L  P  L  E  G

715   TTTAACTATGAATCTATTCTTGGGCAGTGTTGTGAGATGCCAATTGGGTAC
        F  N  Y  E  S  I  L  G  Q  C  C  E  M  P  I  G  Y

766   GTGCAGATACCAGTGGGAATAGCAGGGCCATTGTTGCTTAACGGAAAGGAG
        V  Q  I  P  V  G  I  A  G  P  L  L  L  N  G  K  E

817   TTTTCGGTGCCCATGGCAACCACAGAAGGATGTTTAGTGGCTAGCACCAAC
        F  S  V  P  M  A  T  T  E  G  C  L  V  A  S  T  N

868   AGAGGTTGCAAGGCTATCTATGCTTCTGGTGGTGCTACATGCATTTTGCTT
        R  G  C  K  A  I  Y  A  S  G  G  A  T  C  I  L  L

919   CGTGATGGTATGACCAGAGCACCATGTGTCAGGTTCGGCACAGCCAAAAGG
        R  D  G  M  T  R  A  P  C  V  R  F  G  T  A  K  R

970   GCAGCAGAGTTGAAGTTCTTTGTTGAAGATCCCATAAAATTTGAGTCACTT
        A  A  E  L  K  F  F  V  E  D  P  I  K  F  E  S  L

1021  GCTAACGTTTTCAACCAATCAAGCAGATTTGCCAGATTACAAAGAATTCAG
        A  N  V  F  N  Q  S  S  R  F  A  R  L  Q  R  I  Q

      gtaagtacatgctagctgttaatctgtttcaaataattatgtgtttatagt

      tgttacctgatcggttgtgtttgaatggcag

1072  TGTGCAATTGCGGGGAAGAATCTTTACATGAGATTGTGTTGTAGCACTGGT
        C  A  I  A  G  K  N  L  Y  M  R  L  C  C  S  T  G

1123  GATGCAATGGGAATGAACATGGTGTCCAAAGGTGTACAAAATGTTCTTGAT
        D  A  M  G  M  N  M  V  S  K  G  V  Q  N  V  L  D

1174  TACCTTCAGAATGAATATCCCGACATGGATGTCATCGGTATATCTGGGAAC
        Y  L  Q  N  E  Y  P  D  M  D  V  I  G  I  S  G  N

      gtattagtttttccttaacttctctgctatgcatggtctgttaaaagacta

      ttaaaacaccataactgcagtatggggttatcataaatgtgaatcgaatgg

      gctggaaaatgatagcttaagatataatgaaatttctatcgtttgatctct

      tgaactttagatttatgtgttcgatgcgtgaaatgatagctagataatgaa
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      agataatgaaatttctatcgtttgatctcttgaactttagataatattgtc

      tcctaaagttatctataactggacag

1225  TTTTGCTCGGACAAGAAGCCAGCAGCAGTTAATTGGATCGAGGGCAGAGGA
      F  C  S  D  K  K  P  A  A  V  N  W  I  E  G  R  G

1276  AAGTCTGTAGTTTGTGAGGCAATTATCACAGAAGAGGTGGTGAAGAAAGTT
      K  S  V  V  C  E  A  I  I  T  E  E  V  V  K  K  V

1327  TTGAAAACTGAGGTTGCTGCTCTTGTGGAGCTGAACATGCTTAAAAATCTT
      L  K  T  E  V  A  A  L  V  E  L  N  M  L  K  N  L

1378  ACTGGCTCTGCCATGGCTGGAGCCCTTGGAGGTTTCAATGCCCATGCCAGC
      T  G  S  A  M  A  G  A  L  G  G  F  N  A  H  A  S

1429  AATATCGTCTCAGCTGTGTTTATAGCCACAGGTCAGGATCCAGCTCAGAAC
      N  I  V  S  A  V  F  I  A  T  G  Q  D  P  A  Q  N

1480  ATAGAGAGCTCGCACTGCATCACTATGATGGAGGCTGTAAATGATGGCAAG
      I  E  S  S  H  C  I  T  M  M  E  A  V  N  D  G  K

1531  GACCTCCATATTTCTGTTACAATGCCTTCCATTGAGGTTGGTACCGTTGGA
      D  L  H  I  S  V  T  M  P  S  I  E  V  G  T  V  G

      gtaaaattttctttgtcgttccattcttttaatcatcaagattaatgagt

      tttatgtataagaatcagacttaacacttttaacactttcgagatcagtt

      taaattgatgtagtcgtgagatagtaaggtactgcatgcatgcatcatgat

      gcaggtctg gattcttcaaatctcaatcaaatagttgaaccacttgaaag

      aacttgaaagaaaaaagacaatgaaaggattcttgaaatatctcaatttag

      tgttagcctgtgagaccttacaacgaagcaatgtctttcaccacatatatt

      tatgatcacttggactatatatagacgacaatctcgtataagtatcttgtt

      tcagtttaaactagttgtgaaatcttccaatttcttgttatatttatggta

      tttgcttcgatacag

1582  GGTGGAACTCAGCTTGCATCTCAGTCAGCTTGCTTAAACTTGTTAGGAGTG
      G  G  T  Q  L  A  S  Q  S  A  C  L  N  L  L  G  V

1633  AAAGGTGCCAACAGAGAGGCACCAGGGTCAAATGCAAGGCTCTTGGCTACA
      K  G  A  N  R  E  A  P  G  S  N  A  R  L  L  A  T
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1684  GTAGTAGCTGGTTCGGTTCTTGCTGGTGAACTATCCCTCATGTCAGCTATA
      V  V  A  G  S  V  L  A  G  E  L  S  L  M  S  A  I

1735  TCGTCTGGGCAACTAGTTAATAGCCACATGAAATACAATAGATCTACCAAA
      S  S  G  Q  L  V  N  S  H  M  K  Y  N  R  S  T  K

1786  GATGTCACCAAGGCGTCCTCCtaatcaggggaatacaatacaatccattcc
      D  V  T  K  A  S  S

      cggccttgtatttgaaggtgtacacaggtgatccaagct
```

tional initiation site at the ATTTC sequence 137 bases upstream from the first ATG codon (Fig. 2). The sequences in these regions match well with the eukaryotic consensus sequences for transcriptional (Py---PyA(+ 1)PyPyPyPyPy; Breathnach and Chambon, 1981) and translational (AACAATGGC; Lutcke *et al.*, 1987) initiation. No sequence resembling the TATAA polymerase binding sequence was evident in the region -26 to -34 from the transcription start site. However, a presumptive TATA box showing excellent fit to the eukaryotic consensus (Breathnach and Chambon, 1981) is present 49 bases upstream of the mRNA initiation.

Comparison of Tomato HMG2 to Other HMGR Genes. The deduced amino acid sequence of the tomato HMG2 gene was aligned with the predicted amino acid sequences from *A. thaliana* (Learned and Fink, 1989), human (Luskey and Stevens, 1985), and *S. cerevisiae* (Basson et al., 1988). HMGR protein structure can be divided into three regions: (i) the membrane-spanning region which is conserved between mammalian species but shows no sequence identity with *S. cerevisiae* or *A. thaliana* NH_2-terminus regions, (ii) the highly conserved COOH-terminus region which contains the active site defined as b1 and b2 in hamster HMGR (Liscum *et al.*, 1985), and (iii) the linker region which is least conserved (e.g. *S. cerevisiae* HMG1 and HMG2 show no sequence identity in this region; Basson *et al.*, 1988). Tomato HMG2 shows the high levels of sequence similarity in the COOH-terminus (residues 188 to 602); 91% similarity and 81% identity with *A. thaliana*, 74% and 55% with human, and 75% and 56% with *S. cerevisiae*. Amino acid sequence alignment is shown in Fig. 3. Most conserved are those regions defined as catalytic sites in hamster HMG [(residues 495 to 595 (b1) and

```
Tom  188 DEEIIKSVVQGKIPSYSLESKLGDCKRAASIRKEVMQRITGK//SL/
Ara  172 ....V...ID.V........R...........R.AL..V..R//.I/
Hum  463 .A...QL.NAKH..A.K..TLMETHE.GV...RQLLSKKLSEPS../
Yst  618 NK.VAAL.IH..L.L.A..K....TT..VAV.RKALSILAEA//PVL

Tom  232 //EGLPLEGFNYESILGQCCEMPIGYVQIPVGIAGPLLLNGKEFSVP
Ara  216 //.....D..D............V.YI............D.Y.Y...
Hum  508 //QY..YRDY..SLVM.A...NV.G.MP....V....C.DE...Q..
Yst  663 ASDR..YKNYD.DRVF.A...NV.G.MPL...VI...VIDGTSYHI.

Tom  277 MATTEGCLVASTNRGCKAIYASGGATCILLRDGMTRAPCVRFGTAKR
Ara  261 ..................MFI.....STV.K.......V...AS.R.
Hum  553 ..............R..GLG...SSRV.A.....G.V..LPR.CD
Yst  710 ...........AMR.....N.G....TV.TK.....G.V...PTL..

Tom  324 AAELKFFVEDPIKFESLANVFNQSSRFARLQRIQCAIAGKNLYMRLC
Ara  308 .S.....L.N.EN.DT..V...R........SVK.T.....A.V.F.
Hum  600 S..V.AWL.TSEG.AVIKEA.DST.......KLHTS...R...I.FQ
Yst  757 SGAC.IWLDSEEGQNAIKKA..ST.......H..TCL..DL.F..FR

Tom  371 CSTGDAMGMNMVSKGVQNVLDYLQNEYP//DMDVIGISGNFCSDKKP
Ara  355 ....................E..TDDF.//.................
Hum  647 SRS........I...TEKA.SK.HEYF.//E.QILAV...Y.T....
Yst  814 TT.........I....EYS.KQMVE..GWE..E.VSV...Y.T....

Tom  416 AAVNWIEGRGKSVVCEAIITEEVVKKVLKTEVAALVELNMLKNLTGS
Ara  400 .................V.RG.I.N.....S.............A..
Hum  692 ..I.............V.PAKV.RE....TTE.MI.V.IN...V..
Yst  861 ..I...........A..T.PGD..R....SD.S......IA...V..

Tom  463 AMAGALGGFNAHASNIVSAVFIATGQDPAQNIESSHCITMMEAVN/D
Ara  447 .V..S.........................V...Q.......I./.
Hum  739 ....SI..Y....A...T.IY.. ...A...VG..N...L...SGPT
Yst  908 ....SV.......A.L.T...L.L.......V...N...L.KE.//.

Tom  509 GKDLHISVTMPSIEVGTVGGGTQLASQSACLNLLGVKGANREAPGSN
Ara  493 ...I.....................................ST.S..M.
Hum  786 NE..Y..C......I.......N.LP.Q...QM...Q..CKDN..E.
Yst  953 ./..R...S........I....V.EP.G.M.D....R.PHAT...T.

Tom  556 ARLLATVVAGSVLAGELSLMSAISSGQLVNSHMKYNRSTKDVTKASS
Ara  540 ..R...I...A............AA....R........SR.ISG.TT
Hum  833 ..Q..RI.C.T.M.......A.LAA.H..K...IH...KINLQDLQG
Yst  999 ..Q..RI..CA........CA.LAA.H..Q...TH..KPAEP..PNN
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Fig. 3. Alignment of the deduced amino acid sequences of the COOH-terminal regions from the tomato HMG2 (Tom), *A. thaliana* HMG1 (Ara), human HMGR (Hum), and *S. cerevisiae* HMG1 (Yst). "." represents sequences identical with the tomato HMG2. "/" is used to fill necessary blanks for alignment.

735 to 825 (b2); Liscum *et al.*, 1985]. Of the four potential glycosylation sites (Asn-X-Ser/Thr) suggested in *A. thaliana* (16, 19, 329, 575 amino acid residues, Caelles *et al.*, 1989) only the later two (at residues 345 and 591) are conserved. In the NH_2-terminus region (residues 1 to 187), overall sequence identity with *A. thaliana* HMG1 is less than 45% with numerous gaps evident when optimally aligned. The linker region (117 to 187) shows no sequence conservation and is twenty residues longer than the equivalent region in *A. thaliana*. In contrast, the presumptive membrane spanning regions (residues 44 to 116) shows 78% sequence identity with an additional 17% conservative changes (Fig. 4). The hydropathy plots (Fig. 4) calculated by the method of Kyte and Doolittle (1982) indicate the potential for two membrane spanning helices as indicated for *A. thaliana* (Caelles *et al.*, 1989).

DISCUSSION

We present here the first nucleic acid sequence and gene structure of a HMGR gene from tomato. The coding region of the tomato HMG2 gene consists of 1806 nucleotides of exon and 806 nucleotides of intron and is transcribed into the 2.7 kb-mRNA identified in elicitor-treated tomato cells (Park *et al.*, 1990). The tomato gene shows a high degree of similarity to *A. thaliana* HMG1 both with respect to sequence and gene structure with identical intron locations although the intron themselves show no conservation of sequence or size. In addition, tomato also has two presumptive membrane-spanning regions in the NH_2-terminus portion, which are highly homologous with *A. thaliana* HMG1,

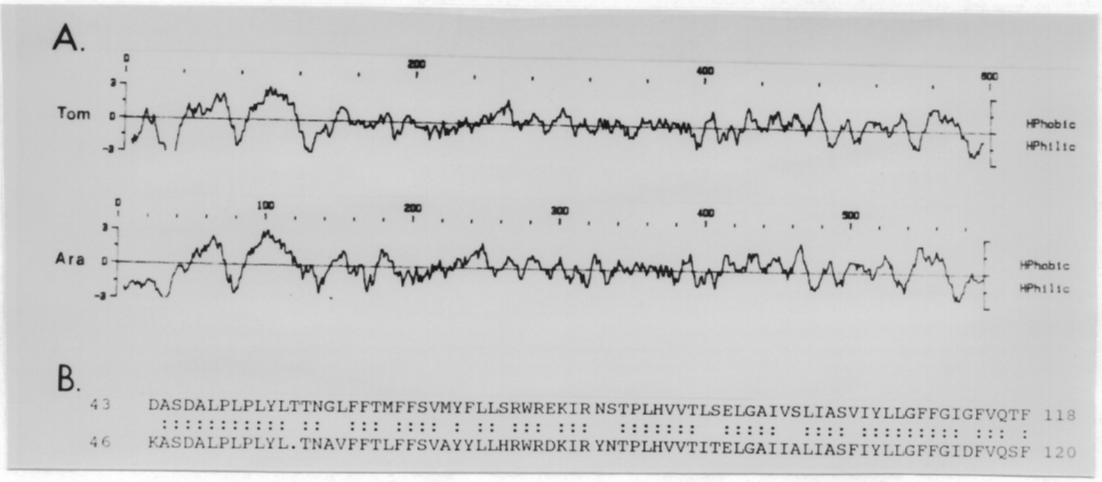

Fig. 4. Hydropathy plots of tomato and *A. thaliana* HMGR. (A). The entire amino acid sequences of the tomato HMG2 and *A. thaliana* HMG1 are plotted (continuous line only) using the algorithm of Kyte and Dolittle (1982) with a window of 9 residues. (B). Deduced amino acid sequence comparison in the NH_2-terminal region representing the putative trans-membrane segment.

compared to seven membrane regions in *S. cerevisiae*, *D. melanogster*, and mammalian systems. Based on these results, it appears that HMGRs are highly conserved among plant species. However, the initial reports of HMGR sequences in both *A. thaliana* (Learned and Fink, 1989) and tomato (Narita and Gruissem, 1989) suggested that there was only a single HMGR gene based on genomic Southern analyses, and Caelles *et al.* (1989) identified two HMGR genes in *A. thaliana* but found they did not cross-hybrdize with each other under relatively stringent hybridization conditions. We observed 3 to 4 HMGR genes in tomato using cDNA sequences from the highly-conserved catalytic region and relatively low stringency hybridization conditions (Park *et al.*, 1990). Thus, these reports suggest a high degree of divergence between HMGR genes within the same plant. Both our tomato clone and HMGR from *A. thaliana* (Learned and Fink, 1989) were isolated using yeast HMG1 as a heterologous probe (Basson *et al.*, 1986). Thus we may have isolated the isogene most similar to yeast and other tomato isogenes may be more divergent in their nucleic acid sequence. An analogous situation exists in the phenylalanine ammonia-lyase (PAL) gene family in plants (Liang *et al.*, 1989). In bean, PAL is encoded by 3 to 4 genes, three of which have been characterized (Cramer *et al.*, 1989). Bean PAL2 gene shows greater sequence identity with the PAL gene from *A. thaliana* than with either bean PAL1 or PAL3 (Cramer *et al.*, 1989; C. J. Lamb, personal communication). The presumptive divergence of the other isogenes within the HMGR gene family may be functionally associated with distinct subcellular locaizations (eg. plastid or mitochondrial) and production of specific isoprenoid end products. The fact that (i) the tomato HMG2 is a elicitor-induced gene and (ii) pathogens primarily induce the activity of HMGR enzyme located in microsomal fraction (Stermer

and Bostock, 1987) suggests that the HMG2 gene is responsible for the synthesis of membrane-bound HMGR enzyme in endoplasmic reticulum. The hamster and human HMGR also lack recognizable signal peptides, although they are clearly integrated membrane proteins at the endoplasmic reticulum. Future work will focus on analysis of promoter to identify elicitor and wound responsible elements and compare them with analogous regions described for other defense-related genes.

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CHAPTER 4

Discussions and Future Directions

HMGR. 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is a very important key enzyme controlling isoprenoid biosynthesis in plants. Extensive studies on HMGR in mammalian systems have provided us with fundamental knowledge of HMGR regulation mechanism at the molecular level. I initiated tomato HMGR studies for the purpose of elucidating HMGR gene expression associated with defense responses in plants. For this, I cloned and characterized a tomato HMGR genomic DNA sequence and tested whether that cloned gene was specifically expressed during defense responses in plants using a tomato cell culture system and fungal elicitors as well as intact plants.

Defense-Related Expression of Tomato HMGR Gene. It was demonstrated that the 2.7 kb-HMGR mRNA of tomato suspension cell cultures is greatly induced by treating with elicitors isolated from tomato pathogenic fungi, *Verticillium albo-atrum* and *Fusarium oxysporum.* The marked induction of HMGR mRNA was transient, rapid and similar to that of defense-related genes previously reported in

plants. Also, wounding of intact tomato tissues results in the induction of HMGR mRNA at comparatively low but significant levels. Use of hybridization probes specific to the HMGR gene cloned and characterized, demonstrated that elevated HMGR mRNA levels in response to wounding or elicitor are due primarily to increased transcripts from this gene. However, the specific probe to the isolated HMGR gene does not show cross-hybridization to mRNA isolated from tomato fruits at any developmental stage, suggesting this gene is associated with defense-related expression and not developmental processes. These results provide strong evidence of differential HMGR expression during defense responses, membrane biogenesis, or carotenogenesis. Southern analyses suggests a tomato HMGR gene family consisting of multiple gene members, suggesting that individual genes may be associated with distinct functions.

HMGR Gene Structure. In overall features, the tomato HMGR gene (HMG2) shows the very similar structure with *Arabidopsis thaliana* HMG1 including size, intron/exon sites, hydropathy profile of deduced amino acid sequences, and two putative membrane-spanning segments in the truncated structure of the NH_2-terminus region in contrast to seven in HMGR from mammalian systems, *Drosophila melanogaster,* or *Saccharomyces cerevisiae.* These results suggest simplified but specific function of tomato HMG2 for adaptation to a variety of functions of the isoprenoid compounds and complex compartmentation.

Future Directions. For studying defense-related expression of tomato HMGR gene, I utilized tomato plant cultivar EP-7 for suspension cell culture system and elicitors isolated from tomato pathogenic fungus *V. albo-atrum.* This system is

appreciated to have several advantages, of which easy maintenance of EP-7 as the cell culture system and rapidity of elicitors in triggering defense-related responses are greatly attractive. However, cell culture system tends to lose specific correlation of intact plant cultivar with a pathogen race. The tomato EP-7 is a cultivar susceptible to *V. albo-atrum*, showing presumably no hypersensitive responses in responses to this fungus. Another weakness in this system is that elicitors isolated from fungal cell wall do not work as effectively or specifically as the elicitors intactly positioned in fungal cell wall. Therefore, the expression of HMGR mRNA in suspension cell cultures treated with elicitors cannot be translated directly to a specific plant-pathogen interaction. Therefore, the initial cell culture/elicitor system, which identified HMGR as a defense-related gene, should be complemented by studying expression of HMGR in host defense responses using well-defined specific compatible or incompatible interactions in the intact plant inoculated with specific fungal races.

In plants, HMGR plays an important role in defense response. My research has determined structural features of the tomato HMG2 gene. To elucidate regulatory mechanisms of HMG2 as a defense-related gene, further research should focus on the 5'-upstream region. Detailed analysis of the 5'-region of HMG2 will be of aide for (i) the explanation of differential expression in defense-response distinct from other functions such as membrane biogenesis and carotenogenesis or (ii) search for the consensus elements especially associated with defense-related expression.

Vita

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