MEMBRANE DOMAIN OF PLANT 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE : TARGETING, TOPOLOGY, AND FUNCTION

by

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ABSTRACT

The rate limiting step in isoprenoid biosynthesis is catalyzed by 3-hydroxy-3-methylglutaryl CoA reductase (HMGR, EC 1.1.1.34). In plants, HMGR is encoded by small gene families whose members are differentially expressed. In tomato, *hmg*2 was previously isolated and sequenced. We report the isolation and sequence analysis of a clone (pCD4) encompassing exon I of tomato *hmg*1 which encodes the putative membrane domain. Sequence comparisons of plant HMGR proteins reveal two hydrophobic stretches within the amino terminus which are highly conserved among species. Using *in vitro* transcription and translation systems, the membrane domain structure of two tomato HMGR isoforms, HMG1 and HMG2, were analyzed. Results from these experiments reveal that tomato HMGRs are targeted to microsomal membranes in a cotranslational fashion that does not involve cleavage of an N-terminal targeting peptide. Membrane topography of HMGR was revealed by protease protection studies, indicating that both tomato HMGRs span the membrane two times such that both the C- and N-termini are located within the cytosol. HMG2 but not HMG1 was glycosylated in the *in vitro* system. Deletion of the *hmg*1 5' untranslated regions and sequences encoding the first six highly charged amino acids resulted in inefficient translation *in vitro*. However, targeting to microsomes was unchanged. HMG1 membrane domain was tagged with a FLAG epitope to facilitate *in vivo* studies. *Agrobacterium*-mediated transformation was used to introduce the tagged *hmg*1 gene into two *Nicotiana tabacum* cell lines, BY-2 and KY-14. The slow growth kinetics of KY-14 prevented effective recovery of transformed lines, however, Northern analyses of BY-2 showed that the *hmg*1 transgene was expressed. Comparisons of BY-2 and KY-14 revealed differences in defense responses to elicitor treatment. BY-2 cells showed minimal defense capabilities, whereas KY-14 cells were rapidly induced as indicated by increased HMGR enzyme activity and browning of the cells. HMGR enzyme activity was decreased in both KY-14 and BY-2 cells following sterol treatment, but the reduction was more pronounced in KY-14 cells. Thus transgenic BY-2 cells may be useful in future *in vivo* immunolocalization studies, but analyses of HMGR transcriptional regulation and regulated degradation will require use of the more responsive KY-14 cells..

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TABLE OF CONTENTS

Title Page i
Abstract.................... ii
Acknowledgments....................iii
Table of Contents....................iv
List of Figures....................vi
List of Tables....................vii
List of Abbreviations....................viii

Chapter I Literature Review....................1
I.1 INTRODUCTION....................2
I.1.1 Isoprenoid biosynthetic pathway in plants.................... 2
I.1.2 Role of HMGR in the pathway 2
I.1.3 Localization of HMGR enzyme activities in plants.................... 4
I.1.4 Plant HMGRs and regulation of their gene expression....................5
I.2 TARGETING AND TOPOLOGY OF MAMMALIAN HMGR....................6
I.2.1 Protein targeting to the endomembrane system 6
I.2.2 Protein targeting to chloroplasts and mitochondria....................9
I.2.3 Protein structure of mammalian HMGR....................11
I.2.4 Role of membrane domain in targeting and topology 11
I.3 STEROL-MEDIATED REGULATION OF MAMMALIAN HMGR 11
I.3.1 Transcriptional down-regulation....................11
I.3.2 Protein degradation....................12
I.4 REGULATION OF PLANT HMGRs....................14
I.4.1 Membrane domain structure....................14
I.4.2 Evidence for transcriptional regulation....................14
I.5 SIGNIFICANCE AND OBJECTIVES OF RESEARCH.................... 16
I.6 REFERENCES....................16

Chapter II The N-Terminal Domain of Tomato 3-Hydroxy-3-Methylglutaryl CoA Reductases....................24

Chapter III *In Vitro* Targeting and Expression of an Epitope-Tagged Tomato HMG1 Membrane Domain in Transgenic Tobacco Cells....................25
III.1 ABSTRACT....................26
III.2 INTRODUCTION....................26
III.3 EXPERIMENTAL PROCEDURES....................28
III.3.1 Cell cultures and sterol or elicitor treatment....................28
III.3.2 Construction of FLAG-tagged vector....................28
III.3.3 PCR amplification and cloning of FLAG-tagged *hmg*1 membrane

domain.. 29
III.3.4 *In vitro* transcription/translation of FLAG-tagged *hmg*1......................29
III.3.5 Western blotting and immunodetection of FLAG-tagged peptides.......29
III.3.6 Construction of plant expression vectors... 31
III.3.7 Transformation of tobacco BY-2 and KY 14 cells................................31
III.3.8 Northern analysis of transgenic cells..31
III.3.9 HMGR enzyme assay..32
III.4 RESULTS..32
III.4.1 Insertion of epitope-tagged HMG1 constructs into microsomal membranes..32
III.4.2 Differential regulation of HMGR activity in BY-2 and KY 14 cell lines..37
III.4.3 Expression of HMG1 tagged transgene in BY-2 cells.......................... 41
III.5 DISCUSSION..41
III.6 ACKNOWLEDGMENTS... 45
III.7 REFERENCES...45

Chapter IV Conclusions and Future Directions..49
VITA.. 53

List of Figures

Figure I.1 A simplified version of plant isoprenoid biosynthetic pathway.........3
Figure I.2 The secretory pathway in plants and animals....................................8
Figure I.3 Types of integral membrane protein topology 10
Figure I.4 Deduced amino acid sequence comparisons of three plant HMGR N-termini...15
Figure III.1 Map of vectors constructed for *in vitro* transcription/translation studies and cell transformation with FLAG-tagged HMG1.............30
Figure III.2 Insertion of FLAG tagged HMG1 (pCD6) into microsomal membranes..33
Figure III.3 Insertion of FLAG tagged HMG1 (pCD7) into microsomal membranes..34
Figure III.4 Protected fragments of *in vitro* synthesized FLAG-tagged HMG1 peptides from pCD6 and pCD7...35
Figure III.5 Western analysis of FLAG-tagged HMG1..................................... 36
Figure III.6 Cellulase-induced browning of KY-14 cells.....................................38
Figure III.7 Induction of HMGR enzyme activity in KY 14 tobacco cell suspension cultures by elicitor...39
Figure III.8 RNA gel blot analysis of *hmg*1 and *hmg*2 transcript levels in cellulase treated KY-14 cells...40
Figure III.9 Expression of FLAG-tagged HMG1 in BY-2 cells..........................43

List of Tables

Table III.1 Effect of 25-hydroxycholesterol on HMGR enzyme activity in tobacco BY-2 and KY-14 cell susprnsion cultures...........................42

List of Abbreviations

Abbreviation	Meaning
HMGR	3-hydroxy-3-methylglutaryl CoA reductase
ER	endoplasmic reticulum
PCR	polymerase chain reaction
PAGE	polyacrylamide gel electrophoresis
Endo H	endo- -N-acetylglucosaminidase H
PP	pyrophosphate
PGR	plant growth regulator
CGN	*cis*-Golgi network
TGN	*trans*-Golgi network
N	amino
C	carboxy
IPP	isopentenyl diphosphate, isopentenyl pyrophosphate
MVA	mevalonate, mevalonic acid
CoA	coenzyme A
SRP	signal recognition particle
PDI	protein disulfide isomerase
BiP	binding protein
PEST	proline, glutamic acid, serine, threonine sequences
LDL	low density lipoprotein
CAT	chloramphenicol acetyltransferase
SRE	sterol response element
NF1	nuclear factor 1
CHO	Chinese hamster ovary
HMGal	HMG-CoA reductase/ -galactosidase fusion gene
NADPH	-nicotinamide adenine dinucleotide phosphate,reduced form

CHAPTER I.
LITERATURE REVIEW

I.1 INTRODUCTION

I.1.1 Isoprenoid Biosynthetic Pathway in Plants

Plants synthesize over 22,000 known isoprenoid compounds (Connolly and Hill, 1992) which are involved in many essential metabolic and regulatory activities. As shown in Figure I.1, these isoprenoid compounds are produced via a common biosynthetic pathway that leads from acetyl-CoA via mevalonate to a central intermediate isopentenyl diphosphate (IPP) (McGarvey and Croteau, 1995). IPP is the basic building block from which these compounds are derived. Structures of the natural products produced by this pathway contain these isoprene (C_5) units covalently linked. This multibranched pathway is one of the most complicated biosynthetic pathways in plants leading to such diverse compounds as photosynthetic pigments, growth regulators such as abscisic acid and gibberellins, phytoalexin antibiotics, sterols, plastiquinone, ubiquinone, isopentenyl-tRNA, and prenylated proteins (Bach, 1987; 1995). These compounds are necessary for a broad range of functions, including cell growth, reproduction, disease resistance, respiration and photosynthesis. Plants must produce these isoprenoid compounds in varying amounts at different developmental stages. Many of the above functions take place in different tissues, cell types, and organelles within the plant. Therefore, cells must be able to precisely regulate this pathway to ensure a constant production of multiple isoprenoids at each stage of growth and development.

I.1.2 Role of HMGR in the Pathway

The initial steps of the isoprenoid pathway involve the fusion of three molecules of acetyl-CoA to produce 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The HMG-CoA is then reduced to yield mevalonic acid in a NADPH-dependent double reduction. This step is catalyzed by mevalonate:NADP oxido reductase, CoA acylating; 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; EC 1.1.1.34) (Rogers *et al.*, 1983). HMGR has been rigorously studied in animals and yeast due to its importance in catalyzing the rate-limiting reaction in cholesterol biosynthesis (Goldstein and Brown, 1990) and is very highly regulated in these systems (Hampton *et al.*, 1996). However, the rate limiting nature of HMGR in plants and its regulation remain controversial (Chappell, 1995; Stermer, 1994). Evidence for the contribution of HMGR as the rate limiting enzyme in isoprenoid biosynthesis has come from several investigators (Narita and Gruissem, 1975; Chappell and Nable, 1987; Stermer and Bostock, 1987) in which they have shown an induction in isoprenoid biosynthesis and a concomitant increase in HMGR activity. However, it has also been demonstrated using pulse-labeling studies with [^{14}C] acetate and [^{3}H] mevalonate, that other enzymes further down the pathway are highly regulated and may also be key control points (Threlfall and Whitehead, 1988; Vogeli and Chappell, 1988; Chappell *et al.*, 1991; Chappell *et al.*, 1995). To more directly evaluate the role of HMGR in regulating carbon flow into plant isoprenoids, Chappell *et al.* (1995) constitutively expressed the hamster HMGR cDNA in tobacco (*Nicotiana tabacum* L.) plants resulting in an increase in total HMGR enzyme activity (3-6 fold) and overall sterol accumulation (3-10 fold). However, only some classes (e.g, sterols) of isoprenoids were affected while carotenoids, phytoalexins and the phytol chain of chlorophyll remained unchanged. There was an over accumulation of an

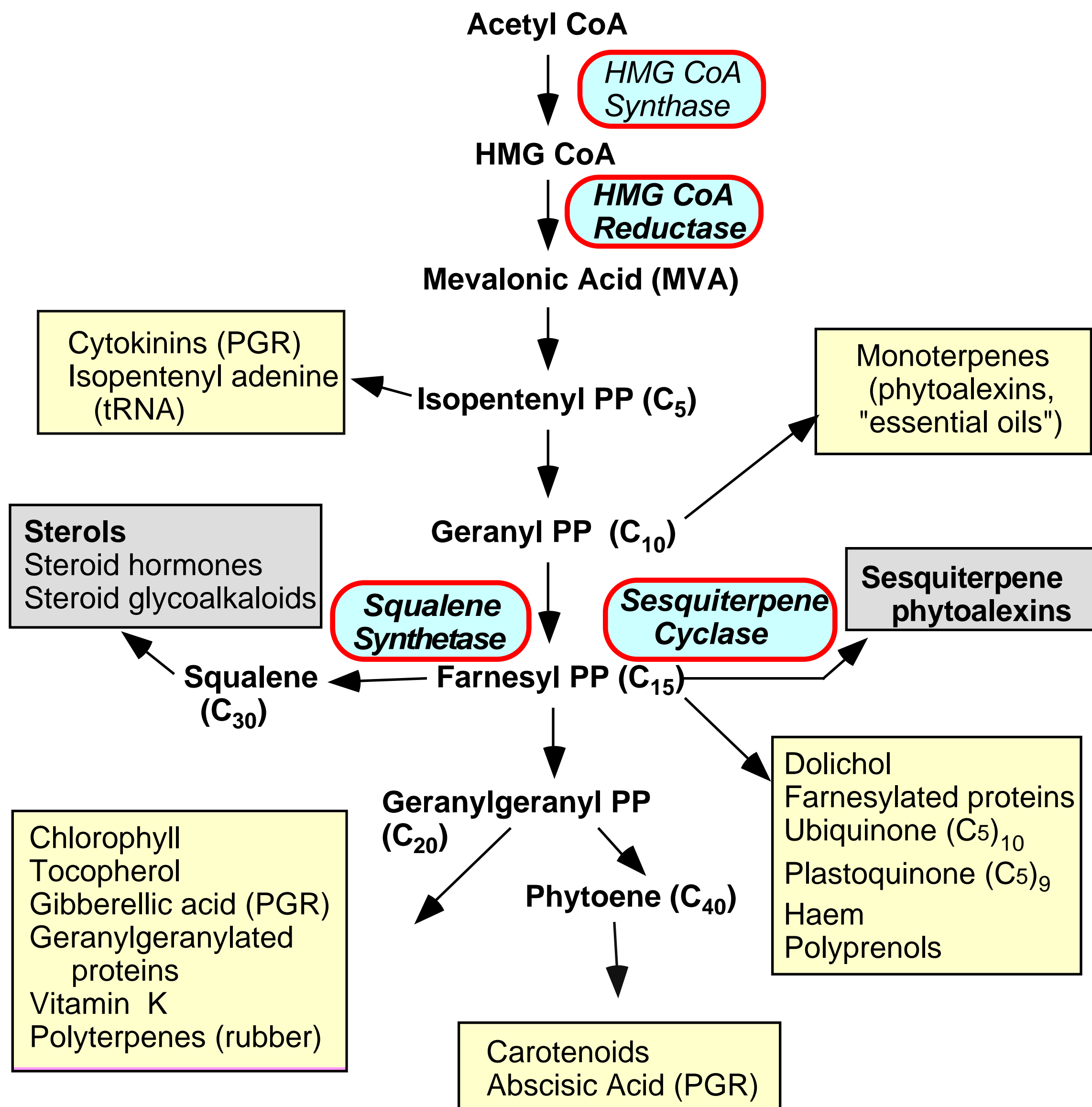

Fig. I.1 A simplified version of plant isoprenoid biosynthetic pathway, highlighting important endproducts. PP, pyrophosphate; PGR, plant growth regulator

intermediate in the sterol pathway, cycloartenol, however the common endproduct sterols (e.g, sitosterol, campesterol, stigmasterol) showed only a 2-fold increase. They suggested that channeling of later enzymes in the pathway may be rate-limiting, and that HMGR, may, in fact, not be the key rate-limiting step in the plant pathway, in contrast to its critical role in animal systems. However, additional studies support the role of specific isoforms of HMGR in mediating pathway flux especially during defense responses.

In Solanaceous plants, sesquiterpene phytoalexin antibiotics are an important determinant of disease resistance, and regulation of HMGR during defense responses has been widely studied (Stoessl *et al.*, 1976). HMGR activity in tobacco cell suspension cultures increases following fungal elicitor or cellulase treatment as did sesquiterpene cyclase, the key branch enzyme leading to sesquiterpene phytoalexin biosynthesis (Chappell *et al.*, 1991). However, a distinct elicitor, pectolyase, induced sesquiterpene cyclase activity and other defense-related proteins but not HMGR. These cultures did not produce the phytoalexin, capsidiol, suggesting that lack of the defense-specific HMGR isoform was rate-limiting. In another study (Threlfall and Whitehead, 1988), tobacco suspension cultures treated with cellulase, had inhibited cell growth. There was a rapid synthesis of sesquiterpenoid phytoalexins, at the same time squalene synthetase activity, the first committed enzyme for sterol biosynthesis, declined rather dramatically. They hypothesized that inhibition of this enzyme by elicitor might operate to channel carbon away from sterol biosynthesis and toward a defense response. These results indicated a highly coordinated regulation of several enzymes in the isoprenoid pathway. In recent results from our laboratory, transgenic plants constitutively expressing tomato *hmg*2, a defense-specific HMGR isoform, showed enhanced resistance to both viral and bacterial pathogens (Yu ,1995; Lång , 1997). Taken together, these results strongly support an important role for plant HMGRs in determining isoprenoid pathway flux and indicate that HMGRs are highly regulated by a variety of developmental and environmental signals such as light, wounding, infection, hormones, herbicides, and sterols (Bach, 1995).

I.1.3 Localization of HMGR Enzyme Activities in Plants

In mammals and yeast, HMGR activity is localized within the cytoplasm associated with the endoplasmic reticulum (ER) where sterol biosynthesis occurs (Brown and Simoni, 1984). The subcellular localization of plant HMGR is controversial, but enzyme activity has been associated with mitochondria (Bach *et al.*, 1986) and chloroplasts (Brooker and Russell, 1975a; Arebalo and Mitchell, 1984; Wilson and Russell, 1992) as well as the ER (Kondo and Oba, 1986; Enjuto *et al.*, 1994). Studies with pea on plastid and cytoplasmic HMGR activites reveal distinctive kinetic and regulatory properties (Brooker and Russell, 1975b; Brooker and Russell, 1979; Wong, *et al.*, 1982). HMGR activites are higher in rapidly growing parts of the plant such as apical buds and roots, but are lower in more mature tissues. Treatment with mevinolin (a competitive inhibitor of HMGR) can slow or inhibit plant growth and development (Gray, 1987; Narita and Gruissem, 1989). HMGR activity is highest in the early stages of tomato fruit development during rapid growth, but decreases during fruit ripening (Gillaspy *et al.*, 1993).

The existence of HMGR activites in different cell compartments supports the hypothesis originally proposed by Goodwin (1963), that different compartments contain separate biosynthetic pathways with all the necessary enzymes for the conversion of acetyl-CoA to the organelle specific isoprenoids. Membranes separating these compartments are impermeable to pathway intermediates. This is in contrast to a second theory proposed by Kreuz and Kleinig (1981) which suggested that IPP is formed solely in the cytosol and subsequently enters the different subcellular compartments for the synthesis of specific isoprenoids.

Although there is some evidence to support cytoplasmic formation of an IPP-pool via mevalonate, other studies suggest an alternative independent formation in plastids. Addition of inhibitors like mevinolin to intact protoplasts decreased sterol formation but had little or no effect on -carotene, plastoquinone and fatty acid formation, indicating a selective inhibition of cytosolic HMGR (Schulze-Siebert and Schultz, 1987). Chloroplasts isolated from guayule (*Parthenium argentatum* Gray) incorporated labeled carbon compounds from [^{14}C] bicarbonate, [2-^{14}C] pyruvate, and [U-^{14}C]-3-phosphoglycerate into isopentenyl diphosphate (IPP) indicating the ability to utilize Calvin cycle intermediates for IPP synthesis via acetyl CoA and mevalonate (Reddy and Das, 1987). Location of plastidic enzymes of IPP synthesis (Wong *et al.*, 1982; Arebalo and Mitchell, 1984) in *Pisum sativum* and *Nepeta cataria* support the concept of separate pathways for IPP synthesis and disagree with the central IPP-pool hypothesis. Evidence for the existence of a novel pathway for the early steps of isoprenoid biosynthesis leading to IPP formation was detected in eubacteria (Rohmer *et al.*, 1993). In this pathway, IPP is synthesized from precursors derived from triose phosphate metabolism and would not require HMG-CoA and MVA. The presence of this pathway in plants could explain the contradictory mevinolin studies, *ie.*, its ability to block cytosolic sterol biosynthesis, but its inability to completely block ubiquinone or plastidic isoprenoid synthesis. Isoprenoid biosynthesis was investigated in green alga *Scenedesmus obliquus* using ^{13}C-labeled glucose and acetate (Schwender *et al.,* 1996). According to labeling patterns, the synthesis of plastidic isoprenoids (*ie.* carotenoids, prenyl side-chains of chlorophylls and plastoquinone) as well as cytosolic sterols were synthesized via glyceraldehyde-3-phosphate/pyruvate and not from acetyl CoA via MVA. The existence of alternative mechanisms for the synthesis of the central IPP or MVA isoprenoid precursor is intriguing and consistent with putative symbiotic origins of plant organelles.

The existence of HMGR activities in various cellular compartments within the plant cell raises the possibility that multiple forms of the enzyme exist. This suggests that multiple HMGR genes might exist or that a single gene encodes for different forms through alternative mRNA processing or post-translational modifications. Discovery of the subcellular localization of HMGR may be important in understanding the regulation of isoprenoid biosynthesis.

I.1.4 Plant HMGRs and Regulation of Their Gene Expression

HMGR is encoded by a single gene in mammals (Chin *et al.*, 1984; Skalnik and Simoni, 1985; Luskey and Stevens, 1985), *Drosophila* (Gertler *et al.*, 1988), *Xenopus* (Chen and Shapiro, 1990), and by two related but divergent genes in yeast designated *hmg*1 and *hmg*2 (Basson *et al.*, 1986). In all plant species studied to date, HMGR is encoded by a small gene family of two

[*Arabidopsis thaliana*; Learned and Fink, 1989; Caelles *et al.*, 1989) or more members [*Lycopersicon esculentum* (tomato); Narita and Gruissem, 1989; Park *et al.*, 1992 , *Solanum tuberosum* (potato); Choi *et al.*, 1992; Stermer *et al.*, 1991; *Hevea brasiliensis* (rubber); Chye *et al.*, 1991; *Raphanus sativus* (radish); Ferrer *et al.*, 1990; *Pisum sativum* (pea); Bach *et al.*, 1991; *Nicotiana sylvestris* (tobacco); Genschik *et al.*, 1992; *Triticum aestivum* (wheat); Aoyagi *et al.*, 1993; and *Catharanthus roseus* (periwinkle); Maldonado-Mendoza *et al.*, 1992], which are differentially expressed during development or in response to stress. Plants regulate HMGR activity at the level of mRNA by differential induction of specific gene family members. In the rubber plant, *Hevea brasiliensis*, HMGR is encoded by a small gene family of three genes, *hmg*1, *hmg*2, and *hmg*3. Expression of *hmg*1 is inducible by ethylene, while *hmg*3 is constitutively expressed (Chye *et al.*, 1992). Also, *hmg*1 is expressed mostly in laticifers where rubber biosynthesis occurs, but *hmg*3 is not cell type specific. In potato which may contain seven or more genes, *hmg*1 is strongly induced in tubers by wounding, whereas *hmg*2 and *hmg*3 mRNAs accumulate at lower levels (Choi *et al.*, 1992). It is interesting that treatment of wounded tubers with arachidonic acid or the fungal pathogen *Phytophthora infestans* suppressed the wound response of *hmg*1, but strongly enhanced transcription of *hmg*2 and *hmg*3 mRNAs. This may suggest that *hmg*1 is associated with the branch of isoprenoid synthesis leading to sterols and sterol glycoalkaloids. Arachidonic acid induces the expression of *hmg*2 and subsequent accumulation of sesquiterpenoid phytoalexins. In tomato, the system studied in Cramer's laboratory, *hmg*1 expression is highest in rapidly growing tissue and is down-regulated by exogenously added sterols (Cottingham and Cramer, unpublished data). Expression of *hmg*1 is also high in immature fruit undergoing rapid cell division (Narita and Gruissem, 1989) and thus may function specifically in sterol biosynthesis and membrane growth. Tomato *hmg*2 shows a pattern of expression activated by wounding, fungal elicitors, and pathogens (Park *et al.*, 1992), but is not involved in fruit ripening or rapid cell growth. Results of these and other studies are consistent with the possible existence of families of isozymes each dedicated to the production of distinct classes of isoprenoids which are independently regulated.

I.2. TARGETING AND TOPOLOGY OF MAMMALIAN HMGR

I.2.1 Protein Targeting to the Endomembrane System

Eukaryotic cells are distinguished from prokaryotic cells by the presence of membrane-enclosed organelles within their cytoplasm These organelles divide the cytoplasm (which is much larger in eukaryotes than bacteria) into discreet subcellular compartments in which specific metabolic processes occur. The resulting subdivision of the cytoplasm allows eukaryotes to function efficiently. Organelles within the endomembrane system consist of the nucleus, endoplasmic reticulum, Golgi apparatus and lysosomes in animals or vacuoles in plants. Other organelles within the cells, such as mitochondria, chloroplasts, and peroxisomes are not part of the endomembrane system.

With the exception of a relatively small number of proteins synthesized in mitochondria and chloroplasts, the rest are synthesized by cytoplasmic ribosomes (Della-Cioppa *et al.*, 1987; Chrispeels, 1991; Bednarek and Raikhel, 1992) and must be transported into or across organellar

membranes to ensure that each subcellular compartment has its required set of proteins. The question arises as to how a nuclear-encoded protein such as HMGR is routed within the plant cell so as to accumulate in specific subcellular locations at the appropriate time.

Initial protein sorting begins while translation (*ie.*, protein synthesis) is still in progress. Proteins destined for the ER, Golgi, lysosomes, plasma membrane or secretion from the cell are synthesized on ER-bound ribosomes. As translation proceeds the polypeptide chains are transported into the ER where folding and further processing occur. From the ER, proteins are transported in vesicles to the Golgi where they are further processed and sorted for transport to lysosomes, plasma membrane or secretion from the cell (Wickner and Lodish, 1985; Chrispeels, 1991). Proteins that move through the secretory pathway (Fig. I.2) are targeted to the ER by a signal sequence which has been shown to reside within the amino acid sequence of the protein itself (Rapoport and Wiedmann, 1985). These sequences can be located anywhere, but are often found at the amino terminus as short hydrophobic stretches of 13-30 amino acids preceded by a stretch of basic residues, e.g., arginine. Sabatini and Blobel (1971) first proposed the "signal hypothesis" which states that proteins contain an encoded signal sequence which triggers attachment of the ribosome to the ER. As the signal peptide on a nascent polypeptide emerges from the ribosome, these sequences are recognized and bound by a signal recognition particle (SRP). The SRP temporarily inhibits translation and targets the complex to the rough ER by binding to a receptor or docking protein on the ER membrane (Blobel and Dobberstein, 1975). The signal sequence is inserted into the membrane, translation continues, and the polypeptide chain is translocated across the membrane. Finally, the signal sequence is usually cleaved by a signal peptidase and the protein is released in the ER lumen. The majority of ER targeted proteins are known to have cleavable signal peptides (Colman and Robinson, 1986), however, some ER proteins such as Sindbis Virus protein PE_2 (Bonatti and Blobel, 1979) and asialoglycoprotein receptor (Spiess *et al.*, 1985) do not.

Once the protein is released into the lumen of the ER, it can either continue through to the Golgi and be secreted or it can be retained in the ER or other endomembrane compartments based on additional targeting signals. The presence of the targeting sequence Lys-Asp-Glu-Leu (KDEL) at the carboxy-terminus is required for ER retention (Munro and Pelham 1987; Rothman and Orci, 1992). If this sequence is deleted from a protein normally retained in the ER (*e.g.,* protein disulfide isomerase or Binding Protein) the mutated protein is transported to the Golgi and subsequently secreted from the cell. If a KDEL or HDEL sequence is added to the carboxy terminus of normally secreted proteins, they are retained in the ER. This signal acts to selectively retrieve proteins from the Golgi and return them to the ER via a recycling pathway. The action of KDEL sequences as both retention/retrieval signals prevents loss of important ER proteins through the bulk flow of proteins in the secretory pathway leading from the ER to the cell surface (Jackson *et al.*, 1993; Schutze *et al.*, 1994).

Proteins can be retained as integral membrane proteins rather than being completely translocated across. Mammalian HMGR is an example of a protein which is targeted to and co-translationally integrated into the ER membrane (Brown and Simoni, 1984). Transmembrane

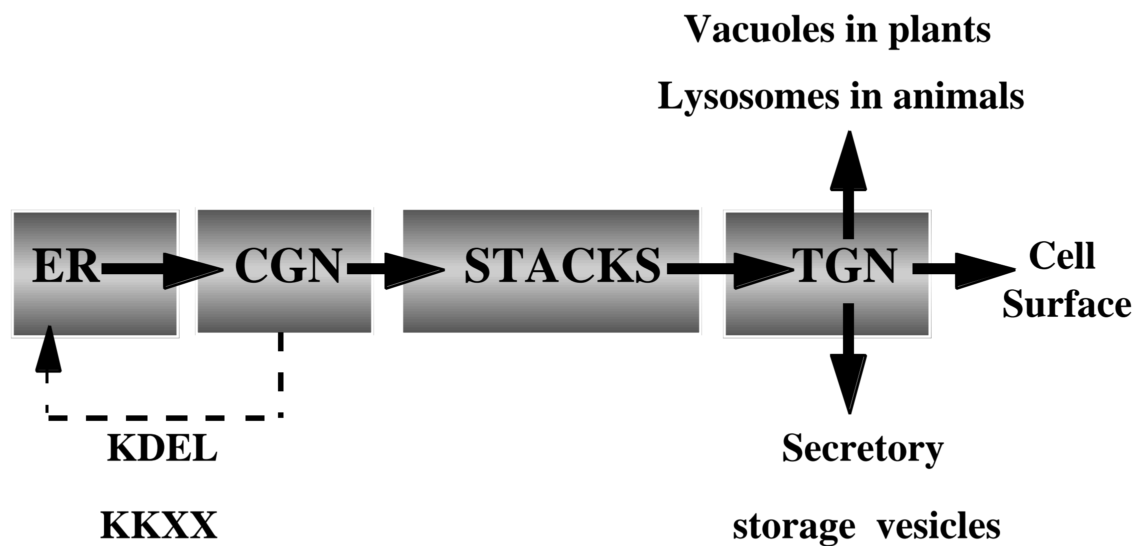

Fig. I.2 The secretory pathway in plants and animals. Protein entry into the endomembrane system requires a hydrophobic signal peptide. Anterograde movement through the pathway is indicated by thick solid arrows between the endoplasmic reticulum (ER), the *cis*-Golgi network (CGN), Golgi stacks, and *trans*-Golgi network (TGN) where the default pathway results in secretion into the extracellular space. Further sorting of proteins to plant vacuoles and animal lysosomes requires additional signals (Gal and Raikhel, 1993; Rothman and Orci, 1992). The dashed arrows indicate retrograde movement of ER-resident proteins which must have a KDEL or KKXX signal for retrieval/retention.

proteins can span the membrane once or several times and are classified into three groups based on their topology (Singer, 1990) (Fig. I.3). Group I proteins span the membrane once and have their N-termini within the ER lumen and their C-termini in the cytosol. Most, but not all of these proteins, have a normal amino terminal signal sequence which is cleaved during translocation. They are anchored by a single transmembrane stretch of hydrophobic residues. Group II proteins span the membrane in the opposite orientation, *ie.* C-termini in the ER lumen and N-termini in the cytoplasm. These proteins are anchored in the ER membrane by an internal signal sequence that is not cleaved. Group III proteins, of which HMGR is an example, span the membrane several times. Proteins with multiple transmembrane spans are inserted as a result of alternating internal signal sequences and stop-transfer sequences. As a result there can be looped domains exposed on both the lumenal and cytosolic sides. Thus, information found within the amino-terminal sequence of a protein in combination with its expression pattern and function may suggest its location within a cell. Because transmembrane proteins do not necessarily have their C-termini in the ER lumen, signals other than the C-terminal KDEL must be used for ER retention. The retention of some transmembrane proteins in the ER is dictated by short N-terminal sequences that contain two lysine or arginine residues (KKXX; Jackson *et al.*, 1993; RRXX; Schutze *et al.*, 1994).

I.2.2 Protein Targeting to Chloroplasts and Mitochondria

Although some proteins are synthesized within the mitochondria and chloroplasts, most proteins are translated on free cytosolic ribosomes and must be imported as completed polypeptide chains in an energy-requiring post-translational process. Import of proteins into both organelles is more complicated than transfer across the ER membrane (Ellis and Robinson, 1987; Keegstra, 1989). Mitochondria have two separate membranes (inner and outer membranes) creating two separate compartments (intermembrane space and matrix) and chloroplasts have three separate membranes (inner and outer membrane and thylakoid membrane) creating three separate compartments (intermembrane space, thylakoid lumen, and stroma). Each compartment, as well as each membrane, contains its own complement of proteins which all need to be sorted. Proteins are targeted to mitochondria via amino terminal sequences of 15-35 amino acids (presequences) that are removed by proteolytic cleavage following their import. These presequences contain several positively charged amino acids. They are recognized by receptors on the surface of the mitochondrion, and are translocated across the outer membrane into the inner membrane at an area where the two are in close proximity. Further translocation requires molecular chaperones since proteins must be partially unfolded before import, as well as energy and an electrical potential which drives membrane insertion of the positively charged presequences. The process in chloroplasts is similar but even more complicated and less well understood. Proteins targeted into chloroplasts have N-terminal sequences of 30 to 100 amino acids called transit peptides which direct the protein across the two membranes of the envelope and are then proteolytically removed. As with mitochondria, this occurs where two membranes are in close proximity. Again energy and molecular chaperones are required for unfolding proteins; however, an electrical potential is not required as there are no positively charged amino

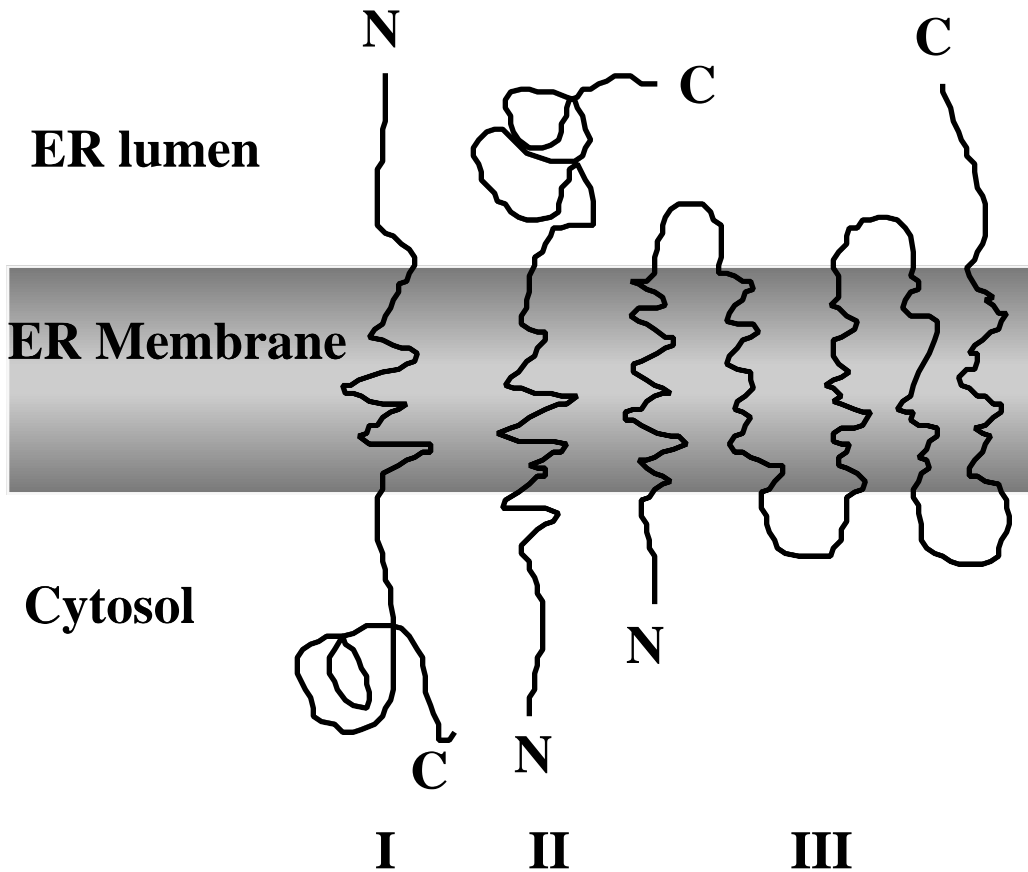

Fig. I.3. Types of integral membrane protein topology. Integral membrane proteins span the membrane via hydrophobic stretches of 20-25 amino acids which can be inserted in a variety of orientations (Singer, 1990). Type I and II proteins span the membrane once, but differ in whether the amino (N) or carboxy (C) terminus is on the cytosolic side. Type III is an example of a protein that has multiple membrane-spanning regions.

acids involved in the transit peptides. Further sorting of proteins within the chloroplast is required (Hageman *et al.*, 1990).

I.2.3 Protein Structure of Mammalian HMGR

Mammalian HMGR is a 97 kDa integral membrane protein of the ER (Brown and Simoni, 1984). The protein can be divided into three regions which include: 1) the N-terminus, 2) the linker region and 3) the C-terminus. The N-terminal region (about one-third) of the protein is extremely hydrophobic. This region is predicted to contain multiple transmembrane spans which bind the protein to the ER membrane (Liscum *et al.*, 1985). The 53-kDa C-terminal region contains the catalytic site of the enzyme. It is more hydrophilic and can be released from microsomes in an enzymatically active form by proteolysis. It therefore projects into the cytoplasm (Liscum *et al.*, 1985).

The region between the N- and C-terminal domains is termed the linker region. It contains the greatest amount of sequence divergence when comparing hamster and human proteins. This region is rich in amino acids characteristic of PEST sequences (proline, glutamic acid, serine, threonine). These PEST sequences are present in many short-lived proteins (Rogers *et al.*, 1983).

I.2.4 Role of Membrane Domain in Targeting and Topology

A model for the secondary structure and membrane orientation of mammalian HMGR was originally proposed using hydropathy analyses and limited proteolysis experiments, which predicted that the N-terminal domain contained seven membrane spanning regions (Liscum *et al.*, 1985) with the C-terminal catalytic site projecting into the cytosol. Each of the seven membrane-spanning regions is separated by a hydrophilic linker. These linkers contain charged residues which comprise loop structures. More recently, a topological model of HMGR has been proposed that consists of eight transmembrane spans (Olender and Simoni, 1992; Roitelman *et al.*, 1992). These studies (Olender and Simoni, 1992), using concanavalin A binding assays for *in vivo* glycosylation of engineered sites in linker segments between spans, as well as specific antibodies to each span, provided strong evidence for the presence of eight membrane spans. Using HMGal, a fusion protein consisting of the entire membrane domain and linker region of Syrian hamster HMGR fused to -galactosidase, the amino terminus (449 residues) was shown to be necessary and sufficient for targeting the protein to the ER (Skalnik *et al.,* 1988). In an *in vitro* translocation system, HMGR was targeted to microsomes in a SRP-dependent manner (Brown and Simoni, 1984) which was taken as evidence of a signal sequence specifically targeting HMGR to the ER. There was no cleavage of a N-terminal signal sequence. Fractionation, endoglycosidase-H sensitivity, and protease protection assays in an *in vitro* transcription/translation system using a mutant HMGR that only contained the first span (39 residues) provided evidence that it was the signal sequence for targeting to the ER. The protein was associated with microsomes, glycosylated and protected from proteolysis in the presence of SRP (Olender and Simoni, 1992).

I.3 Sterol-Mediated Regulation of Mammalian HMGR

I.3.1 Transcriptional Down-Regulation

Mechanisms for multivalent feedback regulation of mammalian HMGR have been rigorously studied and have been found to be very complex (reviewed in Goldstein and Brown, 1990; Edwards *et al.*, 1992; Roitelman and Simoni, 1992; Correll and Edwards, 1994). Steady-state levels of HMGR must be critically maintained within proscribed limits so as to ensure the appropriate amounts of pathway endproducts. This regulation is achieved through a combination of transcription, translation, and protein stability (Brown and Goldstein, 1990). Expression of mammalian HMGR is down-regulated by sterols due to a decline in transcription of mRNA, which causes mRNA levels to fall and enzyme synthesis to decline (Chin *et al.*, 1982; Luskey *et al.*, 1983). Sterols in the form of plasma low density lipoprotein (LDL) and 25-hydroxycholesterol suppress HMGR activity by greater than 95%. This suppression is correlated with decreased synthesis of HMGR mRNA as indicated by a decrease in the rate of incorporation of [^{3}H] uridine into RNA. Much of the initial evidence for transcriptional down-regulation of HMGR was obtained in studies using cultured fibroblasts (Brown *et al.*, 1978; Faust *et al.*, 1979). Human fibroblasts grown in cell culture have low HMGR activities because cell surface receptors bind to low-density lipoproteins (LDLs) found in serum resulting in uptake by endocytosis and subsequent release of cholesterol which inhibits the need for synthesis of mevalonate. If the fibroblasts are transferred to a medium deficient in serum LDL, HMGR activity increases with a concommitant increase in cholesterol synthesis. If LDL is added back to the culture medium, HMGR activity is suppressed but never by 100% (Brown and Goldstein, 1980). About 5% of HMGR activity is mediated by a non-sterol component derived from mevalonate which inhibits translation of the mRNA (Goldstein and Brown, 1990). This is necessary to insure that cells have sufficient HMGR to produce small amounts of MVA that can be preferentially shunted to non-sterol products (e.g. protein prenylation) even if cholesterol levels are high.

Molecular techniques including gene cloning and fusion proteins have made it possible to study the mechanism whereby this transcriptional control occurs. The 5’ flanking region of hamster HMGR (nucleotides -1420 to -23 of the promoter region) and various deletions within this region were fused to a bacterial chloramphenicol acetyltransferase (CAT) reporter gene and transfected into mouse L cells (Osborne *et al.*, 1985). The -513 to -1021 region of the reductase promoter directed complete promoter function and contained multiple copies (5) of the hexanucleotide repeat CCGCCC or its complement GGGCGG which is known as a sterol response element (SRE). Sterols suppressed expression of the chimeric genes by 70 to 80% if the SRE was intact. SREs are also present in other genes such as the SV40 promoter (Fromm and Berg, 1982) and thymidine kinase promoter (McKnight and Kingsbury, 1982). DNA-binding proteins of the nuclear factor 1 (NF1) family have been identified which bind to this *cis*-element in the reductase promoter and facilitate transcription (Gil *et al.*, 1988).

I.3.2. Protein Degradation

Many integral membrane proteins are degraded in the mammalian ER, and HMGR is no exception (Chun *et al.*, 1990). It is degraded ten times more rapidly than other ER proteins (Lecureux and Wattenburg, 1994). One of the physiological functions of ER degradation results

from proof-reading of misfolded and mutant proteins, but correctly made proteins are also degraded by the ER providing another mechanism whereby proteins are regulated in cells.

Both sterols and nonsterols acting together, accelerate the targeted degradation of mammalian HMGR (Nakanishi *et al.*, 1988; Goldstein and Brown, 1990). Insight into the mechanism of HMGR regulation through degradation of the enzyme has come from studies of a line of cultured Chinese hamster ovary (CHO) cells called UT-1 (Chin *et al.*, 1982) which was selected for growth in high levels of compactin, a competitive inhibitor of HMGR. These compactin-resistant cells produced large amounts of HMGR which makes up 1- 2% of the total cell protein. This 500-fold increase in HMGR resulted from a 15-fold increase in transcription of the gene and a decreased rate of degradation of the protein. The cells degrade HMGR slowly when cultured in compactin and in the absence of sterols. If sterols are added, the rate of degradation of HMGR as revealed by pulse-chase experiments with [^{35}S]methionine, is about 2-7 times faster than normal (Faust *et al.*, 1982; Edwards *et al.*, 1983; Nakanishi *et al.*, 1988; Chin *et al.,* 1985; Chun *et al.*, 1990).

It was found from UT-1 experiments that the sterol-mediated degradation rate of HMGR was governed by the membrane domain (Liscum *et al.*, 1985; Gil *et al.*, 1985).
Further dissection of the domains led to a more precise structure responsible for degradation (Skalnik *et al.*, 1988). The membrane domain with spans 4 and 5 deleted was fused to -galactosidase (HMGal). When expressed in CHO cells, the enzyme retained catalytic activity (Jingami *et al.,* 1987). The degradation of this mutant was not accelerated by sterols, and in the absence of sterols, it was less stable than the full-length HMGR. Additional mutant HMGal proteins using Syrian hamster HMGR in CHO cells further delineated the role of the membrane spans and linker region, which lies between the membrane domain and the catalytic site, in the regulated degradation of HMGR (Chun and Simoni, 1992). If they swapped each span independently with the first transmembrane sequence from bacteriorhodopsin, they found that replacing the 4th span had no effect on degradation, but that the 5th-8th spans were important for the regulated degradation of HMGR. Replacement of the 5th and 6th spans with the bacteriorhodopsin transmembrane span resulted in protein being degraded at a normal basal rate, but the rate was not accelerated by MVA, LDL cholesterol or 25-hydroxycholesterol. Replacement of the 7th span resulted in a short-lived protein that was degraded more rapidly in response to MVA but not exogenous sterols. If the 8th span was replaced the basal and MVA-accelerated half-life of HMGR was extended by 5 fold. Replacement of the third transmembrane domain resulted in abnormal ER localization, but had no effect on the regulated degradation of the protein. They found that deletion of the PEST sequences had no effect on degradation, but deletion of the entire linker region resulted in the protein being insensitive to sterol and MVA. There are no features other than hydrophobicity which would indicate why some spans are important and others are not for the regulated degradation of HMGR. No one has deleted specific amino acids to further delineate their possible role in degradation. Yeast HMGR, like the mammalian enzyme, has a large transmembrane domain (7-8 spans), that also functions in end-product-mediated degradation (Hampton and Rine, 1994).

I.4 Regulation of Plant HMGRs

I.4.1 Membrane Domain Structure

The isolation of cDNA and genomic clones of several plant HMGRs has allowed comparisons of their deduced protein sequences across plant species as well as between those of yeast and mammals (Basson *et al.*, 1988; Learned and Fink, 1989; Liscum *et al.*, 1984; Luskey and Stevens, 1985; Park *et al.*, 1992). The general structure of HMGR is similar among plants. As with mammalian and yeast HMGRs, there are three distinct regions which also include a N-terminal transmembrane domain, linker region, and C-terminal domain. The C-terminal portion of the protein, which includes the catalytic domain, is highly conserved across all plant species (74 to 98%) as well as animal and yeast HMGRs (65% identity). The linker region is highly divergent in both size and sequence among all HMGRs in both plants and animals. In common with animal HMGRs is the presence of different versions of PEST sequences. The most striking difference is in the size of the N-terminal domain in plants which contains the putative membrane spanning region (Fig. I.4). Hydropathy profiles of the N-terminus of *Arabidopsis thaliana*, show two hydrophobic sequences. These residues are long enough to span the membrane bilayer, but a controversy exists as to whether there are one or two potential membrane spans (Learned and Fink, 1989; Caelles, *et al.*, 1989). The C-terminal third of plant HMGRs show a wide range of conservation (38 to 97% amino acid identity). However, the putative transmembrane spans of plant HMGRs are highly conserved among plant species (see Figure I.4) which may be a reflection of functional importance. Although the membrane domain of plants is much simpler than that of animals and yeast, this conservation may reflect the importance of this region in anchoring the enzyme to a specific membrane or regulation of the enzyme in response to some physiological or environmental stimuli. More research is needed to precisely define the transmembrane domain in plant HMGRs and its role in the cell.

I.4.2 Evidence for Transcriptional Regulation

Plants use a different strategy than mammals in regulating HMGR. There are multiple forms of HMGR in plants which are distinctly regulated at the gene and protein level (Brooker and Russell, 1975;1979; Choi *et al.*, 1992; Chye *et al.*, 1992; Learned, 1996; Chappell and Nable, 1987; Vogeli and Chappell, 1991; Ji *et al.*, 1992). Unlike mammalian HMGR, there is clear activation of transcription of specific plant HMGRs as
well as suppression or down-regulation as seen in mammalian systems. This coordinated activation or suppression of specific HMGRs serves to channel carbon into sterol synthesis through squalene synthetase or phytoalexins via sesquiterpene cyclase (reviewed in Bach, 1995; Cramer *et al.*, 1993). This is most clearly observed in the defense response. There is a channeling of isoprenoid biosynthesis away from sterol production toward sesquiterpenoid phytoalexins when wounded potato tubers are exposed to fungal elicitors (Chappell and Nable, 1987; Choi *et al.*, 1992). A similar pattern is observed in elicitor-treated tobacco cell suspensions (Vogeli and Chappell, 1991).

Another clear example of transcriptional activation of specific HMGRs is observed in rubber (a *cis*-polyisoprene) biosynthesis. The expression of *hmg*1 mRNA is induced by

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1    M D - V R R R S E E P V Y P S K V F A A D E - K P L K P H K  L.e. HMG2
1    M D - L R R R P P K P P V T N N N N S N G S F R S Y Q P R T  A.t. HMG1
1    M D E V R R R P P K H I V R K D - - - H D G - E V L N S F S  H.b. HMG3

29   K Q Q Q Q Q E D K N T L L I D - - A S D A L P L P L Y L T N  L.e. HMG2
30   S D D D H R R R A T T I A P P P K A S D A L P L P L Y L T N  A.t. HMG1
27   H G H H L P P - - - - - L K P - - S D Y S L P L S L Y L A N  H.b. HMG3

57   G L F F T M F F S V M Y F L L S R W R E K I R N S T P L H V  L.e. HMG2
60   A V F F T L F F S V A Y Y L L H R W R D K I R Y N T P L H V  A.t. HMG1
50   A L V F S L F F S V A Y F L L H R W R E K I R K S T P L H I  H.b. HMG3

87   V T L S E L G A I V S L I A S V I Y L L G F F G I G F V Q T  L.e. HMG2
90   V T I T E L G A I I A L I A S F I Y L L G F F G I D F V Q S  A.t. HMG1
80   V T F P E I A A L I C L V A S V I Y L L G F F G I G F V H S  H.b. HMG3

117  F V S R G N N D S W D E - - - N D E E F L L K E D S R - - -  L.e. HMG2
120  F I S R A S G D A W D L A D - - - - - - T I D D D D H R L V  A.t. HMG1
110  F - S R A S T D S W D V E E Y D D D N I I I K E D T R P T G  H.b. HMG3

141  - C G P A T T L G C A V P A P P A R Q I A P M A P P Q P S M  L.e. HMG2
144  T C S P P T P I V S V A K L P - - - - - - - - - - - - - - -  A.t. HMG1
139  A C A A P S - L D C S L S L P - T K I H A P I V S T T T T S  H.b. HMG3

170  S M V E K P A P L I T S A S S G E D E E I I K S V V Q G K I  L.e. HMG2
159  - - - - N P E P I V T E S L P E E D E E I V K S V I D G V I  A.t. HMG1
167  T L - - - - - - - - - - - - - S D D D E Q I I K S V V S G S I  H.b. HMG3

200  P S Y S L E S K L G D C K R A A S I R K E V M Q R I T G K S  L.e. HMG2
185  P S Y S L E S R L G D C K R A A S I R R E A L Q R V T G R S  A.t. HMG1
185  P S Y S L E S K L G N C K R A A L I R R E T L Q R M S G R S  H.b. HMG3

230  L E G L P L E G F N Y E S I L G Q C C E M P I G Y V Q I P V  L.e. HMG2
215  I E G L P L D G F D Y E S I L G Q C C E M P V G Y I Q I P V  A.t. HMG1
215  L E G L P L D G F D Y E S I L G Q C C E M A I G Y V Q I P V  H.b. HMG3

260  G I A G P L L L N G K E F S V P M A T T E G C L V A S T N R  L.e. HMG2
245  G I A G P L L L D G Y E Y S V P M A T T E G C L V A S T N R  A.t. HMG1
245  G I A G P L L L D G K E Y T V P M A T T E G C L V A S A N R  H.b. HMG3
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Linker

Fig. I.4. Deduced amino acid sequence comparisons of three plant HMGR N- termini. Sequences include *L. esculentum* HMG2 (L.e. HMG2; Park *et al*., 1990), *Arabidopsis thaliana* HMG1 (A.t. HMG1; Caelles *et al*., 1989), and rubber tree (*Hevea brasiliensis*) HMG3 (H.b. HMG3; Chye *et al*., 1991). Sequences identical to tomato *hmg*2 are shaded. Dashes are inserted by the alignment program (DNA STAR MegAlign, Clustal method) to optimize alignment.

ethylene and is highly expressed in laticifers where rubber synthesis occurs, while *hmg*3 is not affected (Chye *et al*., 1992). The *hmg*3 isoform is probably involved in isoprenoid biosynthesis of a "housekeeping" nature, which infers it is constitutively expressed in the plant.

Like mammalian HMGR, specific plant HMGRs are subject to feedback regulation by end-products of the pathway although the role of transcription versus post-translation regulation has not been addressed. More complex control is apparent since environmental stimuli such as light also affect HMGR activity and HMGR transcriptional regulation (Brooker and Russell, 1979; Ji *et al*., 1992; Learned, 1992). After treatment of etiolated pea seedlings with cholesterol or the plant hormone abscisic acid, HMGR activity was reduced, but gibberellic acid had no effect (Brooker and Russell, 1979). These authors also reported that a decrease in HMGR activity of etiolated seedlings was observed following irradiation with red light or continuous white light. More recently, HMGR mRNA has been shown to accumulate in dark-grown *Arabidopsis*, but *hmg*1 was suppressed in response to light (Learned, 1996). Using promoter/reporter gene fusions it was demonstrated that the suppression of *hmg*1 mRNA in response to light was mediated by cis-acting elements within the promoter. While it is clear that plant HMGRs are regulated at the transcriptional level, it is not clear if there is comparable regulation at the level of protein turnover between plants and mammalian systems. Since plants have a much reduced membrane domain as compared to mammalian HMGR, it is interesting to speculate whether or not the domain plays a role in the regulated degradation of plant HMGRs.

I.5 SIGNIFICANCE AND OBJECTIVES OF RESEARCH

The overall objective of this research was to characterize the membrane domain of plant HMGR enzymes by analyzing its role in targeting and orienting the protein within the membrane.

The first specific objective of this research was to isolate and sequence a cDNA clone encoding the putative membrane-spanning region of tomato HMG1 in order to compare this with the analogous region in HMG2 which had previously been isolated (Park *et al*., 1992).

The second specific objective was to characterize the membrane spanning domains of the differentially expressed tomato HMGRs, HMG1 and HMG2, to ascertain their role in targeting and orientation of the protein within the membrane. *In vitro* systems using microsomal membranes for targeting and protease protection assays were performed on these two distinct tomato HMGR isoforms to establish membrane topography, protein insertion mechanisms, and subcellular localization of specific isozymes.

The third specific objective was to tag HMG1 membrane domain sequences with an epitope tag and express it in transgenic *Nicotiana tabacum* BY-2 and/or Kentucky 14 cell lines. These cell lines were used to identify optimal cultures for stable transformation with the FLAG-tagged *hmg*1.

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CHAPTER II

The N-Terminal Domain of Tomato 3-Hydroxy-3-Methylglutaryl-CoA Reductases: Sequence, Microsomal Targeting, and Glycosylation

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CHAPTER III

In Vitro Targeting and Expression of an Epitope-Tagged Tomato HMG1 Membrane Domain in Transgenic Tobacco Cells

III.1 Abstract

The basis for our understanding of HMGR regulatory mechanisms comes from work with mammalian and yeast systems where it has been shown that the membrane domain is responsible for targeting and anchoring the protein within the ER membrane as well as its rapid degradation in response to sterol. In order to more precisely determine signals required in targeting tomato HMG1 to the ER and the final location of HMG1 within the endomembrane system, two separate constructs containing the membrane domain were generated. Both contained sequences encoding a FLAG epitope at the C-terminus of the membrane domain, but lacked the *hmg*1 upstream leader sequences. One construct, pCD6, was designed to eliminate the highly charged amino acids, e.g. 2-6 (M**DVRRR**) to determine their role as potential targeting signals. The other construct, pCD7, encoded the entire membrane domain, beginning at the original ATG codon, thus serving as a control. *In vitro* transcription/translation studies showed that neither change altered targeting to microsomes or orientation of the protein within the membrane.

Two tobacco cell lines were used to compare stable transformation with the FLAG-tagged *hmg*1 constructs for future *in vivo* studies on HMGR regulation. BY-2 cells have been widely used and well characterized, and exhibit fast, synchronous growth. KY-14 cells have been well characterized with respect to defense responses and capsidiol production. Treatment of nontransformed BY-2 and KY-14 cells with sterol resulted in decreased HMGR activity by 3 hr, with KY-14 cells showing the greater reduction. Treatment of BY-2 and KY-14 cells with cellulase, a defense elicitor, revealed differences in defense responses between the cultures. KY-14 cells were maximally induced by 9 hr as indicated by increased HMGR activity, whereas BY-2 cells showed little or no response in enzyme activity. Using *hmg1* and *hmg*2 gene-specific probes, it was shown that cellulase treatment of KY-14 resulted in elevated *hmg*2 mRNA levels indicating activated defense responses.

The construct, pCD7, was cloned into the plant expression vector, pBIB-Kan under the direction of a double enhanced 35S constitutive promoter and used in *Agrobacterium*-mediated transformation of tobacco cells. Although transformation resulted in kanamycin-resistant calli for both cell lines, the slow growth of KY-14 hindered further analysis. However, Northern analysis of transformed BY-2 cells revealed high level expression of the HMG1-FLAG transgene.

III.2 Introduction

The conversion of HMG-CoA to mevalonate is catalyzed by the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) (Bach, 1995). Plant HMGRs studied thus far are encoded by multigene families of two or more members (Learned and Fink, 1989; Caelles *et al.*, 1989; Narita and Gruissem, 1989; Park *et al.*, 1992; Choi *et al.*, 1992; Stermer *et al.*, 1991; Chye *et al.*, 1991; Ferrer *et al.*, 1990; Bach *et al.*, 1991; Genschik *et al.*, 1992; Maldonado-Mendoza *et al.*, 1992). These genes are differentially expressed during development or in response to environmental cues, indicating that particular isozymes might be involved in pathway partitioning leading to the synthesis of specific isoprenoid endproducts (Chappell, 1995a, 1995b; Weissenborn *et al.*, 1995). It has been hypothesized that pathway partitioning may be mediated by protein-protein interactions leading to channeling of intermediates or by

differential subcellular compartmentation (Bach, 1995; Cramer, *et al.*, 1993). Recent *in vitro* studies (Denbow *et al.*, 1996; Campos and Boronat, 1995) have shown that plant HMGR isoforms are primarily targeted to the endoplasmic reticulum (ER), however, their precise location within the endomembrane system and the mechanisms mediating their activities at the transcriptional and post-translational levels remain largely unknown. Based on studies in mammals and yeast, the HMGR membrane domain is a critical determinant of subcellular targeting as well as end-product mediated degradation of HMGR (Goldstein and Brown, 1990; Hampton and Rine, 1994).

Plant HMGR activities have been associated with membrane fractions derived from the ER , plastids, and mitochondria (reviewed by Bach, 1995). We have shown that both tomato HMG1 and HMG2 are targeted to microsomal membranes *in vitro* (Denbow *et al.*, 1996). These results are in agreement with those of Campos and Boronat (1995) who demonstrated that both *Arabidopsis* HMG1 and HMG2 were also targeted to microsomes. Using *in vitro* systems, we demonstrated that sequences within the amino terminus were necessary and sufficient to target both HMGRs to microsomal membranes. These peptides contained two hydrophobic stretches and spanned the membrane twice, positioning both N- and C-termini in the cytosol. These *in vitro* studies, however, did not allow us to determine the exact location of specific isoforms within the endomembrane system. The possibility that specific plant HMGR isozymes are targeted to other organelles or locations within the endomembrane system after insertion into the ER membrane cannot be ruled out. The lack of isozyme-specific antibodies to plant HMGRs has limited research efforts to follow specific isozyme localization within the endomembrane system. Recently, Lång (1997) used a *c-myc* epitope to tag tomato HMG2, the defense specific tomato HMGR isoform, and expressed the construct in transgenic tobacco. HMG2-c-myc was observed in small clusters associated with the ER. Following pathogen treatment with tobacco mosaic virus (TMV), HMG2-c-myc was co-localized with sesquiterpene cyclase in the vacuoles of leaf cells surrounding the TMV lesion. Lång's results support the hypothesis that the defense-inducible isoprenoid biosynthetic enzymes form a distinct "metabolon" for production of sesquiterpenoid phytoalexins (Lång, 1997; Cramer *et al.*, 1993; Bach, 1995). This novel localization to vacuoles suggests that compartmentation may also play a role in pathway partitioning. In contrast to the defense-related *hmg*2, *hmg*1 is coordinately regulated with squalene synthesis and thus, presumably involved in sterol biosynthesis, a function localized to ER in mammals and yeast (Bishop and Bell, 1988). We were interested in determining whether tomato HMG1 is similarly ER-localized and/or differentially targeted compared to HMG2 and in identifying signals involved in specific subcellular targeting.

Signals involving transport and/or retention of plant HMGRs within the endomembrane system can be located in various regions within their amino termini. Comparisons of the membrane domain of plant HMGRs show that in addition to the two membrane spanning domains, the N-terminal first six amino acids *ie.*, MetAspXArgArgArg (where X can be Val, Ile, Leu, or Ser) are highly conserved suggesting functional significance (see Chapter I, Fig. I.4; Chapter II, Fig. II.2). A motif containing two arginines (RRXX, where X can be any amino acid)

has been shown to be involved in retrieval of type II transmembrane proteins that moved on to the Golgi and must be brought back to the ER (Schutze *et al*., 1994). It is tempting to speculate that this sequence might be involved in targeting plant HMGRs to the ER or in ER retrieval. HMGRs that lack this motif may have different locations within the endomembrane system.

In order to test the role of the N-terminal motif (MDVRRR) in targeting and localization within the endomembrane system, constructs encoding the HMG1 N-terminal membrane domain, and a modified N-terminal domain lacking amino acids 2-6, were engineered. Both constructs were modified to encode a C-terminal FLAG epitope tag. The FLAG tagging system (Hopp *et al*., 1988) uses a highly hydrophilic sequence for which monoclonal antibodies are available (IBI Technologies).

In vitro targeting studies were used to determine if elimination of 1) the plant derived *hmg1* leader sequences upstream of the original ATG or 2) the highly charged N-terminal sequences would effect translation and microsomal targeting of HMG1.

The FLAG-tagged full-length *hmg*1 membrane domain was subsequently fused to a strong constitutive promoter (double enhanced 35S) in a binary vector for *Agrobacterium*-mediated transformation for the purpose of developing transgenic tobacco cell lines. Two tobacco cell lines, BY-2 and KY-14 (recently established from cultivar Kentucky 14), were used for transformation. In order to compare the two tobacco cell cultures for studies involving differential regulation of plant HMGRs, BY-2 and KY-14 untransformed cell suspension cultures were tested for the effect of cellulase and sterol treatment on HMGR enzyme activity and mRNA levels.

III.3 Experimental Procedures

III.3.1 Cell Cultures and Sterol or Elicitor Treatment

Two tobacco *Nicotiana tabacum* L. cell suspension cultures were used for stable transformation and sterol or elicitor treatment. BY-2 cells were originally established from the callus induced from a seedling of *Nicotiana tabacum* L. cv. Bright Yellow 2 (Nagata *et al.,* 1992) and were provided by Dr. Richard Cyr (Pennsylvania State Univ.). The tobacco BY-2 cell line possesses exceptionally high growth rates (doubling time=16 hr) and lacks nicotine production. BY-2 cell suspension cultures were grown in Murashige-Skoog (MS) medium as previously described (Qian *et al*., 1996). The other cell line, KY-14, was established from *N. tabacum* L. cv. Kentucky-14 (Chappell, *et al*., 1991) and provided by Dr. Joe Chappell (Univ. of Kentucky). Cell suspension cultures of KY-14 were grown in modified Murashige-Skoog medium (with five-fold higher 2-4-dichlorophenoxyacetic acid than in BY-2 medium) (Lazzeri *et al*., 1985). KY-14 cultures grew with an approximate doubling time of 48 hr and were subcultured every 7 d. Cell cultures in log phase, 3 days after subculturing, were used for all experimental manipulations described. Cellulase (0.1 µg/ml; Sigma Chemical Co., St. Louis, MO) or 25-hydroxycholesterol (100 µg/ml; Sigma) was added to 50 ml cell cultures. Cells were harvested by filtering through Miracloth (Calbiochem, LaJolla, CA) and quickly frozen in liquid nitrogen at various times following treatment.

III.3.2 Construction of FLAG tagged vector

Two complementary oligonucleotides containing sequences encoding the FLAG epitope (DYKDDDDK) (5'-GAGGATCCCGACTACAAGGACGACGATG ACAAG**TAG**GAGCTCGCT-3') (DNAgency, Aston, PA) were inserted into a pSP64poly (A) vector (Promega Corp., Madison, WI). They were designed to contain flanking *Bam*HI and *Sst*I restriction sites (underlined above), an in-frame stop codon (bold), and FLAG sequences (*Bam*HI-FLAG-STOP-*Sst*1). As shown in Fig. III.1, the annealed oligos were digested with *Bam*HI and *Sst*I and ligated into the multiple cloning site of pSP64poly(A) vector (Promega, Corp., Madison, WI). The resulting plasmid, pSP64FLAGpoly(A), was sequenced using Sequenase version 2.0 (U.S. Biochem., Cleveland, OH) as per manufacturer's protocols to verify junctions and orientation of the FLAG sequences (Fig.III.1).

III.3.3 PCR amplification and cloning of FLAG-tagged HMG1 membrane domain

The membrane domain of HMG1 was previously cloned (Chapter 2, Denbow *et al.*, 1996) and the resulting plasmid was referred to as pCD4. The membrane domain of HMG1 was amplified via the polymerase chain reaction (PCR) using 2 ng pCD4 plasmid, 1 μM of each *hmg*1 specific primer and 2.5 U Pfu DNA polymerase (Stratagene, LaJolla, CA) for 25 cycles: 95°C, 1 min; 50°C, 2 min; 72 °C, 3 min). PCR primers were designed to amplify regions that began at the ATG translation start codon or 18 base pairs downstream from the original start codon, which would delete the first highly charged six amino acids. The sense primers 1 (5'-CGCTGCAGATGGACGTTCGCCGGCGACCA-3') or 2 (5'-CGCTGCAGATG CCAGTTAAGCCTTTATGCACA-3') were constructed to contain a flanking *Pst*I restriction site (underlined). The antisense primer 15 (5'GAGAAGAAAACTCACTTGTTGAAC) was previously used in the original cloning of the plasmid, pCD4 (Denbow *et al.*, 1996). The approximately 1 kilobase PCR fragments, which lacked the 188 bp tomato *hmg*1 5'-leader sequences present in the original clone, were then cloned into the newly created pSP64FLAGpolyA vector. The resulting clones were named pCD7 (containing the full-length HMG1 membrane domain beginning at the original ATG but without the original upstream sequences) or pCD6 (which had the first 18 bp deleted and an engineered ATG) (Fig.III.1). These two plasmids were then used for coupled *in vitro* transcription/translation assays and pCD7 was subsequently cloned into a plant expression vector (described below) for transformation of tobacco cells.

III.3.4 *In vitro* transcription/translation of FLAG-tagged *hmg*1

RNA was transcribed *in vitro* from plasmids pCD6 and pCD7 using SP6 RNA polymerase as previously described (Chapter 2; Denbow *et al.*, 1996). Translation of *in vitro* synthesized RNA and subsequent separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was also described previously (Chapter 2; Denbow *et al.*, 1996).

III.3.5 Western blotting and immunodetection of FLAG-tagged peptides

Five nonradioactive translations were combined and separated on a 10% SDS-PAGE gel and proteins were transferred to polyvinylidene difluoride transfer membrane (DuPont NEN) as previously described (Chapter 2; Denbow *et al*, 1996). Filters were incubated overnight at 4°C

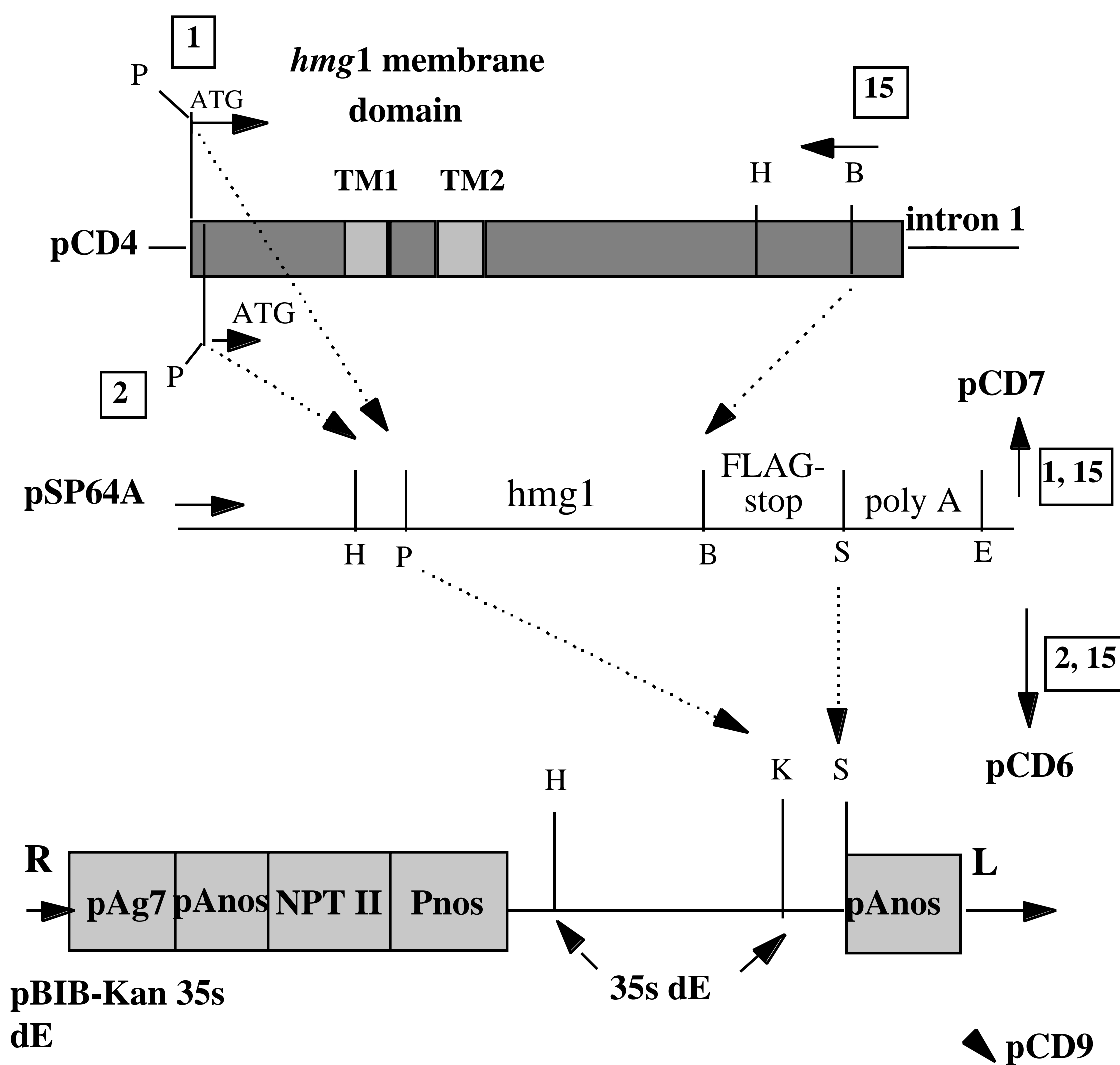

Fig. III.1 Partial map of vectors constructed for *in vitro* transcription/translation studies and tobacco cell culture transformation with FLAG-tagged HMG1 membrane domain. See section III.3.2 and III.3.3 for details of the cloning procedures. Hatched regions show the approximate location of the two membrane spanning domains (TM). Restriction endonucleases: B, *BamH*I; E, *EcoR*I; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *SstI*. Primers used in PCR are designated by squares.

with 10 μg/ml of the anti-FLAG M2 mouse monoclonal antibody (IBI Technologies). The second antibody, horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (Amersham Corp.), was diluted 1:10,000 and detection was done using an ECL Western blotting detection system (Amersham Corp.) according to manufacturer's protocols except that additional blocking agent (5% dry milk) was included in the antibody incubation steps.

III.3.6 Construction of plant expression vectors

The tomato HMG1 membrane domain beginning at the ATG and modified to contain a FLAG epitope at the C-terminus (pCD7) was digested with *Pst*I and treated with Mung-bean nuclease to create a blunt-end. A second digestion with *Sst*I was performed and the resulting fragment was cloned into a derivative of pBIB-Kan (Becker, 1990) which contains a 0.9 kb 35S double enhanced (35S dE) version of the CaMV 35S promoter and a TEV leader sequence (Carrington and Freed, 1990) from pRTL2 (generously provided by Dr. J. Mullet, Texas A&M). The promoter fragment was previously modified to eliminate the ATG within the multiple cloning site (Li *et al.*, 1997). This vector was digested with *Kpn*I and treated with Mung-bean nuclease and subsequently digested with *Sst*I. Ligation of the pCD7 fragment into pBIB-Kan 35S dE resulted in a plasmid referred to as pCD9 (Fig.III.1) which was sequenced to verify cloning junctions as described above.

III.3.7 Transformation of tobacco BY-2 and KY-14 cells

Plasmid pCD9 was mobilized into *Agrobacterium tumefaciens* strain LBA4404 using a direct freeze-thaw method (An, *et al*, 1988). For stable transformation of BY-2 and KY-14 tobacco cells, 5 ml of 3-day old suspension-cultured cells were incubated with 1.0 ml log-phase (10^8) *Agrobacterium tumefaciens* ($O.D._{600} = 1.0$) bearing pCD9 at room temperature for 2 days. Bacterial cells were removed by washing tobacco cells several times with liquid MS medium. Cells were plated on solid MS medium containing 300 μg/ml kanamycin and 500 μg/ml carbenicillin. Calli growing in the presence of kanamycin were visible after 3 weeks at 25°C for both types of cells. Independent calli from BY-2 cells were subsequently transferred to liquid medium and maintained as suspension cultures. Even after six months on solid MS containing kanamycin, KY-14 calli had grown very slowly and remained too small to put into suspension culture or check transgene expression. Expression of full-length HMG1 FLAG-tagged membrane domain in transformed BY-2 cells was confirmed by RNA analysis.

III.3.8 Northern analysis of transgenic cells

Total RNA was isolated from 3 day old early log-phase BY-2 transformed cells using a RNeasy kit (Qiagen) following manufacturer's protocols. Twenty micrograms of total RNA were denatured and separated on 1.2% agarose gels according to Sambrook *et al.*, (1989). RNA was transferred onto a Nytran membrane (Schleicher and Schuell), then hybridized with a ^{32}P-labeled tomato HMG1-specific DNA probe (pCD5; 1 kb *Hind*III fragment of pCD4) in 50% formamide, 6X SSC, 5X Denhardt's solution, 0.5% SDS, and 100 μg/ml of sonicated denatured salmon sperm DNA at 42°C. RNA blots were washed at 65°C in 0.1X SSC, 0.5% SDS for 15 min and exposed to x-ray film overnight. To standardize the RNA loading, membranes were stripped and rehybridized with a probe encoding the pea 23S ribosomal RNA (Yang and Watson,

1993). RNA isolation and Northern blot hydridization were performed similarly for untransformed KY-14 cells before and after treatment with 0.1 μg/ml cellulase. Hybridization probes were generated from the 1 kb *Hind*III fragment of pCD5 (tomato *hmg*1 membrane domain) or the 0.5 kb fragment of pCD1 (tomato *hmg*2; Yu, 1995).

III.3.9 HMGR enzyme assay

Total HMGR activity was measured in BY-2 and KY-14 tobacco cells following treatment with sterol or cellulase using the protocol of Chappell (1995). Specific activity is expressed as nmol MVA/mg protein/hr.

III.4 RESULTS

III.4.1 Insertion of epitope-tagged HMG1 constructs into microsomal membranes

The membrane domain of tomato HMG1 was engineered to contain a FLAG epitope tag at its C-terminus. Two constructs were generated, one that contained the membrane domain beginning at the original ATG codon and the other in which the first eighteen bases were replaced with an ATG start codon yielding an N-terminus with the highly charged amino acids 2-6 (DVRRR) removed. In order to test whether the microsomal targeting and membrane orientation was disturbed by the addition of FLAG or by deletion of 5'-untranslated sequences or the first 18 bases, labeled peptides were synthesized *in vitro* in the presence or absence of dog pancreatic microsomes. Microsomal targeting and N-terminal signal peptide cleavage were assessed by comparing HMGR peptides in the presence or absence of microsomes and/or following proteinase treatment.

In vitro translation of pCD6 and pCD7 resulted in more than one predominate peptide (Figs. III.2 and III.3, respectively). These results are not consistent with those observed for pCD4 (Fig. II.5) which resulted in a single major peptide of 37.5 kD. pCD7 differed from pCD4 only by deletion of the *hmg*1 3'-untranslated leader sequence. After deletion of the upstream sequences, there were three major peptides present (between 30 kDa and the full-length size of 37.5 kDa; *lanes* 3). *In vitro* translation was enhanced in the presence of microsomal membranes (*lanes* 5), and except for one of the extra bands, the peptides were the same size as those synthesized without microsomes, indicating that again no signal peptide was cleaved during synthesis and insertion. Sedimentation of labeled HMGR peptides with microsomal membranes by ultracentrifugation (*lanes* 7) demonstrated that the HMGR peptides were associated with the membranes. Proteinase K treatment of peptides translated in the absence of microsomal membranes resulted in loss of the protein (*lanes* 4). Small protected fragments (Fig. III.4) were evident following digestion of peptides synthesized in the presence of microsomes (*lanes* 3 and 5), confirming a transmembrane configuration. To confirm that the C-termini of the FLAG tagged peptides were still oriented to the cytosol, Western blot analysis of the *in vitro* translation products was performed using the FLAG antibody. The products of pCD7 translation were detected as a band at about 37 kD (Fig. III.5; *lane* 2). All cross-reactivity was lost upon proteinase K treatment of translated products in the presence of the microsomal membranes (Fig. III.5, lane 3) indicating the FLAG epitope was cleaved during synthesis and insertion. HMGR

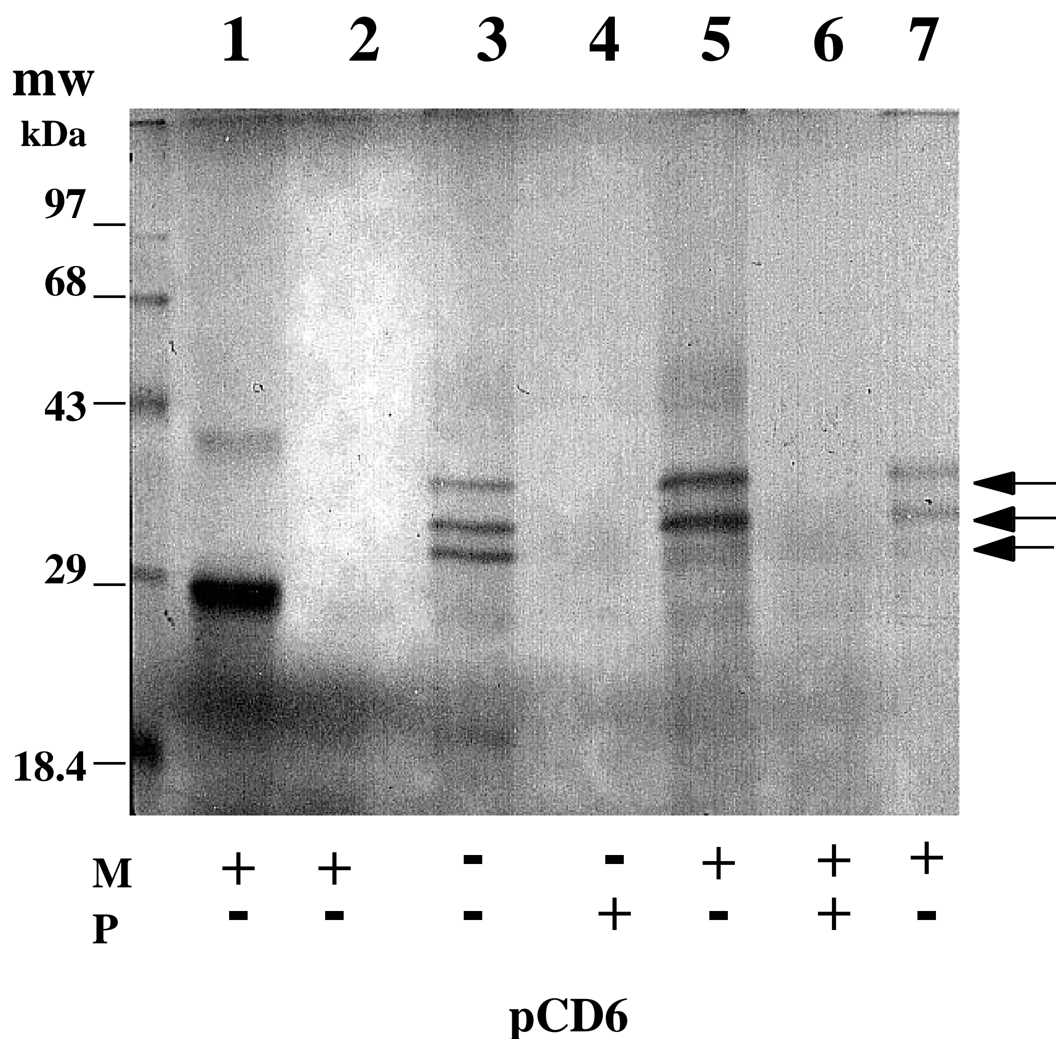

Fig. III.2. Insertion of a FLAG-tagged HMG1 (pCD6) into microsomal membranes. *In vitro* synthesized mRNA encoding an epitope-tagged N-terminal modified HMG1 peptide was translated in the presence of [^{35}S]methionine. Translation was carried out in the presence of dog pancreatic microsomes (*M*) and/or followed by treatment with proteinase K (*P*) as indicated prior to separation by SDS-PAGE. The positions of molecular mass markers are indicated. Bands corresponding to HMGR products are indicated by *arrows*. *Lane 1*, Luciferase control RNA; *lane 2*, no RNA control; *lane 3*, translation of pCD6 mRNA in absence of microsomes; *lane 4*, translation in absence of microsomes; and followed by proteinase K treatment; *lane 5*, translation of mRNA in the presence of microsomes; *lane 6*, translation in the presence of microsomes and followed by proteinase K treatment; *lane 7*, microsomal pellet.

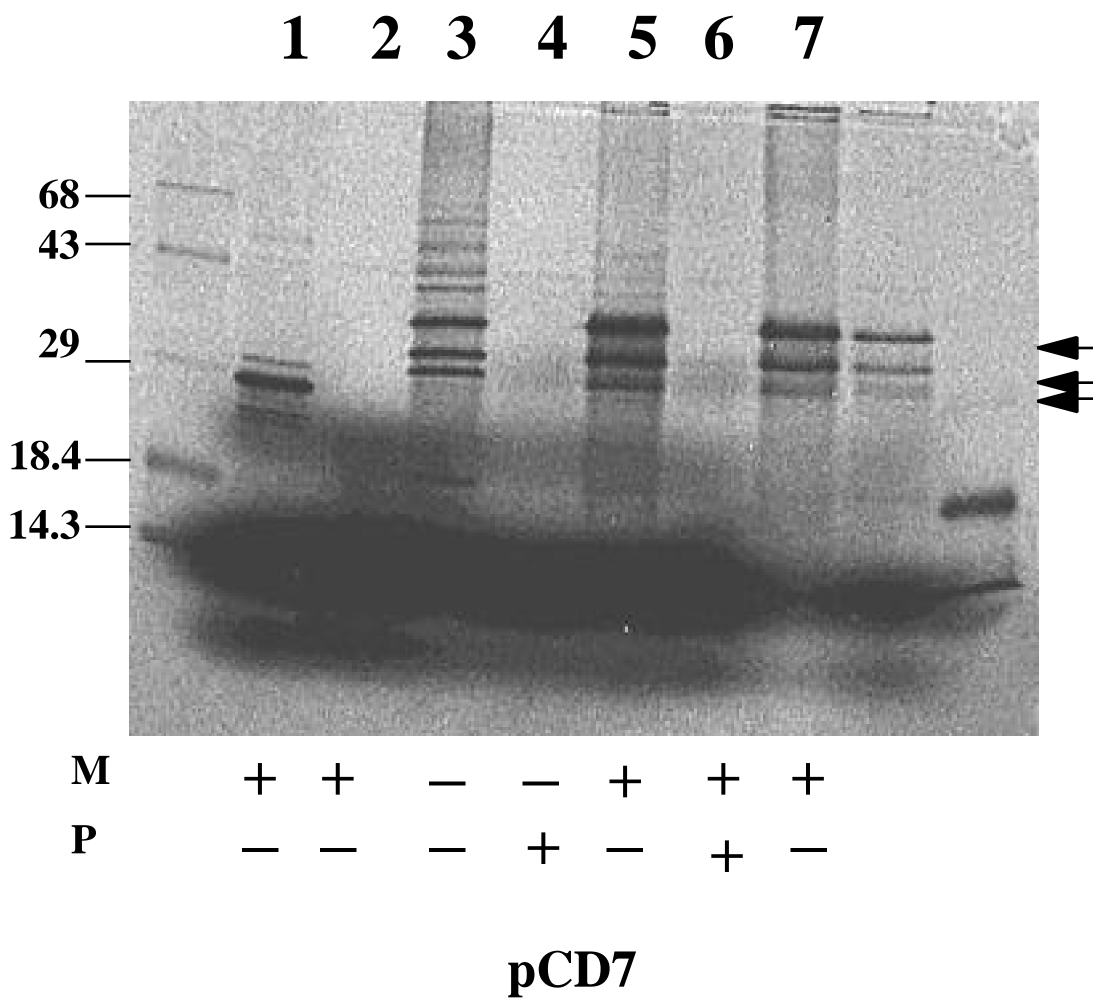

Fig. III.3. Insertion of a FLAG-tagged HMG1 (pCD7) into microsomal membranes. *In vitro* synthesized mRNA encoding an epitope-tagged HMG1 peptide was translated in the presence of [^{35}S]methionine. Translation was carried out in the presence of dog pancreatic microsomes (*M*) and/or followed by treatment with proteinase K (*P*) as indicated prior to separation by SDS-PAGE. The positions of molecular mass markers are indicated. Bands corresponding to HMGR products are indicated by *arrows*. *Lane 1*, Luciferase control RNA; *lane 2*, no RNA control; *lane 3*, translation of pCD7 mRNA in absence of microsomes; *lane 4*, translation in absence of microsomes; and followed by proteinase K treatment; *lane 5*, translation of mRNA in the presence of microsomes; *lane 6*, translation in the presence of microsomes and followed by proteinase K treatment; *lane 7*, microsomal pellet.

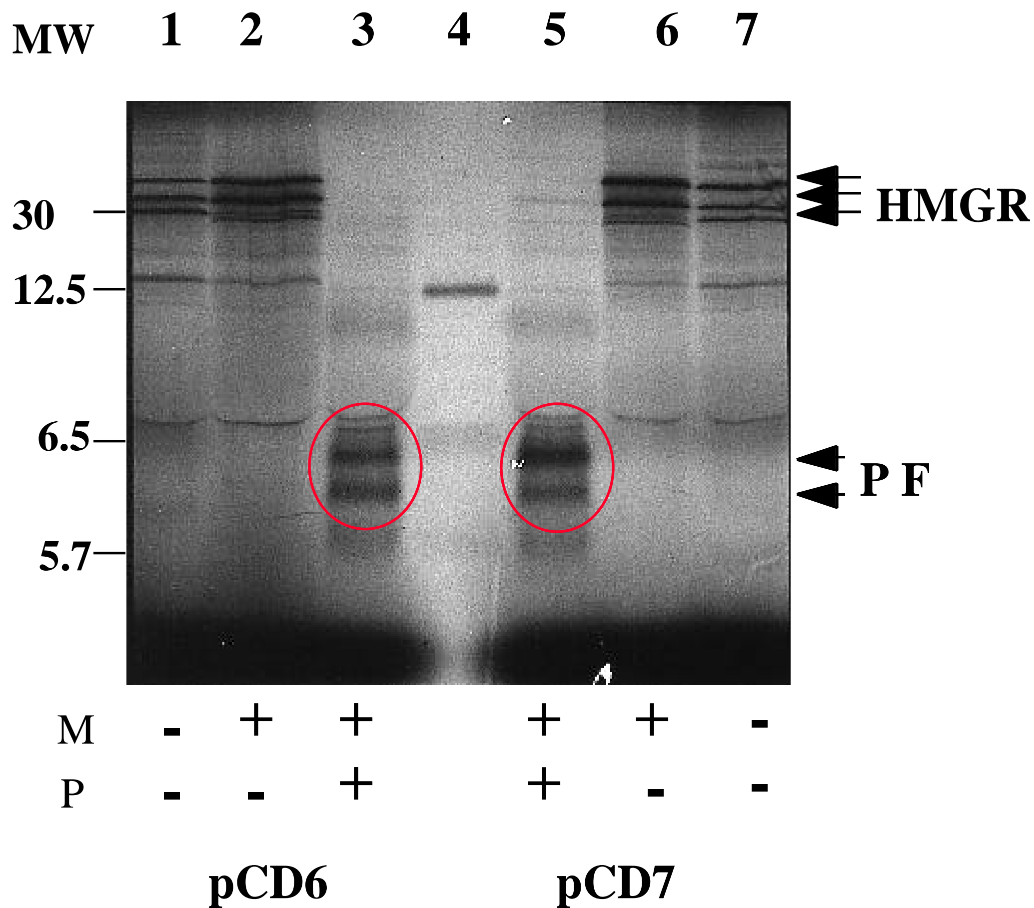

Fig. III.4. Protected fragments of *in vitro* synthesized epitope-tagged HMG1 peptides from pCD6 and pCD7 resulting from microsomal insertion and proteinase K treatment. *In vitro* synthesized mRNAs were translated in the presence of [^{35}S]methionine. Translation was carried out in the presence or absence of dog pancreatic microsomes (*M*). Peptides translated in the presence of microsomes were subsequently treated with proteinase K (*P*) as indicated prior to separation by SDS-PAGE. The positions of molecular mass markers are indicated. Bands corresponding to HMGR products and protected fragments (*PF*) are indicated by *arrows*. *Lanes 1* and *7*, translation of mRNA in absence of microsomes; *lanes 2* and *6*, translation of mRNA in the presence of microsomes; *lanes 3* and *5*, translation of mRNA in the presence of microsomes followed by proteinase K treatment; *lane 4*, molecular weight markers.

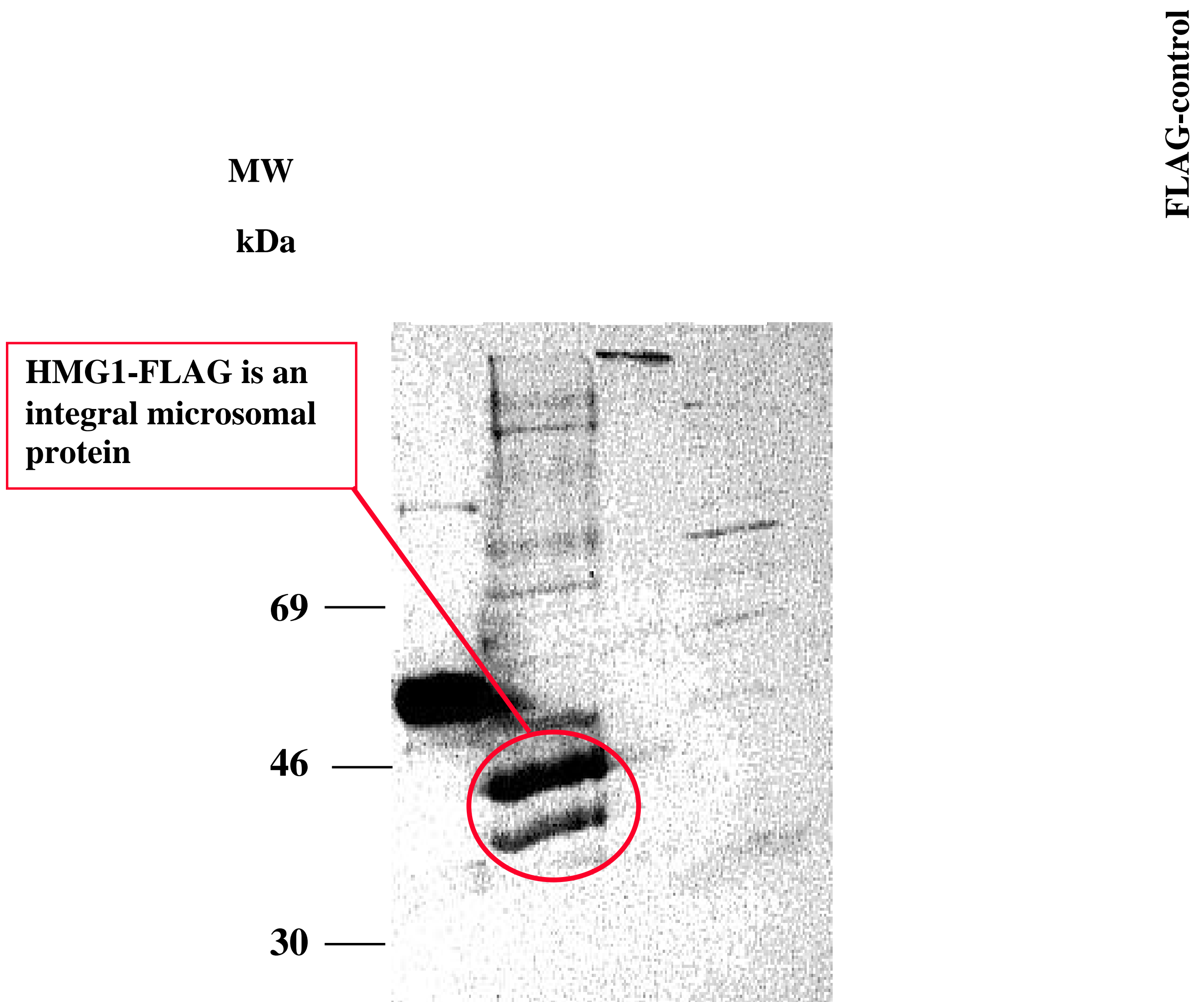

Fig. III.5. Western analysis of HMG1 membrane domain containing an epitope tag at the C terminus. *In vitro* synthesized mRNA encoding FLAG-tagged HMG1 peptides were translated in the presence of dog pancreatic microsomes. Microsomally targeted peptides were separated by centrifugation, treated with proteinase K as indicated, and detected by Western immunoblotting using FLAG M2 monoclonal antibody. Protein standards (Amersham Rainbow protein markers) are indicated. *Lane* 1, FLAG control protein (IBI Technologies); *lane* 2, microsomal pellet; *lane* 3, microsomal pellet treated with proteinase K; *lane* 4, supernatant. Cross-reactive band denoted by circle. Additional gels (not shown) were run to ensure that the protease-protected fragments did not cross-react with the FLAG M2 antibody.

peptides were associated with the microsomal membranes after ultracentrifugation (lane 2). Bands indicating FLAG antibody cross-reactive material were not detected in the size range (< 12 kD) of the protease-insensitive fragments (data not shown). pCD6 was reproducibly less efficient than pCD7 in plasmid yield and *in vitro* transcription/translation, and insufficient product was generated for Western detection (data not shown)..

III.4.2 Differential regulation of HMGR activity in BY-2 and KY-14 cell lines.

Our goal was to stably introduce the FLAG-tagged HMG1 constructs into tobacco cell cultures for subsequent analyses of HMG1 immunolocalization and the role of protein degradation in regulating HMGR activity and pathway flux. In tobacco and potato, both sterols and defense-elicitors appear to down-regulate the HMGR isoform associated with sterol synthesis (Chappell, *et al*., 1991; Threlfall and Whitehead, 1988; Choi *et al*., 1992). Preliminary experiments analyzing the impact of elicitors on induction of host defense responses using the well-characterized tobacco suspension-cultured BY-2 cell line (Nagata *et al*., 1992) suggested that these cells may show only minimal defense response capabilities (Denbow and Lång, unpublished results). We, therefore, initiated studies to compare modulation of HMGR in BY-2 cells with that in the more recently established KY-14 cells that are highly responsive to defense elicitors (Chappell, *et al*., 1991). Both BY-2 and KY-14 cells were tested for defense responses triggered by addition of cellulase. The KY-14 cells rapidly induced host defense responses as indicated by browning of the cells within two hours after adding cellulase (Fig.III.6). In contrast, BY-2 cells remained a bright yellow and HMGR activity levels were essentially unaffected by cellulase treatment over a 12 hour time course (data not shown). Further efforts to elicit cell browning or induction of defense-related HMGR activity in BY-2 cells by using different cellulase concentrations, by altering the time after subculture when elicitor was added, or by adding a crude fungal elicitor from *Fusarium oxysporum* were also unsuccessful (data not shown). Therefore, time course studies following elicitor treatment focused on the KY-14 cells. Treatment of KY-14 cells with cellulase resulted in decreased total HMGR enzyme activity at 3 hr post treatment, followed by the expected increase in activity due to induction of the defense-specific isoform(s) (Fig.III.7). The highest activity (5 fold increase) was observed 9 hr following cellulase treatment. By 12 hr, HMGR enzyme activity returned to basal levels.

Northern analyses were used to assess the effects of elicitor on specific HMGR genes using *hmg*1 (sterol-associated) and *hmg*2 (defense-related) probes. Elicitor-mediated changes in *hmg*1 and *hmg*2 transcript levels are shown in Figure III.8. *hmg*1 mRNA was present in KY-14 cells prior to treatment but was suppressed by cellulase treatment (Fig.III.8A, *lanes* 2 and 3). In contrast, untreated cells (data not shown) lacked *hmg*2 transcript, but showed high levels of *hmg*2 mRNA 9 hr after cellulase treatment (Fig.III.8B, *lane* 3). These results also suggest that the tomato *hmg*1 and *hmg*2 probes function as gene-specific probes for the sterol- versus defense-specific tobacco isoforms.

HMGR activity was also tested in BY-2 and KY-14 cell lines following treatment with the sterol 25-hydroxycholesterol. Both cell lines showed a sterol-mediated decrease in

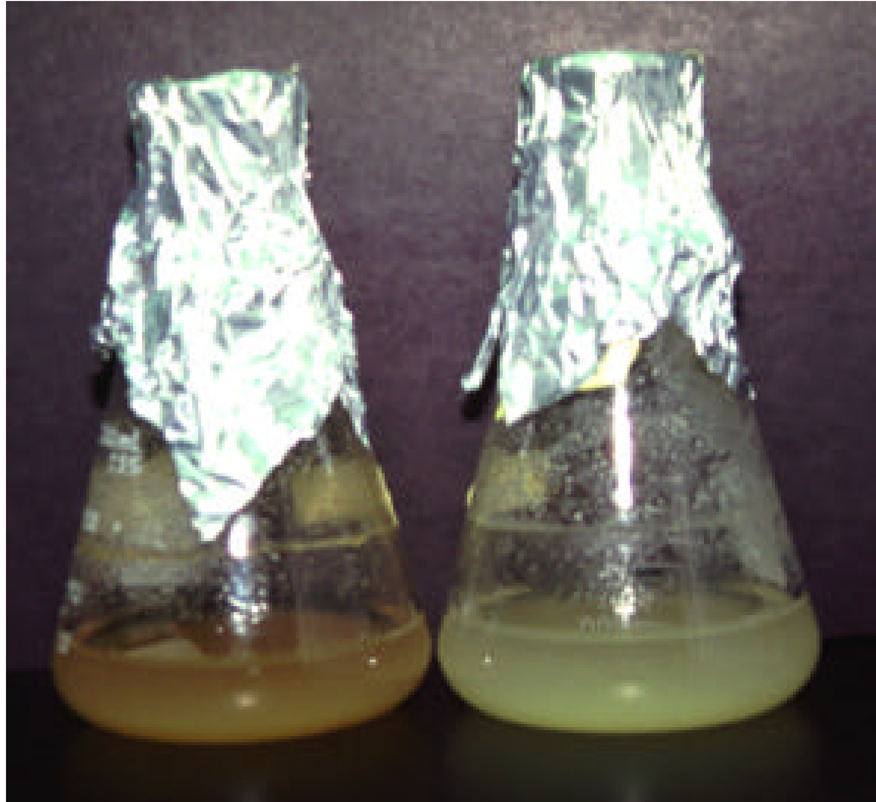

Fig. III.6 Cellulase-induced browning of KY-14 cells. Cells 2 hr after addition of 0.1 μg/ml cellulase (left) and control cells (right).

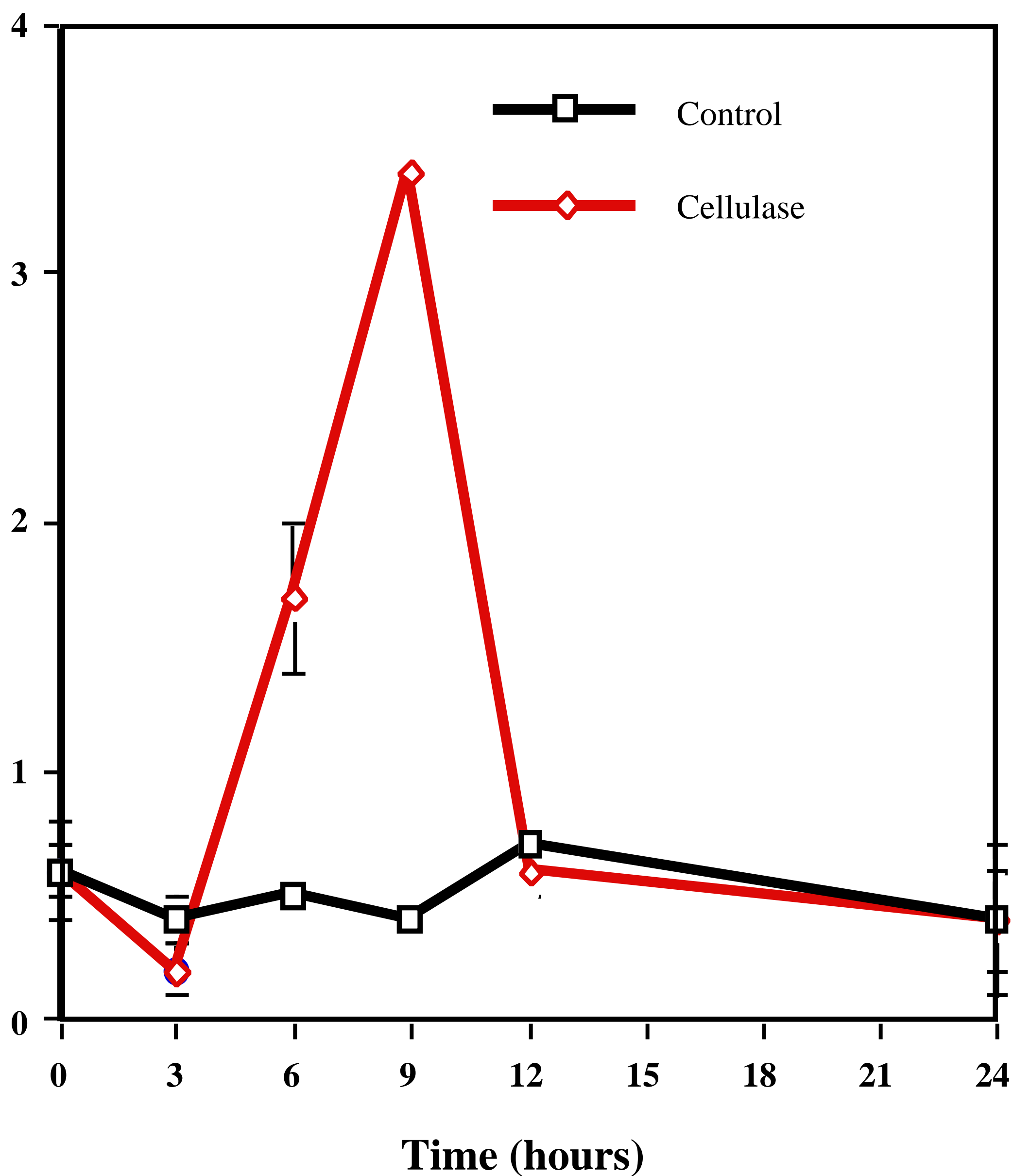

Fig. III.7 Induction of HMGR enzyme activity in KY-14 tobacco cell suspension cultures by elicitor. Three-day-old cultures were treated with 0.1 μg/ml cellulase (0 hr). Enzyme activity was measured in the microsomal pellet of control and treated cells following the protocol of Chappell (1995). Each time point represents two replicates of 3 independent samples (n=6). Error bars represent standard error.

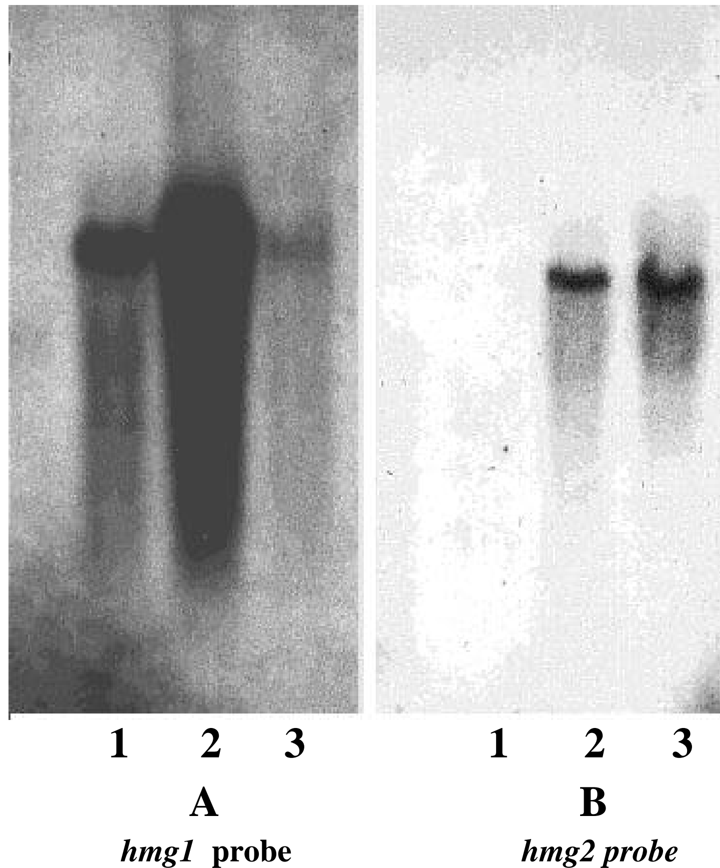

Fig. III.8 RNA gel blot analysis of *hmg*1 and *hmg*2 transcript levels in cellulase-treated KY-14 tobacco cells. Twenty micrograms of total RNA from suspension cell cultures was separated on an agarose gel, transferred to nylon membranes, and hybridized with the ^{32}P-labeled (**A**) pCD5 (*hmg*1) or (**B**) pCD1 (*hmg*2) probes. The two probes were labeled with ^{32}P-dCTP by using a random primer labeling system (Gibco BRL) to similar specific activity. Both RNA blots were exposed to x-ray film for two days. (**A**) *Lane* 1, tomato RNA 9 hr after elicitor treatment; *Lane* 2, KY-14 control RNA; *Lane* 3, KY-14 RNA 9 hr after cellulase treatment; (**B**) *Lane* 1, tomato fruit RNA; *Lane* 2, tomato RNA 9 hr after elicitor treatment; *Lane* 3, KY-14 RNA 9 hr after cellulase treatment. For tomato RNA, either immature fruit of cultivar EP-7 or suspension cultured EP-7 cells treated with *Fusarium oxysporum* cell wall-derived elicitor (Park *et al.*, 1992) were used.

HMGR activity (Table III.1) by 3 hr post treatment but had recovered by 24 hr. KY-14 cells were more responsive to treatment with sterols (67% decrease) and their total HMGR activity remained low from 3 hr post treatment to 12 hr after treatment. In BY-2 cells both the magnitude and duration of the sterol-mediated reduction were less than observed in KY-14 cells. Interestingly, basal HMGR levels in untreated cells were twice as high in the rapidly growing BY-2 cells as that observed in KY-14 cells.

III.4.3 Expression of HMG1 tagged transgene in BY-2 cells.

Although both BY-2 cells and KY-14 cells were used for *Agrobacterium*-mediated insertion of the FLAG tagged *hmg*1 transgene, the KY-14 calli grew too slowly on selective medium for subsequent initiation of suspension cell cultures. However, suspension cultures of the faster growing BY-2 cells were initiated and were analyzed for expression of the transgene. Northern analysis (Fig. III.9) showed that all six independently derived transgenic lines expressed HMG1 FLAG tagged mRNA. The transcript size corresponds to 1.6 kb. The nontransformed controls in lane 7 showed no expression of the transgene.

III.5 DISCUSSION

The subcellular location of HMGR may be critical for the regulation of the isoprenoid pathway in plants. In the present study we focused on three issues: 1) the role of the first six amino acids of HMG1 membrane domain in targeting and topology; 2) the epitope tagging of the membrane domain of HMG1 and subsequent transformation of tobacco cell suspension cultures to be used for future studies on the role of the membrane domain in degradation of the protein, and 3) the effects of sterol and defense elicitors on HMGR activity in tobacco cell cultures.

Our data showed that the presence of the FLAG epitope and removal of the plant leader sequence from HMG1 membrane domain do not affect targeting or insertion into the microsomal membrane. However, in contrast to the single major pCD4 product seen previously (see Chapter 2, Fig. II.5), there were three major translation products observed. There are 10 in-frame ATG codons within the HMG1 sequence (see Chapter II, Fig. II.3), and it would appear the first three were utilized since they correspond to peptides with predicted sizes between 30 and 38 kDa. These constructs eliminated all sequences upstream of the ATG start codon and it may be that the ribosomes no longer can bind appropriately. Constructs encoding *hmg*1 with deletion of amino acids 2 through 6 gave the same banding pattern as the full-length HMG1 membrane domain (minus the *hmg*1 leader). Thus, removal of these residues had no effect on the *in vitro* microsomal targeting. The N-terminal sequences of plant HMGRs are highly conserved with the sequence MetAspXArgArgArg (X can be Val, Ile, Leu, Ser) that resembles a motif which is sufficient for retention of type II membrane proteins in the mammalian ER (Schutze *et al.*, 1994). This motif consists of a pair of arginine residues located at positions 2 and 3, 3 and 4, 4 and 5, 2 and 4, or 3 and 5 of the N-terminus. Not only does the conserved sequence fit this motif; it is positioned in the cytosol which is the expected location for this signal. The retention/retrieval function of this type of motif has not yet been demonstrated for Type I or Type II membrane proteins in plants. The *in vitro* experiments described here addressed ER targeting but not ER

Table III.1 Effect of 25-hydroxycholesterol on HMGR enzyme activity in tobacco BY-2 and KY-14 cell suspension cultures.

Cells	Basal HMGR activity levels [a]	Sterol Treatment [b]	
	nmol MVA/mg/hr	decrease [c]	duration [d]
BY-2	1.1	38%	3 - 12 hr
KY-14	0.6	67%	3 - 6 hr

[a] Enzyme activity was measured in microsomal pellets (Chappell, 1995)
[b] Three-day-old cultures were treated with 100 μg/ml 25-hydroxycholesterol
[c] Values represent the percent decrease at 3 hr from control
[d] Values represent length of sterol-mediated reduction in enzyme activity

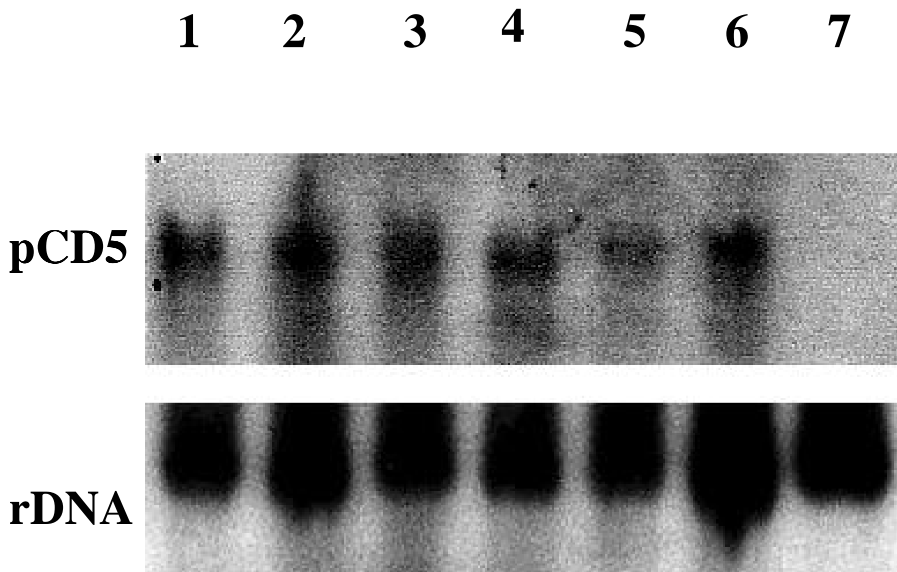

Fig. III. 9 Expression of FLAG-tagged HMG1 in BY-2 cells. Twenty micrograms of total RNA per lane were separated on a 1.2% agarose gel, transferred to a nylon membrane and probed with pCD5 (*hmg*1-specific). Lanes 1 through 6 each represent an independently-derived transformed cell line; lane 7 is the non-transformed control. The blot was stripped and rehybridized with a pea 23S rDNA probe (Yang *et al.*, 1993).

retention/retrieval. Verification of a role of these conserved N-terminal sequences in ER retrieval will require an *in vivo* system. The FLAG-tagged HMG1 clones should function effectively *in vivo* to target the protein to the endomembrane system. Immunolocalization studies using antibodies to the FLAG epitope, should establish whether the RR-motif functions as an ER retrieval signal.

Tobacco cells expressing the tagged HMG1 constructs will also facilitate experiments designed to assess the possible role of the membrane domain in mediating rapid enzyme degradation, a key regulator of mammalian and yeast HMGR activity. By using a specific tagged membrane domain (lacking the HMG1 catalytic activity) driven by a constitutive promoter, complications due to abnormal HMGR activity or transcriptional regulation should be eliminated, permitting direct analysis of membrane domain function in targeting and protein stability. With these future objectives in mind, we introduced the 35S:FLAG-HMG1-membrane domain constructs into BY-2 and KY-14 tobacco cell lines. The rapidly growing BY-2 cells were easily transformed and numerous kanamycin-resistant calli were isolated. Several independently-transformed lines were transferred to liquid medium and expression of the *hmg*1 sequences confirmed by Northern hydridization. The transformed BY-2 cells should be useful for HMG1 immunolocalization and ER-retention studies, and perhaps analyses of sterol down-regulation. However, as discussed further below, their usefulness for protein stability studies or pathway flux following defense activation is clearly limited.

KY-14 cells seemed more responsive to sterol or elicitors based on a comparison of responses by BY-2 and KY-14 cell lines. BY-2 cells show HMGR down-regulation in response to 25-hydroxycholesterol, but could not be induced to turn brown or increase HMGR activity in response to cellulase. This suggests that sesquiterpenoid biosynthesis and other defense responses are not activated. BY-2 cells were selected for rapid growth and highly friable callus and have been maintained in culture for longer than twenty years (Nagata *et al.*, 1992). BY-2 cells cannot be regenerated (Nagata *et al.*, 1992). Defense-response capabilities may also have been lost during this extended culture period. As BY-2 cells cannot be elicited, they are inadequate for studies on regulation of transcription or end-product mediated degradation following elicitor treatment. Although sterols had an affect on both cell lines, the response was more dramatic in KY-14 cells. Consistent with the rapid growth of BY-2 cells and their increased demand for membrane biogenesis, total HMGR activity in BY-2 cells was twice as high as that of the slower growing KY-14 cells.

In contrast to BY-2, KY-14 cells produce large quantities of the sesquiterpenoid capsidiol following treatment with fungal elicitor or cellulase (Chappell and Nable, 1987; Chappell *et al.*, 1991). Cellulase treatment triggered suppression (including transcriptional down-regulation) of HMG1 and induction of HMG2. Thus, KY-14 cells show significant potential for studies on mechanisms of HMGR regulation. However, alternate strategies for recovery of transgenic KY-14 lines will be required. While more than forty independently-derived kanamycin-resistant KY-14 calli continue to be viable, selection in liquid cultures or reduction of kanamycin concentration may be required to obtain better propagation kinetics for stably transformed lines.

Based on our studies, we suggest that BY-2 cells have lost critical regulatory components for mediating HMGR activity and isoprenoid pathway flux and, thus, are inappropriate for studies on transcriptional regulation and regulated protein degradation. BY-2 cells are a good choice, however, for studying localization of HMG1 in the endomembrane system. Overexpression of hmg1 (sterol-specific) in BY-2 cells that grow rapidly, could result in some explicit cellular changes within the endomembrane system. Having two different transformed cell lines, each with their own unique characteristics, should provide the necessary tools for future comparative studies on the differential regulation of specific HMGRs in plants.

III.6 ACKNOWLEDGMENTS

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CHAPTER IV
Conclusions and Future Direction

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC 1.1.1.34) catalyzes the rate limiting step in isoprenoid biosynthesis converting HMG-CoA to mevalonate (Bach, 1995). In mammalian systems, HMGR is encoded by one gene (Chin *et al.*, 1985). In plants, HMGR is encoded by two or more genes (see Chapter I). Animal and plant HMGRs studied to date have comparable structural features. The catalytic domain of both animal and plant HMGRs is highly conserved. A linker region which separates the N-terminal domain from the C-terminal domain is present in both animals and plants, although it is very divergent even among plant species. The N-terminal domain is of considerable interest in this study. In animals and yeast, this domain is very important in targeting and anchoring the protein to the ER membrane (Liscum *et al.*, 1985). It is very large and has been demonstrated to span the membrane eight times in animal systems (Roitleman *et al.*, 1988). It is also important in the sterol-mediated degradation of HMGR in the ER (Chun and Simoni, 1992). The dissimilarities between plant and animal HMGRs are most striking in the size of the N-terminal domain. The plant N-terminal domain is much reduced when compared to the much larger analogous animal domain. We cloned the N-terminal domain (exon I) of tomato HMG1. Analysis of deduced amino acid sequences and comparisons with those of other plant HMGRs, led to the idea that plants contain two potential membrane spanning domains. We demonstrated that tomato HMG1 and HMG2 are targeted and inserted into microsomal membranes by an uncleaved signal sequence in a co-translational process. Results of proteolysis experiments and immunodetection of epitope-tagged HMG2 demonstrated that the protein was oriented in the membrane such that the N- and C-termini were cytosolic and that HMG2 but not HMG1 was glycosylated *in vitro*.

Understanding the regulation of plant HMGR as well as its location within the cell has been a major goal of researchers. In mammals, HMGR is regulated at the levels of transcription, translation, and post-translational modification and by alteration in the rate of degradation of the protein (reviewed by Goldstein and Brown, 1990). Enhanced degradation of HMGR can be induced by both sterol and non-sterol products of the mevalonate pathway, and it is the membrane domain which is responsible for this regulated degradation (Gil *et al.*, 1985; Jingami *et al.*, 1987; Chun *et al.*, 1990; and Chun and Simoni, 1990). This principle was demonstrated using the construction of a fusion protein consisting of the N-terminal domain of HMGR fused in frame to bacterial -galactosidase (Skalnik *et al.*, 1988). Because the construct was transcribed from the SV40 promoter, it was not subject to the transcriptional regulation that characterizes the native HMGR. This made an excellent tool for studying the rate of degradation conferred by the HMGR membrane domain (Chun *et al.*, 1990). In order to initiate analagous studies in plants, the membrane domain of HMG1 was tagged with a FLAG epitope and used to transform BY-2 and KY-14 tobacco cells. Northern analysis confirmed transformation and *hmg*1 mRNA expression in BY-2 cells. The KY-14 cells grew very slowly under kanamycin selection which has limited their use in starting suspension cultures or confirming expression of the *hmg*1 transgene.

To determine which cell lines would be best for future transcriptional and/or protein stability studies, we treated BY-2 and KY-14 cell cultures with sterol or cellulase. Treatment of

both cell lines with sterol resulted in a decline in HMGR activity by 3hr which had returned to normal by 24 hr. Treatment of KY-14 cells with the elicitor cellulase, induced responses as evidenced by browning and induction of defense-specific *hmg*2, whereas BY-2 cells were unaffected. Thus BY-2 cells may not be the cells of choice for future studies on HMGR regulation by elicitors. Native HMGR activity was twice as high in the BY-2 cell as the KY-14 cells and these cells may be suitable primarily for pulse-chase studies to measure sterol-mediated protein degradation or to determine cell locations within the endomembrane system.

In the future, the following experiments need to be done: 1) Determine the role, if any, of the membrane domain in sterol-mediated or elicitor-mediated degradation of HMG1-FLAG tagged protein using pulse-chase protocols with ^{35}S methionine and 2) Determine the role of the membrane domain in determining the specific localization within the endomembrane system of two differentially tagged and expressed HMGRs, HMG1-FLAG and HMG2-c-myc. Because the proteins will be differentially tagged, comparisons can be made within the same cell following treatment with elicitors or pathway endproducts.

Taken together, results of this study have provided significant new information on subcellular targeting of a critical enzyme in the plant isoprenoid biosynthetic pathway. Information learned about targeting as well as our newly developed capacity to study transcriptional regulation and protein stability will give new insights into the complex regulation of this essential pathway in plants.

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