ROLE OF SUBCELLULAR DIFFERENTIATION IN PLANT DISEASE RESISTANCE

by

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Ph.D. Dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PLANT PHYSIOLOGY

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March, 1997
Blacksburg, Virginia

Key words: 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), plant defense, phytoalexins, immunolocalization, vacuole

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(ABSTRACT)

3-Hydroxy-3-methylglutaryl CoA reductase (HMGR, EC 1.1.1.34) catalyzes the reaction from hydroxymethylglutaryl CoA to mevalonate in the isoprenoid pathway. In solanaceous plants, one class of endproducts of the pathway are sesquiterpenoid phytoalexins, antibiotic compounds produced by plants in response to pathogens. We are interested in the role of the defense-inducible isoforms of HMGR in phytoalexin production and disease resistance. Transgenic tobacco, constitutively expressing the defense-inducible tomato *hmgr* isogene, *hmg2*, showed fewer and smaller lesions following tobacco mosaic virus (TMV) inoculation. There is little evidence of phytoalexins acting directly against viruses, but they may reduce the spread of viruses as part of the hypersensitive response resulting in death of the host cell. Transmission electron microscopy of leaf cells of the transgenic plants revealed a larger volume of cytosol and accumulation of electron-dense inclusion bodies within the vacuoles. No structures resembling crystalloid ER or karmellae, caused by overexpression of *hmgr* in mammalian or yeast cells, respectively, were observed. Similar inclusion bodies were found in the vacuoles of wild-type tobacco leaf cells adjacent to necrotic cells in a TMV lesion. Tobacco expressing a truncated (membrane domain) form of *hmg2* did not show enhanced resistance to TMV or any ultrastructural changes, indicating the importance of catalytically active HMG2 in mediating these changes. Sesquiterpene cyclase (a key branch point enzyme controlling sesquiterpene phytoalexin biosynthesis) was not induced and the amount of capsidiol, the tobacco phytoalexin, was not elevated by expression of *hmg2*. After TMV-inoculation, HMGR activity and the amount of capsidiol were higher in the wild-type than in the transgenic plants. Consequently, the enhanced resistance to TMV was not due to constitutive capsidiol production. The transgenic plants may have been able to produce sesquiterpenoid phytoalexins faster due to constitutive *hmg2*-expression and restricted the spread of the virus earlier, so that only a few cells were sacrificed. The subcellular localization of the defense-specific HMG2 isoform was determined by tagging tomato *hmg2* with a c-*myc* epitope, and constitutively expressing the construct in transgenic tobacco plants. In non-induced leaves, MYC-HMG2 was found localized in small clusters associated with the ER. In TMV-inoculated leaves MYC-HMG2 co-localized with sesquiterpene cyclase to the vacuolar inclusion bodies suggesting that they may contain a defense-induced, membrane-associated multienzyme complex dedicated to sesquiterpene production. Our results support the hypothesis of the multibranched plant isoprenoid pathway being partly regulated by pathway partitioning.

This study was funded by Academy of Finland, The Finnish Cultural Foundation, The National Science Foundation, The National Institutes of Health, Alfred Kordelin's Foundation, Kemira Oy Research Foundation, Tiura Foundation and Thanks to Scandinavia Inc.

Acknowledgements

The present study was carried out in the Department of Plant Pathology, Physiology and Weed Science at Virginia Polytechnic Institute and State University, USA.

Thanks to Dr. Eija Pehu I found my way to Blacksburg, VA. I am grateful to have had the opporturnity to mature, as a beginning scientist, and also as an individual, in this lab and with the people around me. My special thanks belong to my advisor, Dr. Carole Cramer, whose insight so many, including me, admire. Back in Finland, as soon as I read the very first proposal of this work, I knew I would love it, and that first impression still has not left me. I wish her all the best in her endeavours, and thank her for letting me have my space to do the work independently and also to spend some time outside the lab enjoying life in the country.

I also give my thanks to the other members of my committee, Drs. Brian Storrie, George Lacy, Elizabeth Grabau and Ruth Alscher, for their encouragement and guidance along the way. I am also thankful to Dr. Deborah Weissenborn, Dr. Donna Hill, Dr. Jingrui Wu, Verlyn Stromberg, Nina Hopkins and Sue Meredith for their kind advice and friendliness.

My thanks also to my fellow students in our lab: Cynthia Denbow, Dafeng Zhou, Hao Ni and Dr. Bill Yu; and friends: Sydha Salihu, Rakesh Chandran, Regina Hanlon, Carla Hegeman, Jia Li, Anne Dorrance, Saba Qusus and Susan Sun.

Finally, I am grateful to my parents for always believing in my abilities, and especially to my father Eero Vanhala for forwarding to me the same faith that he has: "I will do what I wish, others they'll do what they can...". I am forever endebted to all my family, my mother Tuula, my sister Maria, and my grandparents Sylvi, Heikki and Helvi - even if you could not have been here with me, in my thoughts I often was there with you. But most grateful I am to my husband Kai, who made it so much easier just by making me happy.

Dedication

I am dedicating this dissertation to my loving husband Kai; hoping to give you time spent in the lab back in another form.

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Abbreviations

AA	arachidonic acid
ALLN	*N*-acetyl-leucyl-leucyl-norleucinal
ALLM	*N*-acetyl-leucyl-leucyl-methiononal
AMP	adenosine monophosphate
BSA	bovine serume albumin
BCIP	5-bromo-4-chloro-indolyl-phosphate
CaMV	cauliflower mosaic virus
CoA	coenzyme A
CHO cells	Chinese hamster ovary cells
DMAPP	dimethylallyl pyrophosphate
DTT	dithiothreitol
EAS	5-*epi*-aristolochene synthase
Ecc	*Erwinia carotovora* subsp. *carotovora*
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FPP	farnesyl pyrophosphate
GUS	β-glucuronidase
HMG1	3-hydroxy-3-methylglutaryl coenzyme A reductase 1
HMG2	3-hydroxy-3-methylglutaryl coenzyme A reductase 2
HMG-CoA	hydroxymethylglutaryl coenzyme A
HMGR	3-hydroxy-3-methylglutary coenzyme A reductase
HRGP	hydroxyproline-rich glycoproteins
HR	hypersensitive reaction
IB	inclusion body
IPP	isopentenyl pyrophosphate
LDL	low density lipoprotein
MJ	methyl jasmonate
MNL	mevalonate-derived nonsaponifiable lipids
NADH	nicotinamide adenine dinucleotide
NBT	4-nitro blue tetrazolium chloride
PGR	plant growth regulator
PP	pyrophosphate
PQ	plastoquinone
PR	pathogenesis related
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGA	steroid glycoalkaloids
SRE	sterol regulatory element
TMV	tobacco mosaic virus
UQ	ubiquinone

CHAPTER I: LITERATURE REVIEW

I . 1. INTRODUCTION

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC 1.1.1.34) mediates the reductive deacetylation of hydroxymethylglutaryl CoA to mevalonic acid in the isoprenoid pathway. The reaction is essentially irreversible, and it occurs in two steps with the intermediate thiohemiacetal of mevaldic acid being bound to the enzyme during the reaction (Fig. I.1) (Rogers *et al.* 1983).

In animal systems, this is a rate-limiting step for cholesterol biosynthesis, and the enzyme is feedback regulated by cholesterol. In addition to cholesterol synthesis, the mammalian HMGR also takes part in the production of other sterols, isoprenoid compounds such as dolichol, steroid hormones, ubiquinone and isopentenyladenine, and prenyl modifications of proteins (Goldstein and Brown 1990).

In plants, mevalonic acid is used for the synthesis of a much more diverse array of isoprenoid compounds, sometimes called terpenes or terpenoids. Over 22,000 plant isoprenoids have been described, and they are involved in almost all metabolic processes in the plant. This complicated pathway produces compounds for photosynthesis (chlorophylls, carotenoids, plastoquinone), respiration (ubiquinone, cytochrome a), membrane architecture (sterols and triterpenoids), regulation of growth and development (gibberellic acids, abscisic acid, brassinosteroids, certain cytokinins), defense against pathogen attack (phytoalexins), and others (Fig. I.2) (Denbow *et al.* 1996).

We are especially interested in the production of isoprenoid defense compounds, sesquiterpenoid phytoalexins, by this pathway. Phytoalexins are small, antibiotic compounds produced by plants in response to a pathogen. Related plant species tend to produce the same type of phytoalexins, for example isoflavonoid derivatives are produced in the members of Leguminosae; furanocoumarins, isocoumarins and chromones are produced in members of Umbellifereae; and sesquiterpenes are produced in members of Solanaceae (Smith 1996). The main sesquiterpenoid isolated from tomato is rishitin. In potato, rishitin is often the major sesquiterpenoid accumulating after infection, but lubimin, solavetivone, phytuberin, phytuberol, and anhydro-β-rotunol can also accumulate in high concentrations in some interactions and some experimental conditions (Kuc 1982). The main sesquiterpenoid accumulating in tobacco is capsidiol (see Fig. I.3) (Chappell and Nable 1987); others include phytuberin, glutinosone, solavetivone, rishitin, lubimin, and phytuberol (Kuc 1982). Capsidiol adversely affects the molecular structure of fungal membranes (Turelli et al. 1984). In general, phytoalexins are antibiotics with fairly low specificity. They are effective against bacteria, fungi, animal cells, and viruses, as well as plant cells *in vitro* (Smith 1996), but their effectiveness *in vivo* may actually in many cases be dependent on their toxicity on the host cell and their accumulation leading to programmed cell death as part of the hypersensitive response.

Considering the multitude of isoprenoid endproducts in plants, it is not surprising that there are multiple isoforms of HMGR, as well as other enzymes "downstream" from HMGR (*e.g.* sesquiterpene cyclase), all encoded by small gene families. In contrast, HMGR is encoded by only one gene in mammals. Cloned HMGR sequences from plants include two isogenes from tomato (*Lycopersicon esculentum*) (Narita and Gruissem 1989, Park *et al.* 1992), three from potato, (*Solanum tuberosum*)* (Choi *et al.* 1992,

*Several distinct genes in the different isogene classes of *hmgr* exist in potato: seven or more in the *hmg1* class, and one or two genes each in the *hmg2* and *hmg3* classes (Stermer *et al.* 1994).

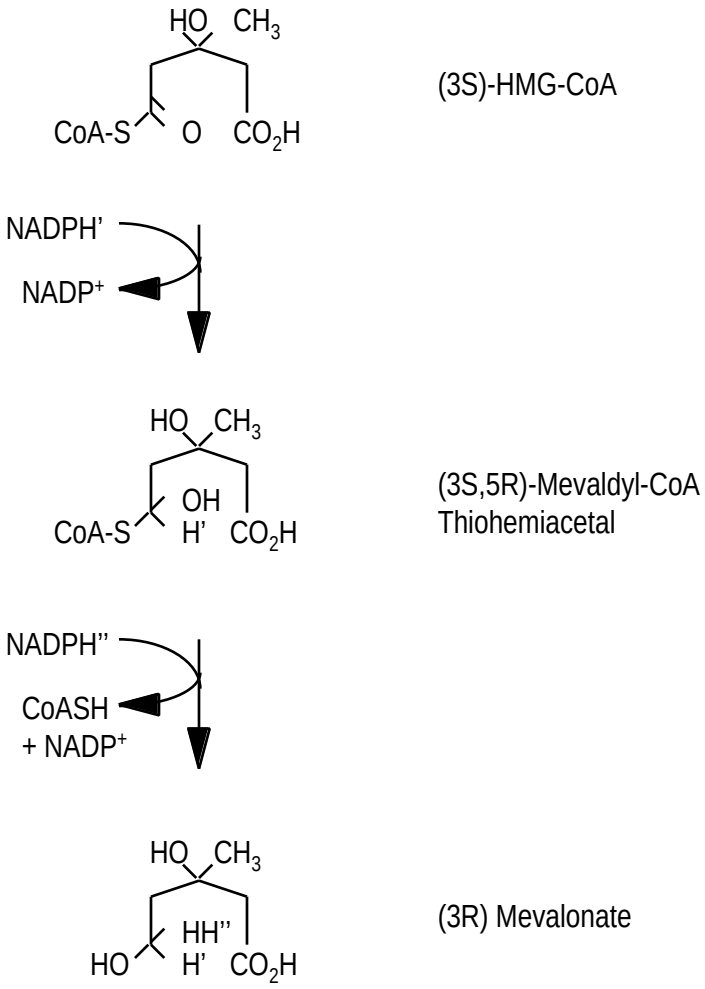

Fig. I.1. Reduction of HMG CoA to mevalonic acid by HMG CoA reductase. (Modified from Rogers *et al.* 1983).

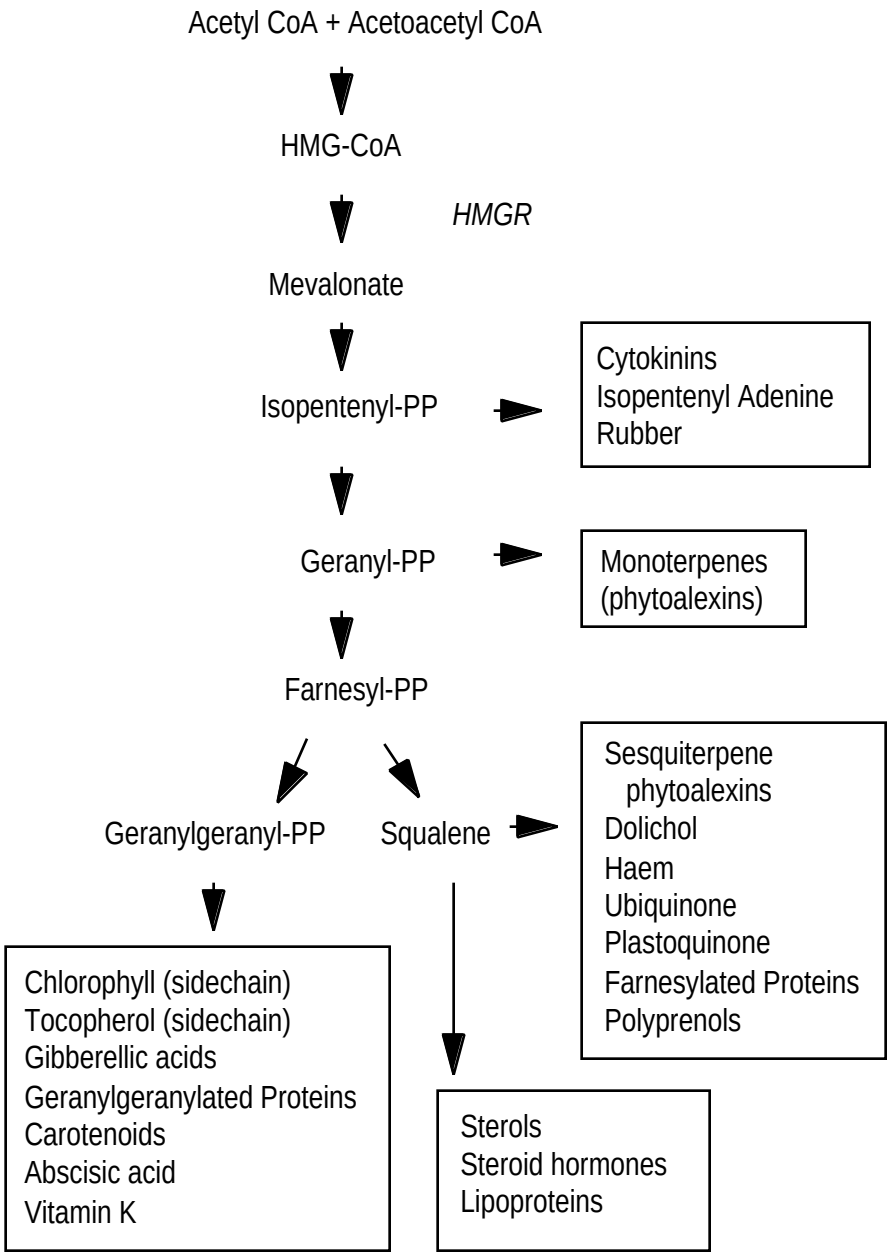

Fig. I.2. A simplified biosynthetic pathway for plant isoprenoids highlighting important end products. PP, pyrophosphate.

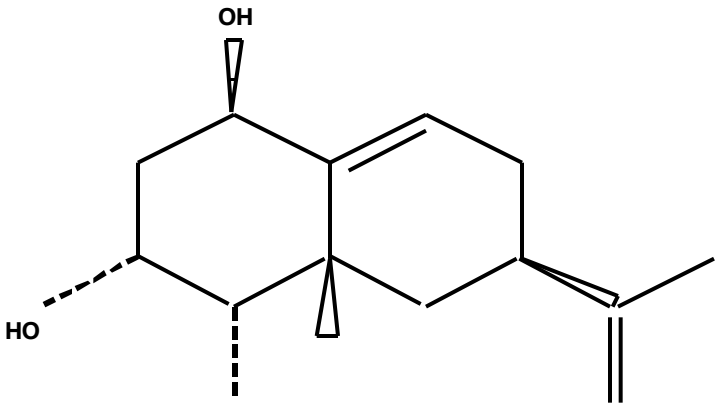

Fig. I.3. The tobacco sesquiterpenoid phytoalexin capsidiol (modified from Kuc 1982).

Stermer *et al.* 1991) and the rubber tree *Hevea brasiliensis* (Chye *et al.* 1991, 1992), and two from arabidopsis (*Arabidopsis thaliana)* (Enjuto *et al.* 1994, Learned and Fink 1989) and radish (*Raphanus sativus*) (Ferrer *et al.* 1990). A single HMGR gene has been cloned from tobacco (*Nicotiana sylvestris*) (Genschik *et al.* 1992), rice (*Oryza sativa*) (Nelson *et al.* 1994), periwinkle (*Catharanthus roseus*) (Maldonado-Mendoza *et al.* 1992), pea (*Pisum sativum*) (Bach *et al.* 1991), and *Camptotheca acuminata*, a Chinese tree that produces the anti-cancer monoterpenoid indole alkaloid camptothecin (Burnett *et al.* 1993), although a multigene family exists in these species. Two genes have been isolated from yeast (*Saccaromyches cerevisiae*) (Basson *et al.* 1986).

The structure of plant HMGRs can be defined in four distinct regions: the highly diverged N-terminal region, the conserved membrane domain, the linker region (also divergent), and the conserved catalytic domain. The C-terminal catalytic domain contains the active site of the enzyme, which is highly conserved between all HMGRs, including the animal and yeast (*Saccharomyces cerevisiae*) enzymes (Caelles *et al.* 1989, Choi *et al.* 1992, Chye *et al.* 1991, Learned and Fink 1989, Narita and Gruissem 1989). The membrane domains of the enzyme are conserved among plants and among mammals; but not between each other. The mammalian HMGR has been shown to be an integral endoplasmic reticulum glycoprotein with an N-terminal membrane domain containing eight probable transmembrane regions (Roitelman *et al.* 1992). Plant HMGRs, however, have two membrane-spanning regions (Enjuto*et al.* 1994) (Fig. I.4), and this domain shows no sequence similarity to mammalian or *S. cerevisiae* regions.

There are several estimates of the number of subunits of HMGR (Rogers *et al.* 1983), but generally, the enzyme seems to exist in a catalytically active dimeric form.

```
Tom2:  1 MD.VRRR.SEEPVYPSKVFAADEKPLKPHKKQQQQQEDKNTLL..IDASDALPLPLYLTNGLF
Tom1:  1 MD.VRRR.PVKPLCTSKDASAGE.PL....KQQQVSSPK........ASDALPLPLYLTNGLF
Ara1:  1 MD.LRRRPPKPPVTNNNNSNGSFRSYQPRTSDDDHRRRATTIAPPPKASDALPLPLYLTNAVF
Hev1:  1 M.....................DTTGRLHHRKHATPVEDRSPTT..PKASDALGVPLYLTNAVF
Hev3:  1 MDEVRRR.PPKHIVRK...DHDGEVLNSFSHGHHLPP.....L..KPSDYSLPLSLYLANALV
Nico:  1 MD.VRRR.SEKPAYPTKEFAAGEKPLKPHK...QQQEQDNSLL..I.ASDALPLPLYLTNGLF
Pot1:  1 MD.VRRR.PVKPLYTSKDASAGE.PLK.......QQEVSSP.....KASDALPLPLYLTNGLF
Pot3:
Rad1:  1 MD.IRRR.PPKPPVN..........NPNRFSNDNDDQPRT.....KASDALPLPLYLTNAVF
Rad2:  1 MD.IRRR.PPKPPVNSNRFLDN.......RSDDDDRRKTLTSP..PKASDALPLPLYLTNAVF
Peri:  1 MD.SRRR.S..PTVTAKA.AAGELPLAPHEGQNQQPS.....I..PRSSDVLPLPLYLANGVF

Tom2: 61 FTMFFSVMYFLLSRWREKIRNSTPLHVVTLSELGAIVSLIASVIYLLGFFGIGFVQTFVSRGN
Tom1: 49 FTMFFSVMYFLLVRWREKIRNSIPLHVVTLSELLAMVSLIASVIYLLGFFGIGFVQSFVSRSN
Ara1: 63 FTLFFSVAYYLLHRWRDKIRYNTPLHVVTITELGAIIALIASFIYLLGFFGIDFVQSFISRAS
Hev1: 42 FTLFFSVAYYLLHRWRDKIRNSTPLHIVTLSEIVAIVSLIASFIYLLGFFGIDFVQSFIARAS
Hev3: 53 FSLFFSVAYFLLHRWREKIRKSTPLHIVTFPEIAALICLVASVIYLLGFFGIGFVHSF.SRAS
Nico: 56 FTMFFSVMYYLLSRWREKIRNSTPLHVVTFSELVAIASLIASVIYLLGFFGIGFVQSFVSRDN
Pot1: 49 FTMFFSVMYFLLVRWREKIRNSIPLHVVTLSELLAMVSLIASVIYLLGFFGIGFVQSFVSRSN
Pot3:  1                         LHVLNFSELVAMVSLIASVIYLLGFFGIGFVQSFVSKGN
Rad1: 44 FTLFFSVAYYLLRRWRDKIRHSTPLHVITITELGALLALVASFIYLLGFFGIDFVQSFISRAD
Rad2: 53 FTLFFSVAYYLLHRWRDKIRYNTPLHVVTVTELGAIVALIASFIYLLGFFGIDFVQSFISRPD
Peri: 52 FTLFFSVMYFLLTRWREKIRNATPLHVVTLSELAALASLIASVIYLVSFFGLDFVQSLIYKPN
```

Fig. I.4.a. Sequence comparison of plant HMGR N termini. Sequences include tomato HMG1 and HMG2 (Tom1, Tom2), potato HMG1 and partial HMG3 (Pot1, Pot3), wood tobacco HMGR (Nico), periwinkle HMGR (Peri), arabidopsis HMG1 and HMG2 (Ara1, Ara2, radish HMG1 and HMG2 (Rad1, Rad2), and rubber tree HMG1 and HMG3 (Hev1, Hev3). Putative membrane domains are in bold, and *N*-linked glycosylation sites within the putative lumenal domain are underlined.

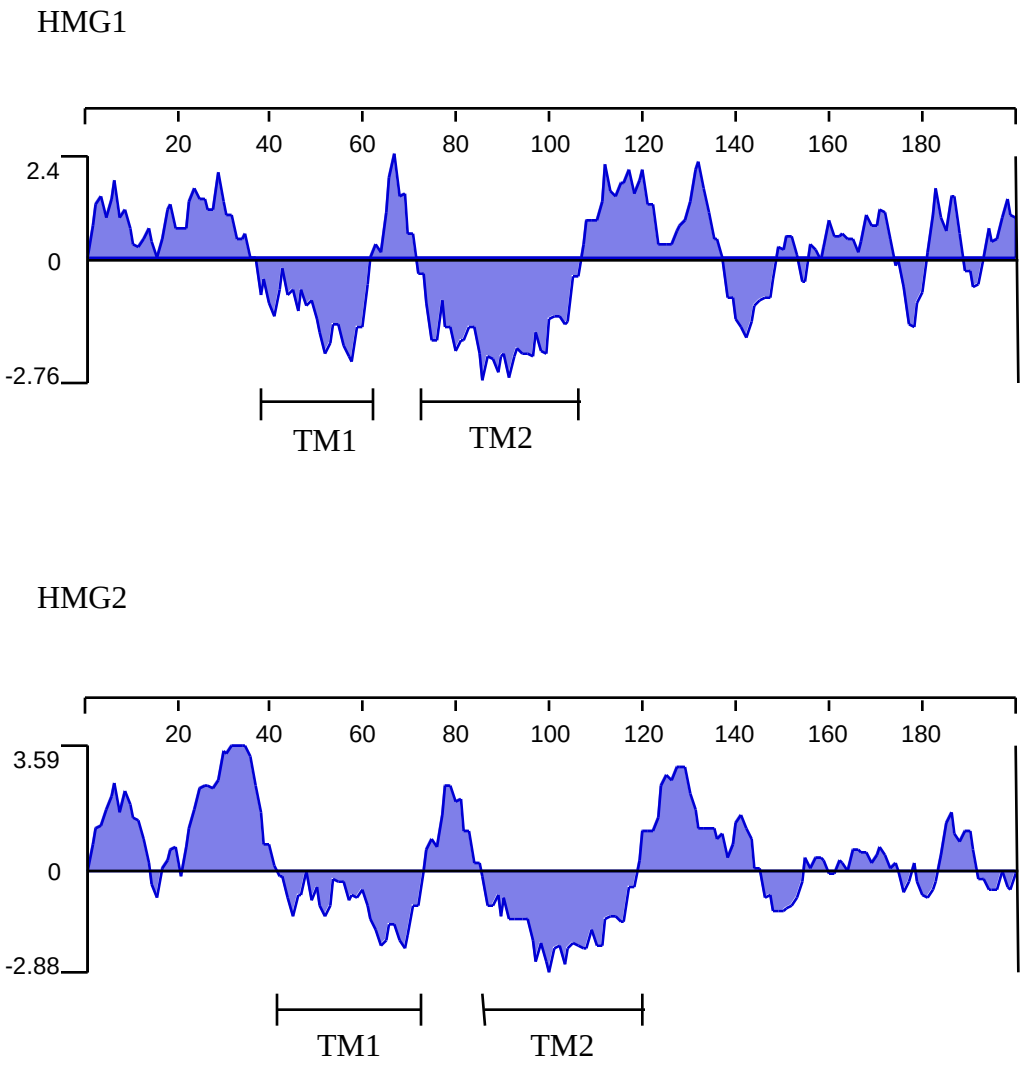

Fig. I.4.b. Hydropathy plot of the N-terminal domains of tomato HMG1 and HMG2 as predicted by Kyte and Doolittle. (Denbow *et al.*1996). TM1 = putative transmembrane domain 1, TM2 = putative transmembrane domain 2. Negative values = hydrophobic residues, positive values = hydrophilic residues.

I. 2. REGULATION OF HMGR EXPRESSION IN PLANTS

I. 2. 1. Developmental expression

The isozymes of HMGR are regulated differently in different parts of the plant and during different developmental stages. As witnessed by mRNA and enzyme activity levels, HMGR expression is usually higher in meristematic, rapidly growing parts of the plant, and in floral tissue. Vegetative tissues usually have very low levels of HMGR mRNA (Choi *et al.* 1992, Chye *et al.* 1992, Genschik *et al.* 1992). In *C. acuminata, hmg1* transcripts were only detected in young seedlings, pollen, stigma and ovules (Burnett *et al.* 1993). Similarly, arabidopsis *hmg2,* when transformed into tobacco, is solely expressed in the root tips, shoot apices and floral tissues, especially pollen (Enjuto *et al.*1995). Potato *hmg1* is expressed in flower primordia and anthers, with lower levels in petals and pistils, whereas *hmg2* is expressed in roots and anthers, and *hmg3* only in anthers (Choi *et al.* 1992).

During fruit and seed development in tomato, *hmg1* is highly expressed during the early stages of rapid cell division and cell expansion (Narita and Gruissem 1989, Gillaspy *et al.* 1993), and *hmg2* is expressed only later during the ripening phase (Gillaspy *et al.* 1993). In maize (*Zea mays*), the highest HMGR activities were found during the stages of rapid cell division in the developing seed. After the seeds had reached maturation stage, endosperm HMGR activity had decreased to one fifth of the maximal activity, and embryo activity to half (Moore and Oishi 1993).

A likely explanation for the high levels of HMGR in meristematic tissues may be an increased need for mevalonate for sterol biosynthesis. It also could be related to protein prenylation, an isoprenyl modification of proteins connected to cell growth and division. In pollen, high levels of HMGR activity might reflect the synthesis of specific isoprenoid compounds prior to pollen tube germination. (Enjuto *et al.* 1995).

A special case of isoprenoid biosynthesis is the synthesis of rubber in *H. brasiliensis*, where a specific isogene (*hmg1*) is highly expressed in the latex producing cells, the laticifers (Chye *et al.* 1992).

I. 2. 2. Wound- and pathogen- induced expression

In addition to expression in meristematic and rapidly dividing cells, HMGR is also highly expressed in injured and pathogen-infected tissues, where increased amounts of sterols are probably needed for membrane repair and production of isoprenoid phytoalexins is needed for protection against the invaders. Different plants accumulate different classes of phytoalexins. Sesquiterpenoid phytoalexins that derive from HMG CoA are abundant in Solanaceae, but absent in Leguminosae, which accumulate isoflavonoid and ptreocarpan phytoalexins instead (Kuc 1982). The first experiment correlating increased HMGR activity and phytoalexin accumulation after wounding and pathogen treatment was done with sweet potato root tissue. Suzuki *et al.* (1975) reported increases in HMGR activity after slicing and infection of sweet potato plant (*Ipomoea batatas*) roots by the fungal pathogen *Ceratocystis fimbriata*, together with concomitant formation of the furanoterpene phytoalexin, ipomearmarone. Since then, HMGR genes have been isolated from several plant species, and it is now known that specific isogenes of HMGR are activated by wounding and pathogen challenge.

The expression pattern of different isogenes in response to wounding and infection has been studied in most detail in potato tubers. Wounding (slicing) induces the

expression of three isogenes, *hmg1*, *hmg2* and *hmg3*. The rapid increase in *hmg1* expression after wounding may be involved in synthesis of wound-related compounds such as glycoalkaloids. The expression of *hmg2* and *hmg3* are further induced if the wounded tissue is inoculated with *Phytophthora infestans*, or treated with arachidonic acid (AA), an elicitor present in the lipids of *P. infestans* (Choi *et al.* 1992). Interestingly, pathogens and AA are both also known to activate sesquiterpene cyclase, which is a key branch enzyme in sesquiterpene phytoalexin synthesis (Zook and Kuc 1991). On the other hand, induction of *hmg1* gene expression following wounding is suppressed by elicitor or pathogen treatments (Choi *et al.* 1992, Yang *et al.* 1991), as is squalene synthetase activity, a key enzyme for sterol biosynthesis. Thus, it seems that during pathogen attack, isoprenoid intermediates are effectively shunted into phytoalexin synthesis.

Isogenes in other plant species are induced similarly to a certain extent. In tomato, *hmg2* mRNA levels are elevated in response to wounding or fungal elicitors (Park *et al.* 1992, Yu 1995). The exception to the rule in the isogene nomenclature is arabidopsis *hmg2*, which is not induced by wounding. So far, there has not been a report of a wound- or pathogen-inducible isoform of HMGR in arabidopsis, but this is not completely surprising, since arabidopsis does not produce isoprenoid phytoalexins.

The *hmg1* gene of *C. acuminata* is regulated similarly to potato *hmg1* (Burnett *et al.* 1993). By studying translational fusions of *C. acuminata hmg1* promoter to the reporter gene β-glucuronidase (GUS) in transgenic tobacco, it was shown that *C. acuminata hmg1* is induced by wounding, and this induction is suppressed by methyl jasmonate (MJ). MJ was speculated to be a signal molecule for pathogen recognition that would be released by lipoxygenase after AA treatment. However, Choi *et al.* (1994) were able to show that plants discriminate between these two signals: in potato, AA and MJ treatments induced different *hmgr* genes. Low concentrations of MJ further increased the wound-inducible expression of *hmg1* and lowered *hmg2* expression, and high concentrations of MJ suppressed *hmg1* expression but did not affect *hmg2*, whereas AA strongly suppressed *hmg1* and strongly induced *hmg2*. These results support placement of *hmg1* and *hmg2* within discrete steroid and sesquiterpenoid phytoalexin biosynthetic channels.

Studies on elicitor-inducible expression of HMGR have also been done with cell suspension cultures. For example, *P. infestans* cell wall fragments, as well as cellulase from *Trichoderma viride*, increased HMGR enzyme activity in tobacco cell cultures (Chappell *et al.* 1991), and cell wall elicitor from the fungal plant pathogen *Magnaporthe grisea* induced rice *hmgrI* mRNA expression in suspension cultures (Nelson *et al.* 1994). Incidentally, rice *hmgrI* was not induced by wounding.

Table I.1. Summary of cloned plant *hmgr* sequences and their expression.

Species	**HMGR gene**	**Expressed in:**	**Induction :**
Lycopersicon esculentum	*hmg1*	immature fruit,	...
		low constitutive	
	hmg2	mature fruit, pollen	wounding, pathogen
Solanum tuberosum	*hmg1*	low constitutive, flowers	wounding, pathogen,
			suppressed by elicito
	hmg2	roots, anthers	wounding, pathogen
	hmg3	anthers	wounding, pathogen
Nicotiana sylvestris	*hmgr*	constitutive, roots,	wounding, pathogen
		protoplasts	
Arabidopsis thaliana	*hmg1*	low constitutive	...
	hmg2	root tips, shoot apices,	...
		pollen	
Hevea brasiliensis	*hmg1*	laticifers	ethylene
	hmg2	...	...
	hmg3	constitutive expression	...
Catharanthus roseus	*hmgr*	constitutive expression	...
Camptotheca acuminata	*hmg1*	seedlings, pollen, stigma,	wounding
		ovules	
Raphanus sativus	*hmg1*	...	...
	hmg2	seedlings	...
Triticum aestivum	*hmgr10*	low constitutive / callus	...
	hmgr18	constitutive / callus	...
	hmgr23	roots, callus	...
Oryza sativa	*hmgr1*	suspension cell culture	pathogen

I. 2. 3. Regulation by light

The levels of *hmgr* mRNA accumulate in dark-grown or etiolated seedlings, or in mature plants after a dark-treatment. Recently, Learned (1996) studied the effect of light on arabidopsis *hmg1*, and found that the accumulation of *hmg1* mRNA by dark-treatment (about five times higher after 24 hours in darkness) can be suppressed by exposing the plants to continuos white light. They were able to show that the suppression is mediated by *cis*-acting elements in the arabidopsis *hmg1* promoter, and so is likely to be controlled at the transcriptional level.

Interestingly, suppression of *hmg1* mRNA required continuous illumination, and was dependent on the amount of light perceived during the illumination period, being strongest in complete darkness and lowest in normal light levels. As brief pulses of light were not capable of suppressing *hmg1*, and the plants did not respond to far-red light, it is questionable that this reaction would involve phytochrome. Instead, the authors propose that *hmg1* may not be responding to light as a direct signal for photomorphogenesis, but is somehow able to sense metabolic changes dependent on light as an energy source. An earlier report on the effect of bleaching herbicides alleviating the regulatory effect of light on maize HMGR activity supports this view (Ji and Hatzios 1992). Bleaching herbicides disrupt chloroplasts and inhibit carotenoid biosynthesis, obviously leading to problems in photosynthesis.

There is a discrepancy in the reports of the effects of light on plant HMGR which probably comes from the different isoforms present in plants. Levels of both *hmg1* and *hmg2* mRNA are elevated in dark-grown arabidopsis plants, but in wheat (*Triticum aestivum*), for example, where partial clones of *hmgr* have been isolated, mRNA from one clone (HMG 18) accumulates to a higher level in the light, whereas mRNA from another clone (HMG 23) accumulates preferentially in the dark (Aoyagi *et al.* 1993). Also, by measuring HMGR enzyme activity, it has been noted that the plastid and microsomal HMGR activities respond differently to light. The HMGR activity in the plastid fraction was doubled after dark-grown pea-seedlings were treated with red light for 5.0 minutes and returned to darkness for 1.75 hours (Wong *et al.* 1982). Conversely, the microsomal HMGR activity declined rapidly, and stayed low for the next 24 hours (Brooker and Russell 1979). The same kind of differential response has been observed in the HMGR activities of the microsomal and heavy membrane fractions of radish seedlings (Bach and Lichtenthaler 1984).

Whether the above mentioned putative metabolic signal would be able to mediate the perception of light to these different isoforms of HMGR (which might reside in different compartments of the cell), remains a hypothetical question. The signal would not likely be any endproduct of the pathway(s), but it is possible that the commitment of a common intermediate (such as acetyl-CoA) to isoprenoid biosynthesis could act as a regulating factor connecting photosynthetic carbon assimilation and isoprenoid biosynthesis (Learned 1996).

I. 2. 4. Protein phosphorylation

HMGR activity can also be modulated post-transcriptionally by reversible protein phosphorylation. The mammalian HMGR is known to be inactivated by phosphorylation of a regulatory site (Ser872 in the human enzyme) by an AMP-activated protein kinase. The kinase is stimulated by 5'-AMP, and is itself regulated by reversible phosphorylation (Fig. I.5), being inactivated by dephosphorylation and reactivated by a kinase kinase (Clarke and Hardie 1990, Ingebritsen 1983). The sequence around the serine residue is

conserved in many species, including plants, suggesting that inactivation HMGR by a kinase is probably evolutionally an old event.

A homologue for HMGR kinase has also been found in plants, and the discovery of HMGR kinase was the first direct evidence for a protein kinase cascade in higher plants (MacKintosh *et al.* 1992). Like the mammalian AMP-activated protein kinase, the plant kinase is regulated by reversible phosphorylation. However, the plant enzyme is not stimulated by AMP. There is evidence linking calcium/calmodulin complexes and regulation of HMGR by phosphorylation in plants (Vögeli *et al.* 1992, Wititsuwannakul *et al.* 1990), but more work is needed to elucidate the whole cascade. Possibly, still unidentified stimuli for the cascade could be hormones (*e.g.* abscissic acid), isoprenoids (stigmasterol, cholesterol) and even light, since all these mediate inactivation of HMGR activity.

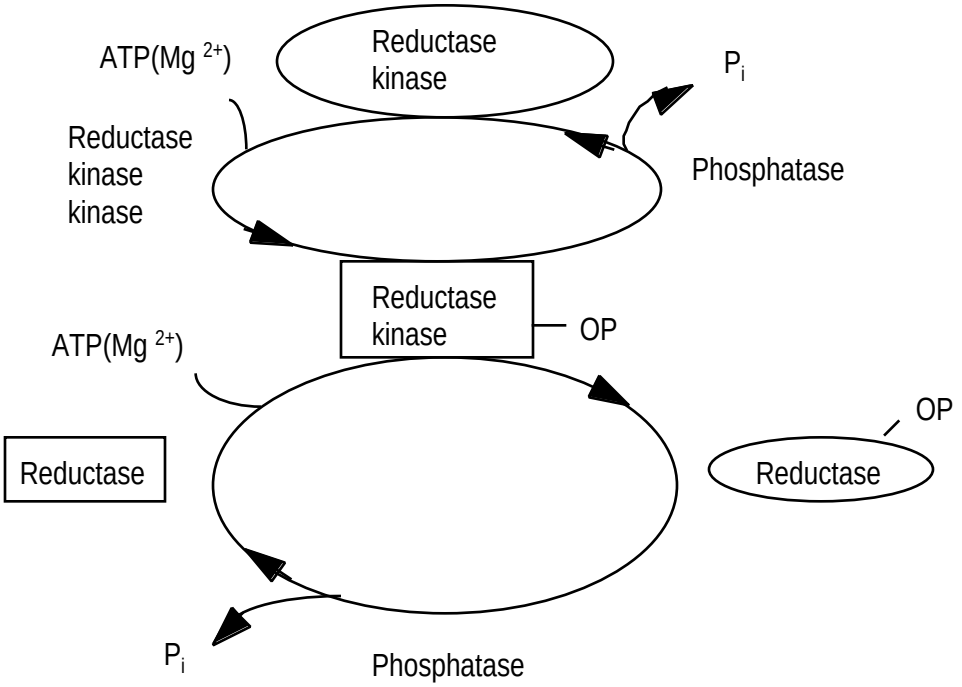

Fig. I.5. Phosphorylation and dephosphorylation of HMGR. (Modified from Ingebritsen 1983).

I. 2. 5. Feedback inhibition by isoprenoids

The single HMGR gene in mammals is regulated transcriptionally and post-translationally by sterols, namely cholesterol and low density lipoproteins (LDL), and a sterol regulatory element (SRE) has been identified in the promoter region of the gene (Osborne *et al.* 1988). It has been suggested that, in the presence of sterols, proteins binding to the SRE actively repress transcription of HMGR (reviewed by Goldstein and Brown 1990).

In yeast, which has two isozymes of HMGR, HMG1 is feedback regulated similarly to the mammalian HMGR by mevalonate-derived compounds early in the pathway. In contrast, HMG2 appears to be modulated by molecules synthesized late in the pathway, after the level of squalene. This feedback regulation coordinates with "contra-regulation" by oxygen, so that the two isoforms are regulated in opposite fashion by the same stimulus. The requirement of oxygen divides the isoprenoid pathway into two parts, the early part up to squalene being independent of oxygen, and the production of the latter products depending on it. When oxygen tension in the cells is high, the late products of the pathway are abundant, and HMG1 is the predominant isoform. When oxygen tension is low, early products accumulate, and HMG2 is the predominant isoform. Regulated degradation plays a third part in this scheme; HMG1 is extremely stable, but rapid degradation of HMG2 is induced by mevalonate-derived early products of the pathway. Thus, the yeast HMG2 resembles a stress protein that is specifically produced at anaerobiosis and is destroyed when the stress is removed (Hampton *et al.* 1996).

Some sterols and nonsterol isoprenoids inhibit HMGR activity in plants, but very little is known about the mechanism, or even about the level of regulation by these compounds in plants. As mentioned earlier, the mevalonate pathway in plants produces several important isoprenoids and isoprenoid-derived compounds compared to just one major endproduct, cholesterol, in animals, thus making feedback regulation of HMGR conceivably more complicated in plants. So far, there have been only a few reports on isoprenoid feedback regulation of HMGR in plants. For example, Russell and Davidson (1982) observed changes in HMGR activity in pea seedlings that were sprayed with different compounds. Stigmasterol or cholesterol reduced HMGR activity, whereas sitosterol had no effect. Isoprenoid growth regulators either inhibited activity (abscisic acid) or increased it (zeatin and gibberellin), while the non-isoprenoid growth regulator 2,4-D had no effect. However, 2,4-D in the growth medium of carrot suspension cell cultures did increase HMGR activity (Nishi and Tsuritani 1983). All and all, it is not clear if the effect of isoprenoid growth regulators on HMGR activity is a form of feedback regulation or just a manifestation of their hormonal action (Stermer *et al.* 1994).

I. 2. 6. Protein degradation

There are few studies reported on degradation of HMGR in plants. In the mammalian system, however, the regulated degradation of HMGR has been the target of extensive research because of health-related interest in being able to regulate cholesterol biosynthesis *in vivo.*

Normally, most ER proteins have half-lives of about two days, whereas, the half-life of rat liver HMGR is only 2-4 hours (Gil. *et al.* 1985). In Chinese hamster ovary (CHO) cells, degradation of HMGR is enhanced by mevalonate or LDL (Inoue *et al.* 1991). A special CHO cell line called UT-1, which contains up to 500-fold more HMGR embedded in enlarged, crystalloid ER (Chin *et al.* 1982), degrades the enzyme as well as the membranes when treated with sterols (Gil *et al.* 1985). In the yeast (*S. cerevisiae*),

these membranes are called karmellae, and their degradation has been shown to occur through a differentiation process into self-degradative organelles (Lum and Wright 1995), a process which might be representative of how ER membranes are turned over in general.

As for HMGR itself, it is known that the mammalian 8-span membrane domain is both necessary and sufficient for regulated degradation. Gil *et al.* (1985) showed that a catalytically active, truncated form of HMGR missing the membrane domain showed slower degradation than the holoenzyme, and that degradation was not accelerated in the presence of LDL, sterols, or mevalonate. On the other hand, a translational fusion between the membrane domain and β-galactosidase contained the needed information for ER localization and regulated degradation of the protein (Skalnik *et al.* 1988). Several studies have since been done to further define the important regions in the membrane domain. Recently, Kumagai *et al.* (1995) identified the second membrane spanning domain of hamster HMGR as important for regulated degradation by introducing a sea urchin HMGR membrane domain::β-galactosidase fusion into CHO cells. The fusion protein was stably expressed, and its degradation was not accelerated by sterols, unless the second membrane domain from sea urchin HMGR was replaced with the corresponding hamster domain. Sekler and Simoni (1995) have also found that introduction of a functional *N*-glycosylation site between spans 5 and 6 of the membrane domain eliminates regulated degradation. This would suggest that the second membrane domain is not the only region responsible for the degradation, and additionally, the process may take place on the lumenal side of the ER membrane.

It has been proposed that sterols or compounds derived from mevalonate might bind to the membrane domain and thereby act as a signal for accelerated degradation (Gil *et al.* 1985, Skalnik *et al.* 1988). Whether this would make HMGR more susceptible to a more general degradation machinery, or if there exists a specific mechanism for degradation of HMGR, still remains a question. What is known, is that cycloheximide abolishes mevalonate-dependent degradation of hamster HMGR, suggesting that a short-lived protein is involved in mediating the signal (Chun *et al.* 1990).

Apart from the sterol-regulated degradation, all HMGRs (including many plant HMGRs) also contain so called PEST sequences which are common to rapidly degraded proteins (Rogers *et al.* 1986). These sequences, rich in proline, glutamic acid, serine and threonine, lie in the linker region between the membrane domain and the catalytic domain of HMGR. Deletion of the PEST sequences from hamster HMGR had little effect on sterol regulated degradation (Chun and Simoni 1992), but it does not rule out the possibility that they could be important in another context.

In addition to the PEST sequences, one of the few facts about degradation of HMGR that could possibly prove interchangeable between animals and plants, is the finding that sterol- regulated degradation and basal degradation of HMGR can both be completely inhibited with two cysteine protease inhibitors, *N*-acetyl-leucyl-leucyl-norleucinal (ALLN), and *N*-acetyl-leucyl-leucyl-methiononal (ALLM) (Inoue *et al.* 1991). HMGR activity recovered from potato extracts was shown to be 10-fold higher if cysteine protease inhibitors were included in the extraction, while other types of inhibitors had no effect (Stermer *et al.* 1994). This is in agreement with experience from our laboratory, where we are using leupeptin as a routine precaution while isolating microsomes for HMGR analysis.

I. 3. SUBCELLULAR LOCATION OF PLANT HMGR

I. 3. 1. The endomembrane system

The endomembrane system, which consists of endoplasmic reticulum (ER), Golgi apparatus, partially coated reticulum, endosomes, secretory vesicles, vacuoles, tonoplast, plasma membrane, and transport vesicles, functions inside the cell to target newly made proteins to their destinations.

Soluble proteins entering the plant endomembrane system are synthesized with a 13-30 amino acid long signal peptide that is cleaved cotranslationally as it crosses the ER membrane. (A short peptide is also used to translocate proteins into mitochondria, chloroplasts and peroxisomes, but these are usually called transit peptides.) Upon reaching the lumen of ER, the proteins fold, form oligomers, and are subjected to modifications such as disulfide bond formation, *N*-linked glycosylation and prolyl hydroxylation. From the ER, the default destination for the proteins is out of the cell through the endomembrane system, unless they contain specific targeting signals to retain them in ER or Golgi, or target them to vacuoles or the plasma membrane (reviewed by Bednarek and Raikhel 1992, Chrispeels 1991). Analogous to animal systems, retention inside plant ER is mediated through the C-terminal amino acid sequences KDEL and HDEL (Lewis and Pelham 1992, Munro and Pelham 1987). Nothing is so far known about the retention of proteins in the plant Golgi. Sorting to vacuoles occurs after the Golgi in the partially coated reticulum with the help of at least three types of signals in the polypeptide, located either in the C- or the N- terminus, or within the mature protein. (Gal and Raikhel 1993).

Membrane proteins probably follow the same route to various endosomal compartments, but usually they do not contain a cleaved signal sequence for entry into the ER. Instead, the hydrophobic transmembrane domains of integral membrane proteins act as anchors in the membrane (Chrispeels 1991). The exact mechanism of insertion into the membrane is not known. Motifs for ER retention have been identified in membrane proteins of animal cells. Type I membrane proteins are efficiently retrieved back to the ER from post-ER organelles if they contain the KK motif, two lysine residues close to the C-terminus (Jackson *et al.* 1993). Type II membrane proteins are retained in ER by a somewhat similar RR motif, two arginines near to the N-terminus of the protein (Schutze *et al.* 1994).

I. 3. 2. Targeting and topology of plant HMGR

The mammalian HMGR is an integral endoplasmic reticulum (ER) glycoprotein, the catalytic domain of which resides on the cytosolic side of the ER membrane. In plants, the subcellular location of HMGR isoforms remains controversial. At the moment, two opposing views exist about the location of the isoprenoid pathway in the plant cell. The first one proposes that the whole pathway from synthesis of isopentenyl pyrophosphate (IPP) occurs independently in cytosol, plastids and mitochondria. According to the other, IPP is synthesized solely in the cytosol and imported into the organelles for synthesis of specific isoprenoid endproducts. (See Fig. I.6).

In support of the fully separate pathways in different organelles, HMGR enzyme activity has been localized to both plastids and mitochondria in addition to the cytosol (ER) (Bach 1986, 1987, Brooker and Russell 1975, Camara *et al.* 1983, Schulze-Siebert 1987), and where purified, the different organellar forms of the enzyme have distinct kinetic parameters. However, none of the cloned HMGR genes seem to fit the plastidic or mitochondrial categories, because no putative transit signals to these organelles have so

far been identified in any plant HMGR sequences. The possibility remains, that other, unknown isoforms of HMGR could be targeted to plastids and mitochondria, as isoprenoid synthesis does involve these organelles in addition to the cytosol. Farnesyl pyrophosphate (FPP) and sesquiterpenes, as well as triterpenes and phytosterols are synthesized in the cytosol and in the cytosol/ER boundary, but monoterpenes and diterpenes, carotenoids, chlorophylls, prenylquinones and tocopherols are synthesized in plastids, and ubiquinone biosynthesis occurs partially in the mitochondria (Fig. I.7) (Kleinig 1989, McGarvey and Croteau 1995).

A novel pyruvate/glyceraldehyde 3-phosphate non-mevalonate pathway leading to IPP has also recently been found in several bacteria and in the green alga *Scenedesmus obliquus* (Rohmer *et al.* 1993, Schwender *et al.* 1996), raising the possibility that chloroplasts might possess a completely different pathway of IPP biosynthesis from the acetate/mevalonate pathway. Mevinolin, a highly specific inhibitor of mevalonate and sterol biosynthesis (Alberts *et al.* 1980), blocks sterol biosynthesis in higher plants but does not affect chlorophyll and carotenoid biosynthesis. Unless mevinolin is unable to cross the chloroplast membranes, a different pathway for IPP synthesis may exist in the chloroplast which cannot be blocked by mevinolin (Schwender *et al.* 1996).

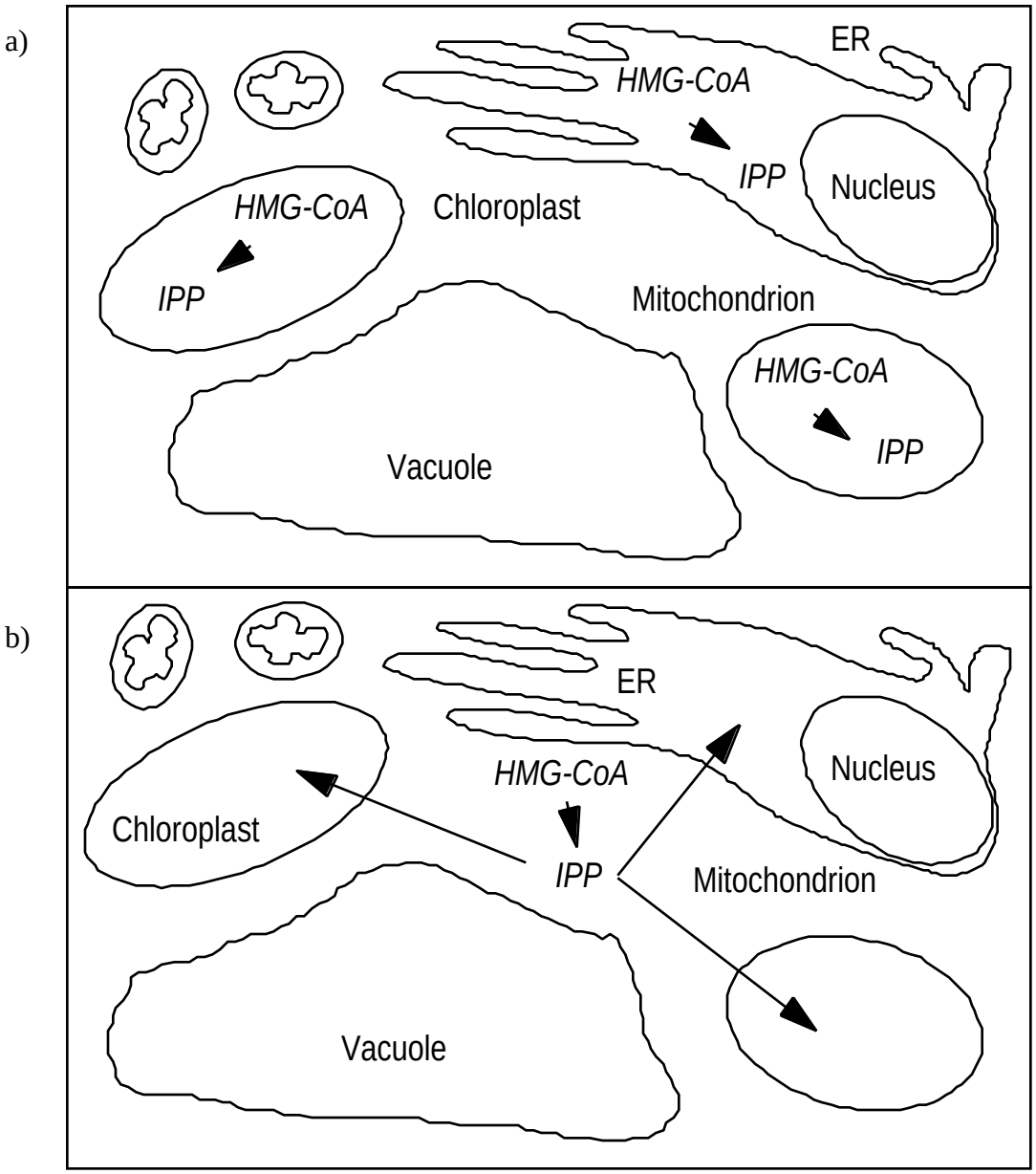

Fig. I.6. Two models of the location of isoprenoid pathway(s) in plant cells.
a) IPP and its derivatives are synthesized independently in each organelle.
b) IPP is synthesized in the cytosol and imported into organelles for synthesis of specific isoprenoid end products. IPP; isopentenyl pyrophosphate.

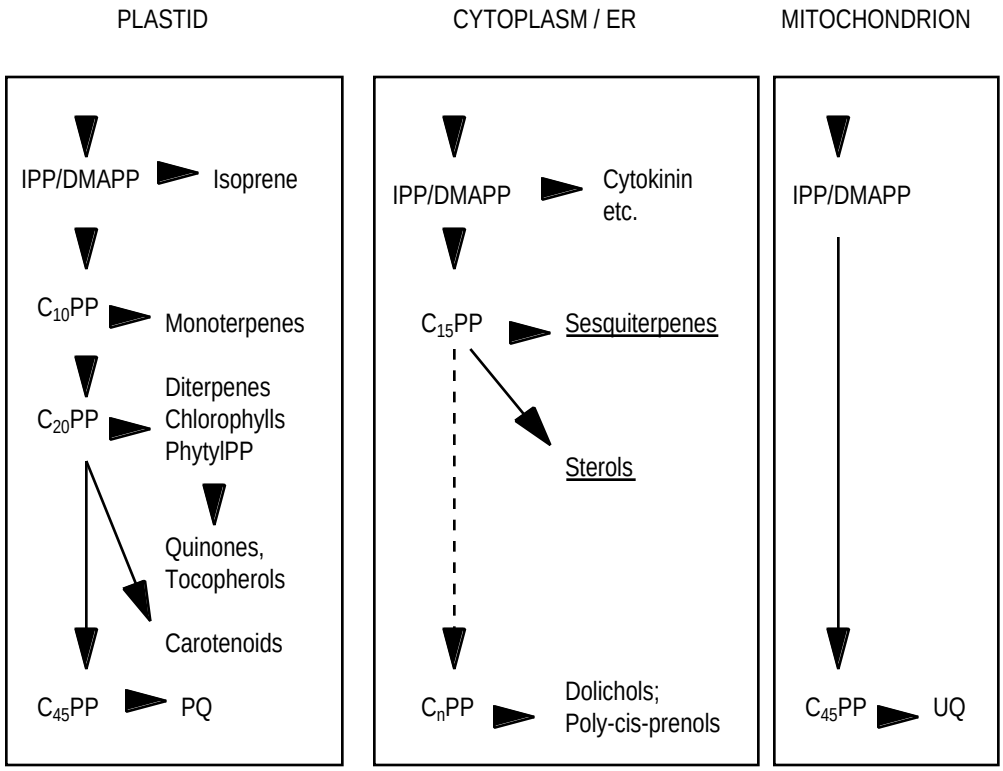

Fig. I.7. Compartmentation of isoprenogenic pathways in the plant cell. IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; PP, pyrophosphate; PQ, plastoquinone; UQ; ubiquinone. (Kleinig 1989).

Moreover, Heintze *et al.* (1990) found that chloroplasts in young barley tissue were capable of synthesizing IPP, but in mature tissue they relied on imported cytosolic IPP. So, the origin of IPP might even differ according to the developmental stage and tissue type of the cell.

Nevertheless, all cloned HMGRs appear to fall in the cytosolic category. Two studies specifically show that arabidopsis HMG1 and tomato HMG1 and HMG2 are inserted into microsomal membranes *in vitro* (Enjuto *et al.* 1994, Denbow *et al.* 1996). In all three proteins the membrane domains function as internal signal sequences, which are recognized by the signal recognition particle, and inserted cotranslationally into microsomal membranes without cleavage of a signal peptide. The proteins span the membrane twice, leaving both the N-terminal end and the C-terminal catalytic domain positioned on the cytosolic side, and only a short hydrophilic peptide on the lumenal side of the membrane (Fig. I.8).

Mammalian HMGR has eight transmembrane regions, all completely divergent from the plant domains (Roitelman *et al.* 1992). Several of these regions have been shown to be important in the regulated degradation of HMGR in response to sterols, *e.g.* cholesterol (see I.2.6 Protein degradation). As plants do not utilize outside sources of sterols as mammals do, this type of down-regulation of HMGR probably does not play a major part in plants. Consequently, the more elaborate membrane domain sructure

present in mammals might not be necessary for plant HMGRs. The fact that all the cloned plant HMGRs have the same kind of domain structure with two conserved potential membrane spanning regions (see Fig. I.4) suggests a similar microsomal location for all of them.

The *in vitro* targeting and topology of arabidopsis and tomato HMGRs in microsomes and the absence of recognized transit signals indicate that plant HMGR is targeted to the ER membrane. Furthermore, the N-termini of most plant HMGRs have the consensus sequence MetAspXArgArgArg (where X can be Val, Ile, Leu or Ser), which fulfills the requirements for an RR-type ER retention motif (two arginine residues at positions 2 and 3, 3 and 4, 4 and 5, 2 and 4, or 3 and 5 from the N-terminus) (Schutze *et al.* 1994).

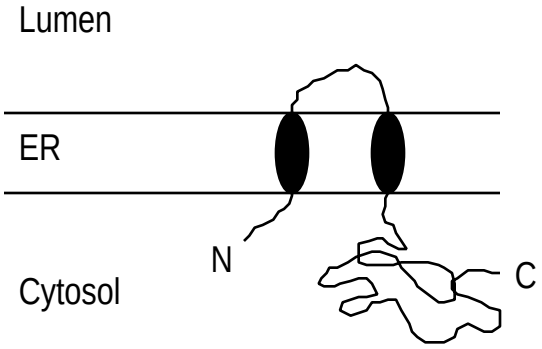

Fig. I.8. Orientation of tomato HMG1 and HMG2 in microsomal membranes. The proteins span the membrane twice, leaving both the N-terminal and the C-terminal catalytic domains positioned on the cytosolic side, and only a short hydrophilic peptide on the lumenal side of the membrane (Denbow *et al.* 1996).

I. 3. 3. Location of different isoforms

Even if all known plant HMGRs were targeted to the ER, it has been suggested that another form of compartmentalization could exist between the different cytosolic isoforms. Several studies have pointed out that isoprenoid biosynthesis can be rapidly redirected from sterol and steroid biosynthesis to accumulation of sesquiterpenoid phytoalexins when cells are challenged with pathogens or elicitors. Associated with the changes in the levels of these products are changes in the activities of HMGR, squalene synthase, and sesquiterpene cyclase. This would seem to suggest that separate branches of the pathway lead to sterols versus sesquiterpenoid endproducts. (Fig. I.9). Do these branches both exist in the cytosol, and if so, are they are physically separated?

The earliest report of the two branches acting separately came from studies on potato tubers. Tjamos and Kuc (1982) showed that eicosapentanoic acid and arachidonic acid (AA) extracted from the pathogenic fungus *Phythophthora infestans* elicited the accumulation of sesquiterpenoids and at the same time inhibited the accumulation of

steroid glycoalkaloids. Similarly, the synthesis of sesquiterpenoid phytoalexins was increased and the rate of sterol synthesis was decreased in tobacco cell cultures that were incubated with *Phytophthora parasitica* cell wall fragments (Threlfall and Whitehead 1988, Vögeli and Chappell 1988). Slicing of potato tubers increased squalene synthetase activity (sterol branch), but if the discs were then treated with AA or *P. infestans*, squalene synthetase activity was suppressed, and sesquiterpene phytoalexins rishitin and lubimin accumulated in the tissue (Zook and Kuc 1991). HMGR activity precedes both the accumulation of sesquiterpenoid phytoalexins (Chappell *et al.* 1991, Chappell and Nable 1987, Ôba *et al.* 1985, Stermer and Bostock 1987, Suzuki *et al.* 1975, Vögeli and Chappell 1988), and the accumulation of sterol and steroid glycoalkaloids (SGA) (Choi *et al.* 1992).

The relationships between different isoforms of HMGR have also been studied by observing mRNA levels of specific isoforms of HMGR during wounding or elicitor treatment. In concert with Zook and Kuc (1991), Choi *et al.* (1992) reported that mRNA levels of potato *hmg1* were strongly induced by wounding, but the induction could be suppressed by treatment with AA or *P. infestans.* Conversely, *hmg2* and *hmg3* mRNA levels were induced by wounding, but strongly enhanced by AA or the pathogen. Transcript levels of potato *hmg1* and *hmg2* also responded differently to methyl jasmonate (see I. 2. 2. Wound- and pathogen- induced expression).

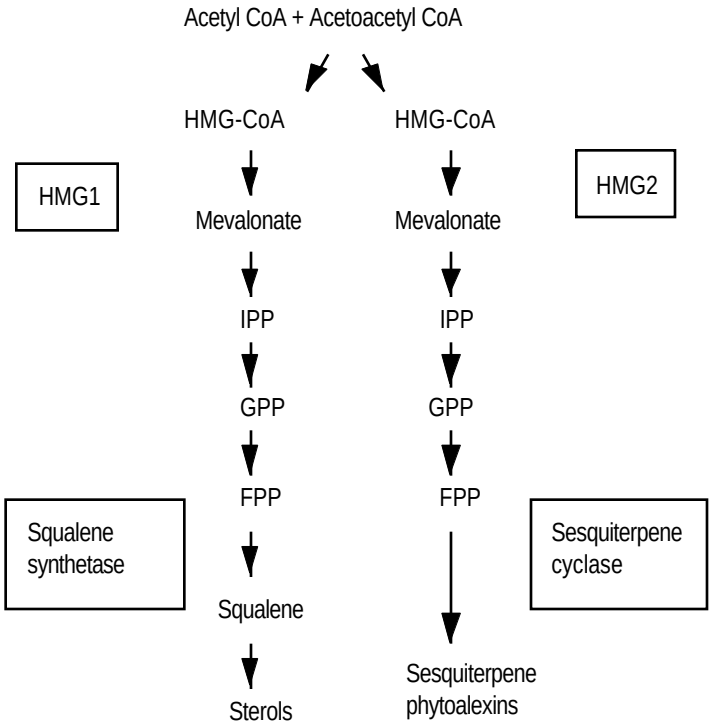

Fig. I.9. Model for a bipartite isoprenoid pathway in plants.

Several exciting studies have also been done in yeast (*Saccharomyces cerevisiae*), which has two isozymes of HMGR, HMGR1 and HMGR2. Casey *et al.* (1992) present a strong argument supporting physiological compartmentalization of the yeast isozymes. They looked at the effect of available sterols and unsaturated fatty acids on HMGR1-or HMGR2- mediated mevalonate metabolism using null mutants of *hmg1* and *hmg2*. When mevalonate was formed via HMGR1, its metabolism to mevalonate-derived nonsaponifiable lipids (MNL) was limited by palmitoleic acid availability, was not affected by oleic acid, and was strongly inhibited by ergosterol. In sharp contrast, HMG2-mediated MNL production was unaffected by palmitoleic acid, inhibited by oleic acid, and only moderately decreased in the presence of ergosterol. Because the regulatory interactions above are directly related to the source of mevalonate, they suggest that the two isozymes and subsequently two pathways are not only separate but also physiologically compartmentalized in such a way that they are unable to share enzymes and intermediates.

The study of Wright *et al.* (1988) also supports distinct compartmentalization of yeast HMGR1 and HMGR2. They looked at the ultrastructure of yeast cells that were genetically engineered to overproduce yeast HMGR1 or HMGR2. Normally, yeast HMGR is localized in the ER. In yeast cells that were overproducing HMGR1, abnormal, large stacks of paired membranes were seen around the nucleus and HMGR was shown to be localized in these membrane structures by immunolabeling. However, in the cells that were overproducing HMGR2, these kind of structures could not be found. Instead, in a small percentage of cells, stacks of membrane closely associated with the plasma membrane were observed. Thus, it seems likely that the isozymes are associated with distinct compartments within the ER.

I. 3. 4. Does glycosylation play a role in targeting ?

In mammals, glycans play a role in the targeting of lysosomal proteins. A mannose-6-phosphate group in the N-linked glycan of a protein targets it to the lysosome with the help of recyclable receptors in the Golgi. (Kornfeld and Mellman 1989). In yeast and plants, glycans have not been found to affect targeting in the endomembrane system. Wilkins *et al.* (1990) showed that glycans are not directly involved in targeting proteins to the vacuole. This does not exclude the possibility of glycans acting as targeting signals in other types of proteins in plants.

Certain plant HMGRs contain a N-linked glycosylation site in the short lumenal segment of the protein between the two membrane spanning regions (Fig. I.4). It is interesting to note that this site is not conserved in all isoforms of plant HMG-CoA reductase. The isoforms that contain the glycosylation site are the ones associated with production of secondary isoprenoid compounds, such as rubber (rubber tree HMG1), or phytoalexins (tomato HMG2, the tobacco and periwinkle HMGRs). The elicitor-inducible HMGR gene from rice makes an exception by lacking the glycosylation site, although the sequence for rice HMGR is very divergent in the transmembrane and lumenal region overall compared to dicots. The other genes that lack the glycosylation site are more of a housekeeping nature; tomato HMG1, arabidopsis HMG1 and rubber tree HMG3 are constitutively expressed and involved in membrane biogenesis and growth (Denbow *et al.* 1996).

There are examples of endomembrane differentiation during the production of secondary isoprenoid compounds like phytoalexins and rubber (Snyder and Nicholson 1990, Chye *et al.* 1991). It is enticing to speculate about the importance of glycosylation sites in the targeting of HMG-CoA reductase. The sites exist only in isoforms that are associated with production of specialized secondary compounds that possibly would need

to be sequestered away in a separate compartment inside the cell. So far it has been impossible to localize the different isoforms using immunochemistry, because there are no antibodies available that could differentiate between the plant HMGR isoforms due to their high similarity.

I. 3. 5. Metabolic channels

What is the cytosol like? The common view of the cell as a "bag of enzymes" still persists, although it has been estimated that the concentration of protein in the cytosol is closer to the concentration of protein in crystals than in dilute solutions (Fulton 1982). The so-called quinary structure, the interactions between macromolecules such as actin and histones (McConkey 1982), also occupies some space. In this context, channeling becomes a very appealing means of controlling metabolism. In metabolons, the reaction sequence is much more efficient when the intermediates do not diffuse in the bulk-phase. The transit time from one active site to another is faster, and higher concentrations are attained with fewer molecules. The reactions are segregated from competing enzymes, and unstable substrates can be acted on quickly. Furthermore, regulation of sequential steps in an enzyme complex can take place through allosteric effects. All in all, the time required for a new steady state is reduced. (Ovádi 1991, Srere 1987).

Indirect evidence from *in vitro* studies with pure enzymes support the existence of metabolons in several pathways, among which are glycolysis, nucleotide synthesis, urea biosynthesis, steroid biosynthesis, fatty acid oxidation, Krebs TCA cycle, amino acid metabolism and macromolecular biosynthesis (Srere 1993). In plants, flavonoid biosynthesis may be facilitated by a multienzyme complex (Hrazdina and Wagner 1985, Hrazdina and Jensen 1990, 1992).

If the two membrane-bound, cytosolic isozymes of HMGR (HMG1 and HMG2) are physically separated so that they cannot compete for HMG-CoA in the same environment, they could be involved in distinct metabolic channels. This idea has been brought up by several authors recently (see Bach 1995, Chappell 1995, Denbow *et al.* 1996, Stermer *et al.* 1994). For instance, when the hamster HMGR gene was engineered into tobacco, only the levels of sterols (eg. the intermediate cycloartenol) were increased, and carotenoid and phytol levels were not affected (Chappell *et al.* 1995). This indicates that the mammalian (hamster) HMGR cannot act as a rate-limiting enzyme for overall isoprenoid biosynthesis in transgenic tobacco, but instead controls only a subset of sterol endproducts.

However, HMGR is not the only regulatory point in the pathway. Instead of the the isoprenoid pathway being regulated at just one "rate-limiting" point in the pathway (*e.g.* HMGR in mammals), it seems that the branches are co-ordinately regulated by several enzymes. Two candidates for major regulatory enzymes in the pathway that lie downstream of HMGR are squalene synthetase and sesquiterpene cyclase (5-*epi*-aristolochene synthase), leading from FPP to sterol and sesquiterpene phytoalexin biosynthesis, respectively (Facchini and Chappell 1992, Vögeli and Chappell 1988, 1990, Vögeli *et al.* 1990) (see Fig. I.9). In general, it has been suggested that the single enzyme "rate-limiting" paradigm should be more or less discarded (Srere 1994), as most pathways share what is called "distributive" control. Although one step can have a higher flux control coefficient than others, it is not usual that one step has exclusive control. In order to increase the flux through a pathway by increasing the amount of the "rate-limiting" enzyme all the other enzymes would have to be in excess! Metabolons fit the model of "distributive" control well; even in loose complexes of sequential enzymes the increase in one enzyme cannot increase the amount of the entire complex. (Srere 1993). HMG2 is an integral membrane protein and 5-*epi*-aristolochene synthase (EAS) is apparently a

cytoplasmic enzyme (Facchini and Chappell 1992); it remains to be shown if these enzymes are part of a specific metabolic channel, or even if they are localized in the same subcellular compartment.

I. 3. 6. Cytoplasmic responses to pathogen ingress

The hypersensitive reaction (HR) is the major, basic resistance response of plant to infection and colonization by pathogens. In HR, the cells in and around the infection site collapse and die in an attempt to kill the attacking organism by sacrificing a few cells and thereby restricting the colonization to these cells. Significant progress has recently been made in understanding mechanisms of recognition, signalling, gene activation, and production of defense-related compounds and proteins in the HR (reviewed by Bent 1996, Dangl *et al.* 1996, Hammond-Kosack and Jones 1996, Ryals *et al.* 1996). Executing the repertoire of responses that plants have against pathogens requires a multitude of cellular changes.

With available microscopic techniques, it has been possible to observe the cytoplasmic responses of the host to a pathogen in live cells. Kitazawa *et al.* (1973) recorded the response of protoplasm of potato cells to infection by *P. infestans*. By using epidermal cells of young unfolded leaflets or cells on cut surfaces of mature leaf petioles they were able to follow the cytoplasmic streaming of the host cell during penetration by the fungus. They found that cytoplasm accumulated more actively around penetration sites in combinations with an incompatible race of *P. infestans* than in those infected by a compatible race. Also, in the incompatible combination, protoplasmic streaming stopped soon after the fungus had penetrated the cells whereas in the compatible interaction, the streaming did not stop at least for 22 hours after inoculation.

More recently, two other studies were done to observe cytoplasmic responses of barley (*Hordeum vulgare*) coleoptile cells to the fungi *Erysiphe pisi* and *Erysiphe graminis*. The prepenetration stages of infection of a pathogen (*E. graminis*) and a nonpathogen (*E. pisi*) species of the fungus were examined using a time-lapse video system and microinjected silicon oil droplets. Cell cytoplasm was clearly responding to the presence of the fungus: cytoplasmic strands were seen under the appressoria and extending away from it, and the movement of the droplets indicated active cytoplasmic streaming near the appressoria. The number of strands increased between appressorial maturation and attempted penetration, and it was suggested that the appressoria of the fungus might release signal(s) upon maturation, which could induce physiological changes in the cytoplasm of the host cell already prior to penetration. No differences were found between the incompatible and the compatible interaction. (Kobayashi *et al.* 1990, 1993).

A study using transparent potato epidermal cells inoculated with *P. infestans* also showed major rearrangements within the cytoplasm at the penetration site, where callose and autofluorescent material were laid down. First, cytoplasmic aggregates were seen to form at the penetration site and around intracellular fungal structures. The nucleus was translocated to the penetration site and cytoplasm became granulated, until finally cytoplasmic streaming stopped, and the protoplast collapsed together with the fungal haustorium. Surprisingly, if the invasion stopped at an early stage of these events, the host restored its normal cytoplasmic activity (*i. e.* the nucleus left its position and aggregation in the cytoplasm dissolved), and the cell survived. Hypersensitive cell death resulted only if the fungus had developed an identifiable haustorium. The only difference between the incompatible and compatible interaction was in the number of cells that responded to the attack; in the incompatible one it was slightly higher. These results suggest that the HR does not develop until the most immediate, highly localized barrier

has failed, and that the outcome of compatible and incompatible interactions is probably determined by a small difference of how many cells responded to the infection (Freytag *et al.* 1994, Schmelzer *et al.* 1995).

Several electron microscopic studies on fixed tissues show the same kind of cytoplasmic reorganization; an increase in cytoplasmic content and activity (Beckman *et al.* 1991, Benhamou 1995, Mould *et al.* 1991, Shi *et al.* 1991, 1992), which is probably indicative of a high metabolic rate in the host cells. At the time of the attack, a lot of newly made proteins and compounds are called for. Most of them are probably needed to secure the physical barriers, *e. g.* to strengthen cell walls, and to plug sieve pores and plasmodesmata. Lignin, callose (β-1,3-glucan), and hydroxyproline-rich glycoproteins (HRGPs) often accumulate at the site of infection together with proteins such as chitinases, β-1,3-glucanases, cellulases, pectinases, and fructosidase (Beckman *et al.* 1991, Benhamou 1995, Benhamou *et al.* 1989, 1990a, b, and c, 1991, Benhamou and Cote 1992, Tahiri-Alaoui *et al.* 1993).

Other compounds are probably responsible for the "chemical barriers", such antagonists to pathogens as phenolics and phytoalexins. Accumulation of various substances in response to pathogens is clearly seen in electron micrographs. Some of these deposits are of lipoidal nature, for they stain heavily with osmium and lipid stains. The deposits can be extracted from fixed tissue with organic solvent treatment, and similar deposits in eggplant parenchyma cells were found to bind a gold-complexed lipase. (Shi *et al.* 1991, 1992, Mould *et al.* 1991, Benhamou 1995). It is probable that these deposits contain terpenoid phytoalexins, which are also known to be lipophilic. In a study of cotton (*Gossypium barbadense*) plants inoculated with *Fusarium oxysporum* f. sp. *vasinfectum* it was noted that the substances developed more rapidly and accumulated to a greater extent in resistant than in susceptible plants (Shi *et al.* 1991). Other deposits have been shown to bind a laccase-gold complex, indicating the presence of phenolic compounds (Benhamou 1995). Likewise, phenolic compounds accumulate for example in *Colletotrichum trifolii* -resistant alfalfa (*Medicago sativa*) plants but not in susceptible ones (Baker *et al.* 1989).

A striking example of phytoalexin accumulation in response to a pathogen was provided by Snyder and Nicholson (1990). Juvenile sorghum (*Sorghum bicolor*), synthesizes two phytoalexins, apigenidin and luteolinidin, as a response to attempted infection by the fungus *Colletotrichum graminicola*. This fungus causes a disease called anthracnose. Both resistant and susceptible cultivars synthesize the phytoalexins and resist the fungus, but as the susceptible cultivars grow older, they lose the ability to respond rapidly to fungal infection. Sorghum offers an excellent chance to observe phytoalexin production during pathogen challenge, because the compounds that are made are visible pigments that differ in color from other components of the plant. In their experiment, the first leaf of 6-day old seedlings was inoculated with *C. graminicola* spores. Twenty hours later, mature appressoria which are the structures from which penetration occurs, had formed. By 22 h, colorless inclusions were seen in the epidermal cells beneath the fungal appressoria. The inclusions moved towards the point of attachment of the appressorium, and became orange-red, the same color as the isolated phytoalexins, apigenidin and luteolinidin. With time the inclusions had enlarged from less than 1 μm in diameter to 20 μm or larger. At 30 h, the inclusions became even more intensely pigmented, and colorless inclusions could no longer be found. Finally, at 45 h, the inclusions released their contents into the cytoplasm and the cell died. Later, pigment was also synthesized in the cells immediately surrounding the infection site. The synthesis of the phytoalexins probably occurred inside the inclusions, because they were first clear and only later became pigmented. In a later report (Snyder *et al.* 1991) it was

demonstrated that the potential level of accumulated phytoalexins was substantially higher than the level required for fungitoxicity.

In other plant species, such as cotton, phytoalexins can be visualized by their autofluorescense. Essenberg *et al.* (1992) found that the microscope filter sets designed for fluorescein detection are selective for autofluorescent cells in cotton as well. Cotton leaves were infiltrated with the bacterial pathogen *Xanthomonas campestris* pv. *malvacearum*, and the development of yellow-green autofluorescence was observed. A few fluorescent cells were seen one day after inoculation, after which the number of the cells increased and clusters of cells started to appear. Later, these cells developed brown pigmentation. The presence of phytoalexins in the autofluorescing cells was confirmed by fluorescence-activated cell sorting; an average strongly fluorescing cell contained 40 times as much 2,7-dihydroxycadalene, 10-25 times as much lacilene, and 10 times as much 2-hydroxy-7-methoxycadalene as an average less fluorescent cell.

In conclusion, plant cells have a repertoire of actions they take when a pathogen attempts to invade. Most of them are used nonspecifically against several types of pathogens, as witnessed by the events that happen in and immediately around the challenged cells, and which are generally considered as "hallmarks" of HR. However, in interactions between different plant and pathogen species it seems that the weight given to separate components of the HR response is different. For example, in some species phytoalexins and phenolic compounds are not produced in susceptible plants at all, and so it would seem that they are an extremely important part of HR in the resistant plants. In other interactions, plugging of vessels might be more effective than phytoalexin production in restricting the spread of the pathogen. In the end, the outcome, resistance or susceptibility, may boil down to the properties of the host and the pathogen in question. One critical aspect in these events is certainly their timing. Resistance to many pathogens is probably determined by how many cells develop the response, and how fast it happens.

I. 4. SUMMARY

Tomato HMG2 has potential to be an excellent marker for studying subcellular differentiation in plant disease resistance. HMGR is a key enzyme in the plant isoprenoid pathway, which produces phytoalexins, antimicrobial compounds that may play an important role in disease resistance. HMG2 is an isoform of HMGR encoded by a wound- and pathogen-inducible member of a multigene family of *hmgr*s in tomato. Expression of *hmgr* genes is also regulated by light and the developmental stage of the plant. Post-transcriptional regulation of HMGR involves protein phosphorylation, and may involve protein degradation and feedback inhibition by isoprenols.

HMG2 is a membrane protein and it may prove rewarding to follow its route through the endomembrane system to its final location in nonstressed versus pathogen-challenged cells. This can be done by immunocytochemistry. However, for immunodetection it is necessary to first introduce an epitope sequence into *hmg2* to be able to use a commercial antibody that recognizes the epitope in the chimeric gene product. There are no antibodies available that can distinguish between the different isoforms of HMGR due to their high similarity.

HMG2 also contains a putative *N*-glycosylation site in between its membrane-spanning regions (on the ER lumen side of the membrane), which enables us to study the importance of glycosylation to targeting of membrane proteins in plants through deletion of this site.

In the long run, the fact that the branches of the plant isoprenoid pathway (HMG1 and HMG2) leading to different endproducts such as sterols and sesquiterpene

phytoalexins might be channeled may also lead to interesting views about the control of metabolism. The different isoforms of HMGR may be localized in different subcompartments of the cell to furnish the channels.

I. 5. LITERATURE CITED

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I. 6. PRESENT STUDY OBJECTIVES

The present study concentrates on the localization of tomato HMG2 during pathogen challenge. Our hypothesis is that tomato HMG2 and its defense-related products are localized in specific compartments inside the cell and delivered to the site of pathogen interaction, whereas HMG1 is probably associated with sterol biosynthesis, and does not respond to elicitors or pathogen challenge in the same way as HMG2 does.

The objectives of this work are:

1) To develop methods to tag tomato HMG2 differentially from HMG1. This is necessary because antibodies that will distinguish between HMG1 and HMG2 are not presently available.

2) To determine the subcellular location of HMG2 in nonstressed cells and in cells expressing a defense response.

3) To monitor subcellular differentiation and its role in disease resistance.

CHAPTER II: ENHANCED RESISTANCE TO TOBACCO MOSAIC VIRUS IN TOBACCO PLANTS CONSTITUTIVELY EXPRESSING TOMATO *hmg2* IS ASSOCIATED WITH CHANGES IN LEAF MESOPHYLL CELL ULTRASTRUCTURE BUT NOT WITH CONSTITUTIVE CAPSIDIOL PRODUCTION

The information presented in this chapter will be incorporated into a manuscript [Lång, S. S., Wu, J., and Cramer, C. L. (1997)], to be submitted to Molecular Plant-Microbe Interactions.

II. 1. ABSTRACT

Transgenic tobacco, constitutively expressing tomato *hmg2*, which encodes a defense-related isoform of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), showed fewer and smaller lesions following tobacco mosaic virus (TMV) inoculation. Transmission electron microscopy was utilized to address if expression of *hmg2* caused overproduction of membranes or other ultrastructural changes in tobacco leaf mesophyll cells. No structures resembling crystalloid ER or karmellae, caused by overexpression of *hmgr* in mammalian and yeast cells, respectively, were observed. However, significant changes in cell ultrastructure were noted, including a larger volume of cytosol (including the endomembrane structure) and accumulation of electron-dense, osmiophilic inclusion bodies inside the vacuole. The same characters were observed in tobacco cells adjacent to necrotic cells in TMV lesions. Transgenic tobacco plants constitutively expressing a truncated (membrane domain) form of *hmg2* did not contain the vacuolar inclusion bodies or show other ultrastructural changes. These plants were not more resistant to TMV than the wild-type, indicating the importance of a catalytically active *hmg2* in mediating these changes. In biochemical analyses, TMV-induced increase in total HMGR activity and total capsidiol amount per gram of leaf were found to be lower in the plants expressing tomato *hmg2* than in the wild-type. Sesquiterpene cyclase was not induced by expression of tomato *hmg2* in non-inoculated leaves. These results confirm that constitutive production of capsidiol, the tobacco sesquiterpenoid phytoalexin, does not play a role in the *hmg2*-mediated enhanced resistance to TMV. However, constitutive availability of the rate-limiting HMGR may facilitate more rapid generation of defense compounds leading to effective lesion limitation. A speculative model of the advantage of speed in phytoalexin production due to expression of HMG2 is discussed. The composition of the vacuolar inclusion bodies and their role in HR is unknown.

II. 2. INTRODUCTION

Phytoalexin accumulation is one of the inducible defense mechanisms usually associated with the hypersensitive reaction (HR) (Smith 1996). The major phytoalexins that accumulate in solanaceous plants, sesquiterpene isoprenoids, are a small group of end-products among a vast number of other compounds synthesized by the isoprenoid pathway. Many of these compounds have critically important functions in the plant, including chlorophylls, carotenoids, and plastoquinone (photosynthesis), ubiquinone and cytochrome a (respiration), sterols and triterpenoids (membrane architecture), gibberellic acids, abscisic acid, brassinosteroids, and certain cytokinins (regulation of growth and development), and phytoalexins (defense against pathogens) (Bach 1995, Chappell 1995).

The enzyme 3-hydroxy-3-methylglutaryl coenzyme reductase (HMGR, EC 1.1.34) catalyzes an early step from hydroxymethylglutaryl CoA to mevalonic acid in the pathway. Plant *hmgr*-genes constitute a multigene family encoding differentially

regulated HMGR isozymes. For example, tomato *hmg1* is constitutively expressed and associated with sterol biosynthesis (Narita and Gruissem 1989), whereas *hmg2* is induced by wounding and pathogens, presumably associated with phytoalexin biosynthesis, (Cramer *et al.* 1993, Denbow *et al.* 1996, Park *et al.* 1992).

Whether HMGR is a rate-limiting step for isoprenoid biosynthesis in plants, is controversial. When Chappell *et al.* (1995) constitutively expressed *hmgr* from Chinese hamster ovary (CHO) cells in transgenic tobacco plants, total HMGR activity in the plants increased 3- to 6-fold, but the amount of end-product sterols only increased 2-fold, and a sterol biosynthetic intermediate, cycloartenol, accumulated more than 100-fold. Moreover, the levels of other isoprenoids, *e.g.* sesquiterpene phytoalexins, did not change. This led the authors to suggest that other, later enzyme(s) in the pathway could be controlling the flux to end-product sterols.

In tobacco (*Nicotiana tabacum*), sesquiterpene cyclase (5-*epi*-aristlochene synthase or EAS) is the key enzyme catalyzing the first committed step, cyclization of farnesylpyrophosphate (FPP), specific to the phytoalexin biosynthetic branch. EAS is a soluble, probably cytoplasmic enzyme with several (12-15) isoforms encoded by a partially clustered gene family (Facchini and Chappell 1992). In tobacco cell suspension cultures, EAS is induced after treatment with elicitors, such as cellulase and cell wall fragments from the fungus *Phytophthora parasitica* (Vögeli and Chappell 1988, Vögeli and Chappell 1990, Facchini and Chappell 1992). The induction of EAS is co-ordinated with increase in HMGR activity, preceding accumulation of sesquiterpenoid phytoalexins.

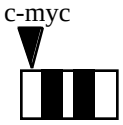
We are interested in the regulatory role of HMGR in the production of phytoalexins in the plant defense response. We have found that expressing the defense-specific isogene *hmg2* from tomato in transgenic tobacco plants renders them more resistant to rotting by *Erwinia carotovora* subsp. *carotovora* (*Ecc*), and decreases lesion size in a resistant interaction with tobacco mosaic virus (TMV) (Yu *et al.* 1997, unpublished results). Since the most interesting data from this study originated from one single plant (plant 2421), we retested this plants' response to TMV, and included two sets of new plants, expressing either the full-length tomato *hmg2* (SL7-plants) or the membrane domain of tomato *hmg2* (SL2-plants) in the experiments. The *hmg2*-sequences in both the SL7- and SL2-plants were tagged with a sequence encoding a human c-*myc*-epitope for future immunolocalization studies. The results suggest that HMG2 plays a critical role in defense and that the defense-specific isoform may, in fact, function in a rate-limiting capacity for synthesis of defense-related isoprenoids. Ultrastructural and biochemical analyses were utilized in order to understand the basis of *hmg2*-mediated resistance to TMV.

II. 3. RESULTS

II. 3. 1. Transgenic plants expressing *hmg2* restrict spreading of TMV lesions

Yu *et al.* (1997) found that compared to the wild-type, a tobacco plant (plant 2421) expressing the full-length tomato *hmg2* cDNA driven by an enhanced CaMV 35S promoter showed 34% less tissue loss following *Ecc* inoculation in a maceration assay. The size of *Ecc* lesions on detached leaves was reduced by 12%, and the area of the lesions was reduced by 21%. The area of TMV lesions was reduced by 42 to 47%. In the current study, we included additional constructs, shown in Table II.1, to further delineate the effect of HMG2 expression on TMV interactions. SL2-plants express the membrane domain of tomato *hmg2* driven by an enhanced CaMV 35S promoter ($35S^{DE}$). SL7-plants express the full-length tomato *hmg2* driven by the same promoter. In both these

Table II.1. hmg2-construct used in the study. See methods and material for details.
a) The expression of the transgenes was driven by a double enhanced version of the CaMV 35S promoter and tobacco etch virus 3'-untranslated leader sequence (Carrington and Freed 1990).
b) The c-*myc*-tagged *hmg2* membrane domain contains the first 690 bp of tomato *hmg2* cDNA. A 39 bp fragment encoding a 13 aa c-*myc* epitope (Evan *et al*. 1985) was inserted at the *Bgl*II site in tomato *hmg2* (Denbow *et al*. 1996). The c-*myc*-tagged *hmg2* contains the full-length (1806 bp) tomato *hmg2* cDNA.
c) Dark boxes represent transmembrane spans in the membrane domain, shaded boxes represent the catalytic domain.
d) HMGR activity was assessed in young leaf tissue (not fully expanded, top leaves of tobacco plants), where the 35S promoter is active. Values represent a range of several independent determinations of leaf material from individual transgenic lines.

Plant	Promoter[a]	Coding region[b]	Transgene product[c]	HMGR activity[d] (nmol/mg/h)
Xanthi	-	-		0.5-0.9
SL2	$35S^{DE}$	*c-myc* -tagged *hmg2* membrane domain		0.5-0.9
SL7	$35S^{DE}$	*c-myc* -tagged full-length *hmg2*		1.3-1.5
2421	$35S^{DE}$	full-length *hmg2*		1.0-7.0

constructs, the N-terminus of *hmg2* was tagged with a human c-*myc*-epitope sequence to facilitate detection of the *hmg2* gene product (see Fig. III.1). Plant 2421 contains the full-length *hmg2* gene without the epitope under the control of $35S^{DE}$.

The tobacco cultivar used in the study (Xanthi nc) is resistant to TMV and non-transgenic plants develop necrotic lesions typical of the hypersensitive response (Fig. II.1a). The response of tobacco plants expressing tomato *hmg2* to TMV was studied by surface inoculation of excised leaves and incubation for five days at room temperature. Lesions on plant SL2-6, the plant expressing the non-catalytic membrane domain of *hmg2*, were similar to those on wild-type leaves (Fig. II.1b). Lesions on plants expressing full-length *hmg2* were more sharply defined and darker brown in color (Fig. II.1c). The number and size of lesions were smaller in plant 2421, which had earlier been identified with the highest level of HMGR activity among the transformants. However, there was a great degree of variation between individual plants started from shoots from the same

plant 2421 T_0-parent. Shoot 2421-1 developed only a few pinpoint-sized lesions (Fig. II.1d), shoot 2421-3 exhibited a decreased number of fairly large, dark-brown lesions (Fig. II.1e), and shoot 2421-2 showed lesion development comparable to the wild-type, but the color of the lesions was strikingly dark brown, similar to the SL7-plants (Fig. II.1f). The dark lesions also had more chlorosis in the surrounding tissues than the wild-type lesions. Three leaves from each shoot were inoculated with TMV and they all gave consistent phenotypes. We do not have an explanation to why different shoots from the same plant (plant 2421) developed different sized lesions, but suppression of the transgene to a different degree in different shoots may be one possibility.

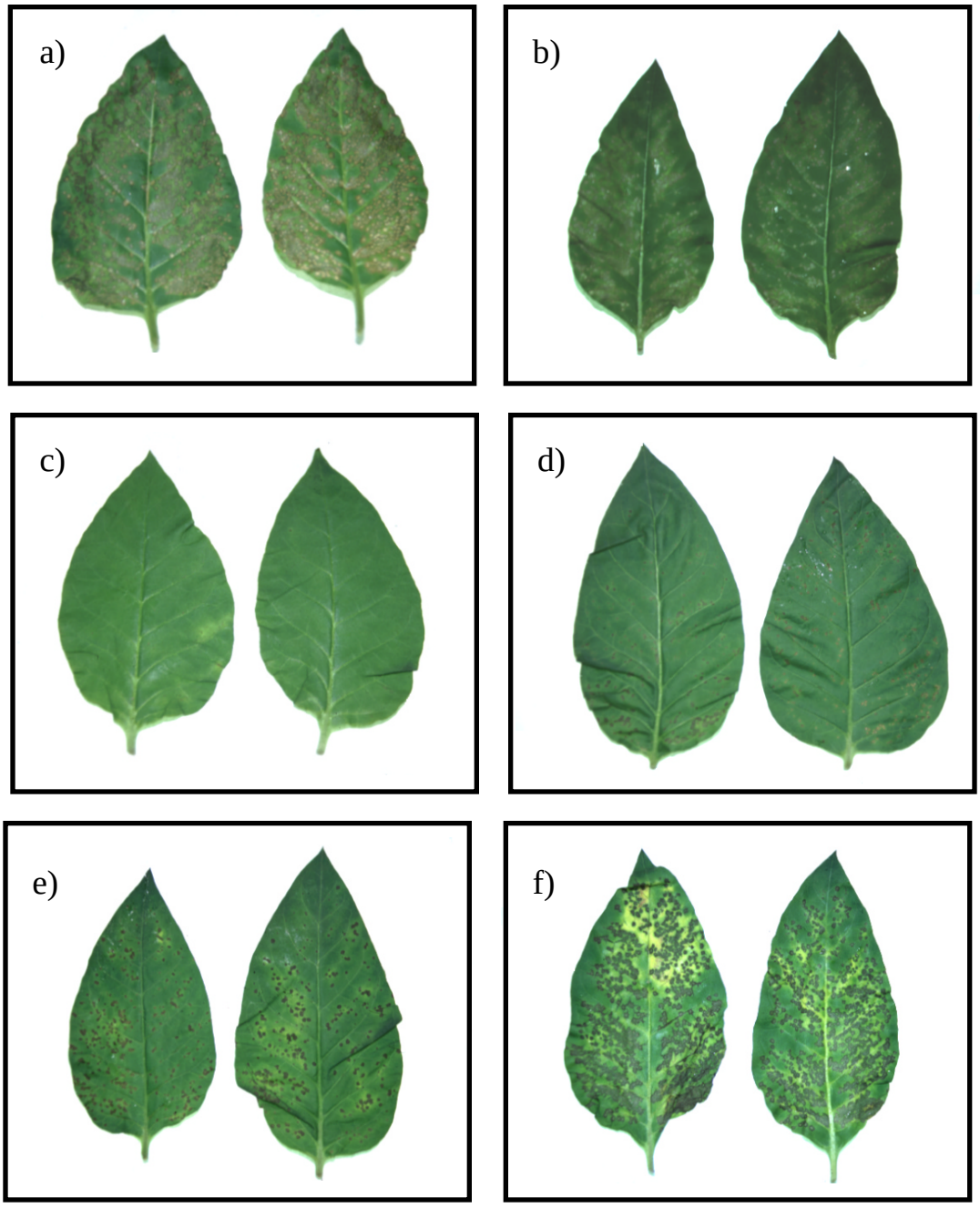
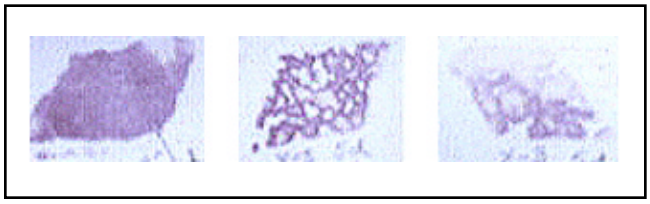


Fig. II.1. TMV-lesion phenotype on excised leaves of wild-type and the *hmg2*-transgenic tobacco plants 5 d after inoculation with 50 µg/ml of TMV strain U1.

a) Lesions on the wild-type Xanthi.
b) Lesions on the SL2-plants (expressing MYV-HMG2md).
c) Lesions on plant 2421-1 (expressing the full-length tomato *hmg2* without an epitope).
d) Lesions on plant 2421-3 (expressing the full-length tomato *hmg2* without an epitope).
e) Lesions on plant 2421-2 (expressing the full-length tomato *hmg2* without an epitope).
f) Lesions on SL7-plants (expressing MYC-HMG2).
Plants 2421-1, 2421-2, and 2421-3 are individual plants regenerated from shoots from the original plant 2421.
Note the darker brown color of lesions on the transgenic plants expressing the full-length constructs, and the smaller size of lesions on plant 2421-1.

a)



b)

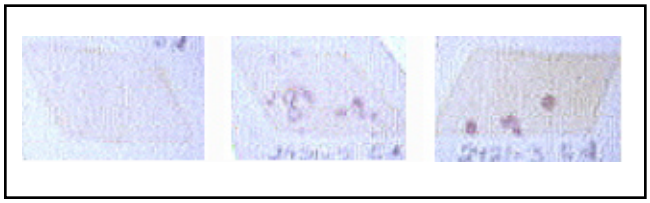


Fig. II.2. Tissue blot immunoassay of pieces of tobacco leaf 5 days after inoculation with 50 µg/ml TMV strain U1.
a) Wild-type Xanthi, three different leaves. The lesions on the wild-type consisted of dried, dead tissue which did not leave a print on the blotting paper. In the blot at the far left, the juices from surrounding tissues spread to cover the lesion areas as well.
b) From left to right: plant 2421-1, plant 2421-3, and plant 2421-2 (all expressing the full-length tomato *hmg2* without the c-*myc*-epitope sequence). Purple color indicates the presence of a rabbit anti-TMV antibody detected by a secondary, alkaline phosphatase-conjugated goat anti-rabbit antibody. In the transgenic leaves, the virus is restricted to the area of the lesions.

II. 3. 2. Vacuolar inclusion bodies are found in TMV-inoculated leaves and non-inoculated leaves of the *hmg2*-expressing plants

In mammalian and yeast (*S. cerevisiae*) systems, overexpression of *hmgr* results in novel structures inside the cell consisting of stacked membranes of ER. In CHO cells, which accumulate up to 500-fold levels of HMGR, the resulting membranes are called crystalloid ER (Chin *et al.* 1982). In yeast (*S. cerevisiae*), with approximately 10-fold higher HMGR levels, they are called karmellae (Wright *et al.* 1988). Ultrastructure of plants used in the study was analyzed by electron microscopy to determine whether expression of a plant *hmgr* gene, *hmg2*, causes a similar phenomenon in plant cells. No structures resembling the ordered crystalloid ER or karmellae were found in transgenic or in wild-type leaf tissue.

However, compared to the wild-type Xanthi, *hmg2*-expressors showed other morphological changes. In the plants expressing full-length *hmg2*, the cytosol occupied a larger volume inside the cells, and the endomembrane structure seemed enlarged (Fig. II.3a,b). Most notably, electron-dense inclusion bodies (IBs) had accumulated within the vacuoles. The IBs stained intensely with osmium, which suggests they are of lipophilic nature (Fig. II.3c,d). These changes were not observed in SL-2 plants expressing the construct without the catalytic domain of *hmg2*. (Fig. II.3e).

Ultrastructural analyses of wildtype and *hmg2*-expressing plants five days after inoculation with 50 μg/ml of TMV strain U1 revealed that the same features were found in nontransgenic tobacco cells adjacent to necrotic cells in the lesion (Fig. II.3f). This result suggests that expression of *hmg2* was inducing defense-related changes in cell morphology in the absence of pathogen. In order to address whether *hmg2*-expression leads to continuously activated host defenses, we analyzed HMGR activity and the levels of capsidiol and sesquiterpene cyclase in non-inoculated and TMV-inoculated leaves.

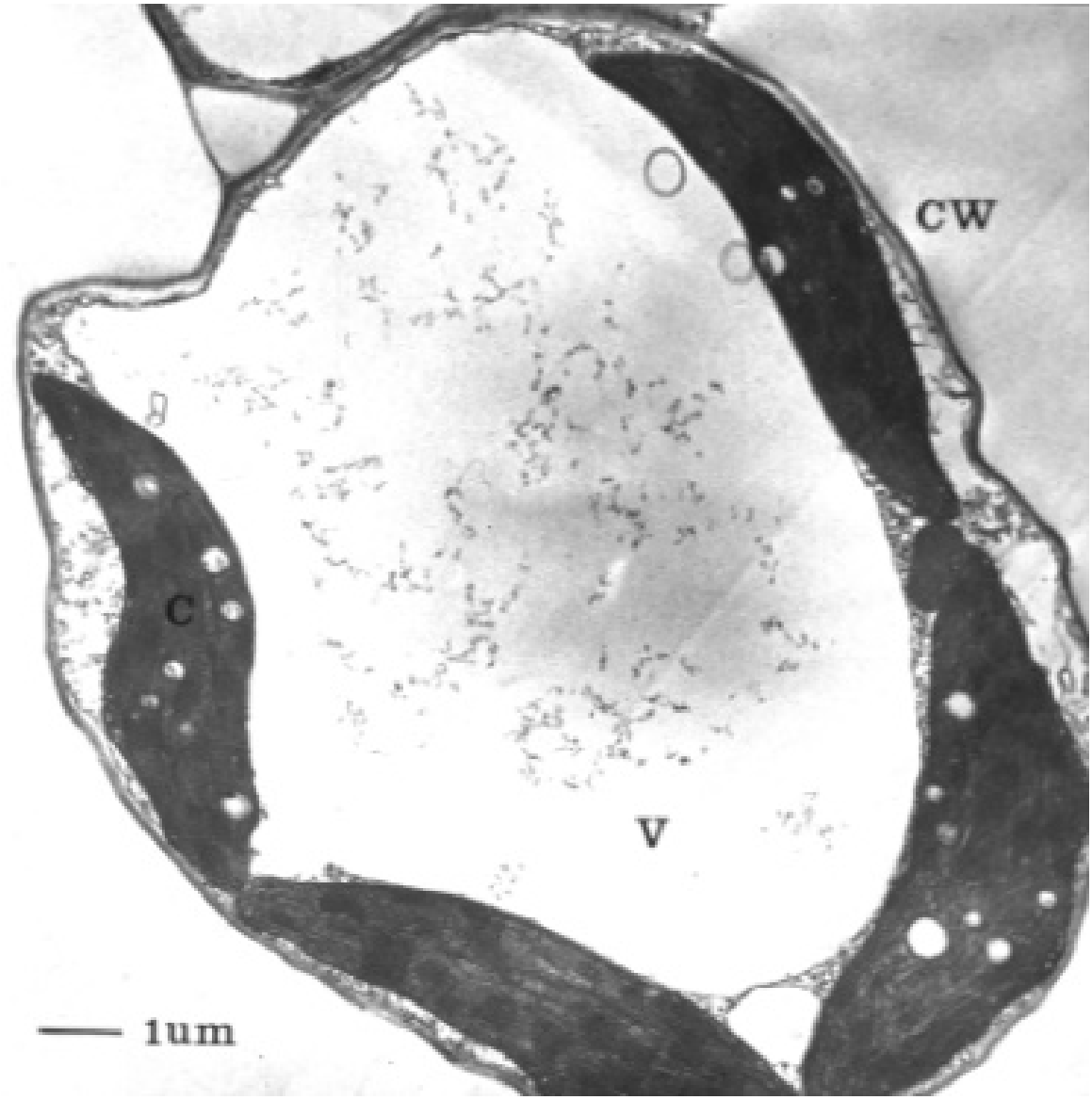


Fig. II.3a. Electron micrograph of a wild-type Xanthi leaf mesophyll cell from a healthy plant. Bar =1 μm. CW, cell wall; V, vacuole; C, chloroplast. Note density of the cytoplasm and absence of inclusion bodies in the vacuole.

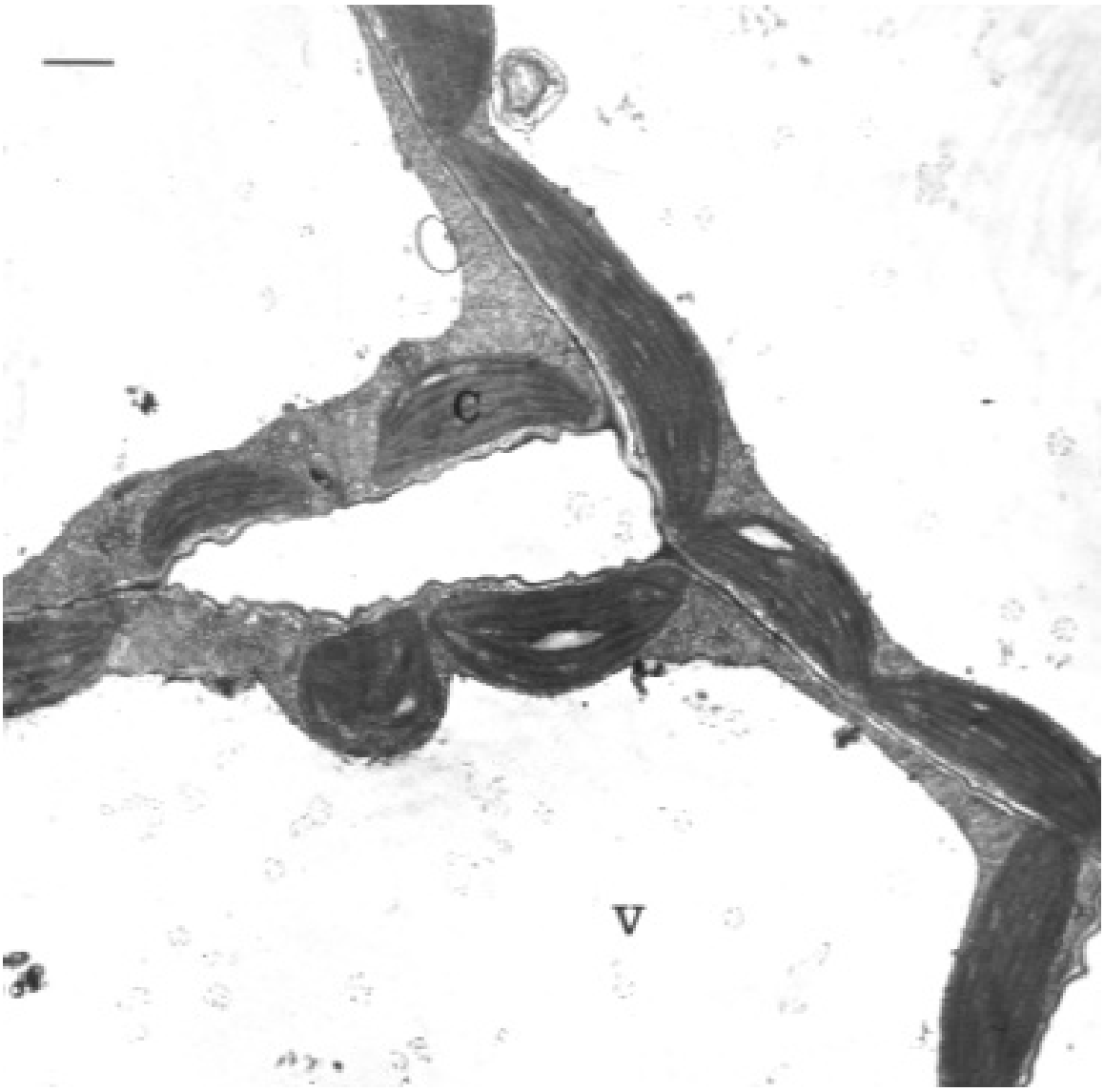


Fig. II.3b. Electron micrograph of leaf mesophyll cells from plant SL7-5 (expressing the full-length, c-*myc*-tagged tomato *hmg2*). Compared to the wild-type cells, cells in SL7-plants showed thickened cytoplasm consisting of increased amounts of endomembrane. Bar = 1 μm. V, vacuole; C, chloroplast.

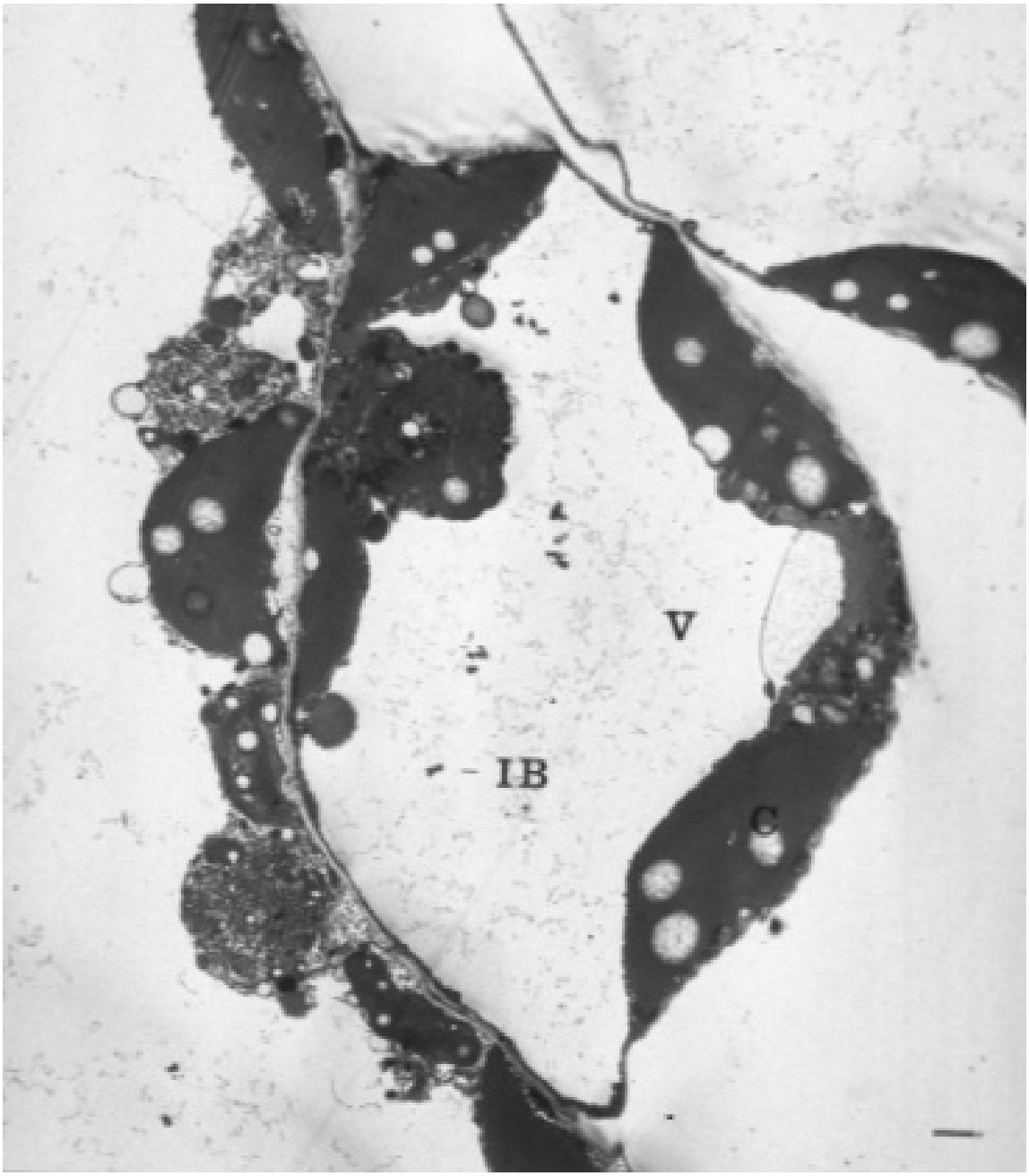


Fig.II.3c. Electron micrograph of a leaf mesophyll cell from plant 2421 (expressing the full-length tomato *mg2* without the c-*myc*-epitope sequence) exhibiting morphological changes (enlarged endomembrane structure and dense inclusion bodies within the vacuole) similar to the changes in SL7-plants (expressing the full-length, c-*myc*-tagged tomato *hmg2*. Bar = 1 μm. V, vacuole; C, chloroplast; IB, inclusion body.

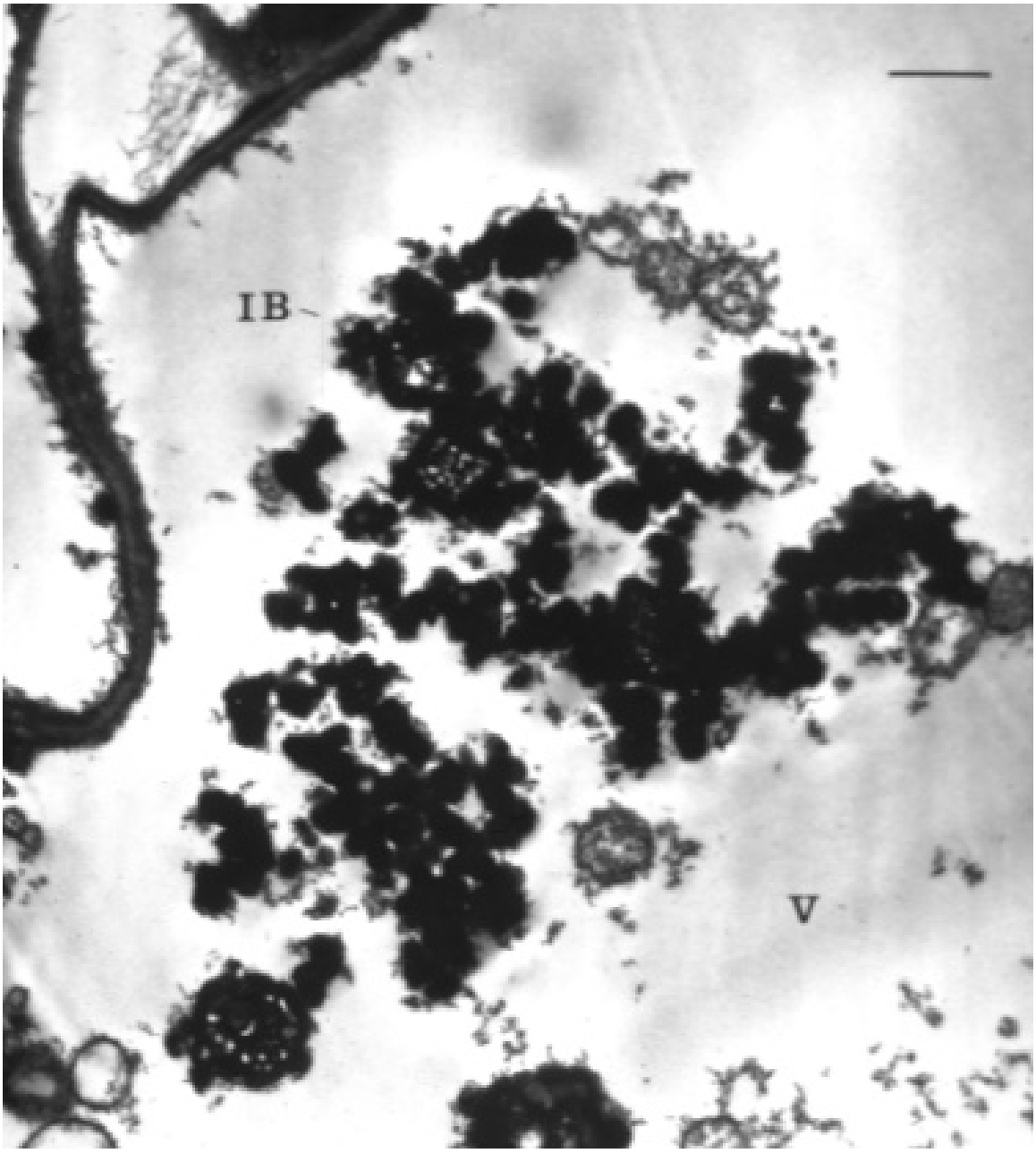


Fig. II.3d. Electron micrograph of a leaf mesophyll cell from plant SL7-28 (expressing the c-*myc*-tagged, full-length tomato *hmg2*) showing electron-dense inclusion bodies within the vacuole. Bar = 1 µm. V, vacuole; IB, inclusion bodies.

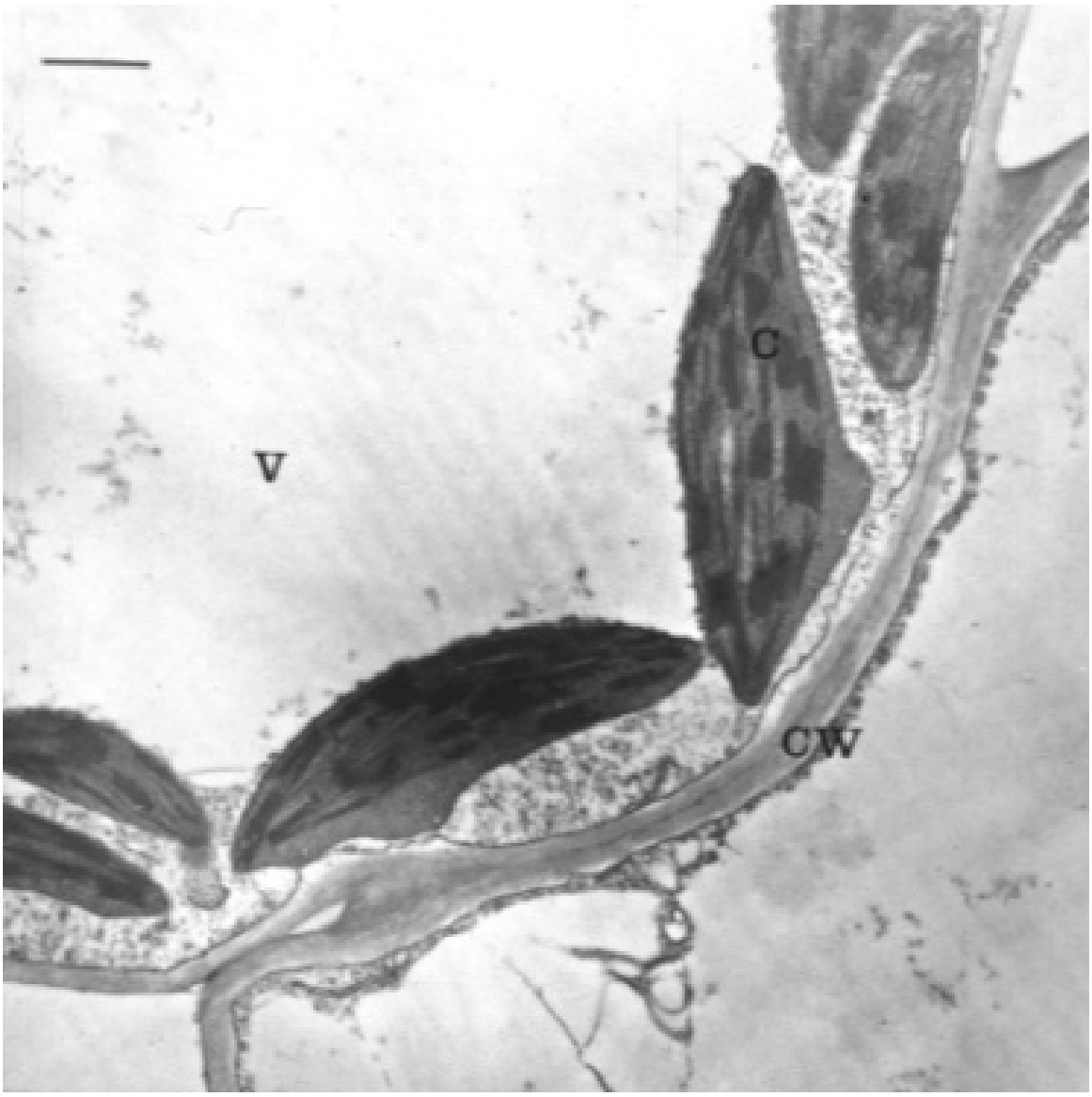


Fig. II.3e. Electron micrograph of a leaf mesophyll cell from the plant SL2-8 (expressing the c-*myc*-tagged membrane domain of tomato *hmg2*, without the catalytic domain). The morphological changes observed in transgenic plants (enlarged endomembrane structure and vacuolar inclusion bodies) were not detected in leaf mesophyll cells from SL2-plants. Bar = 1 μm. V, vacuole; CW, cell wall; C, chloroplast.

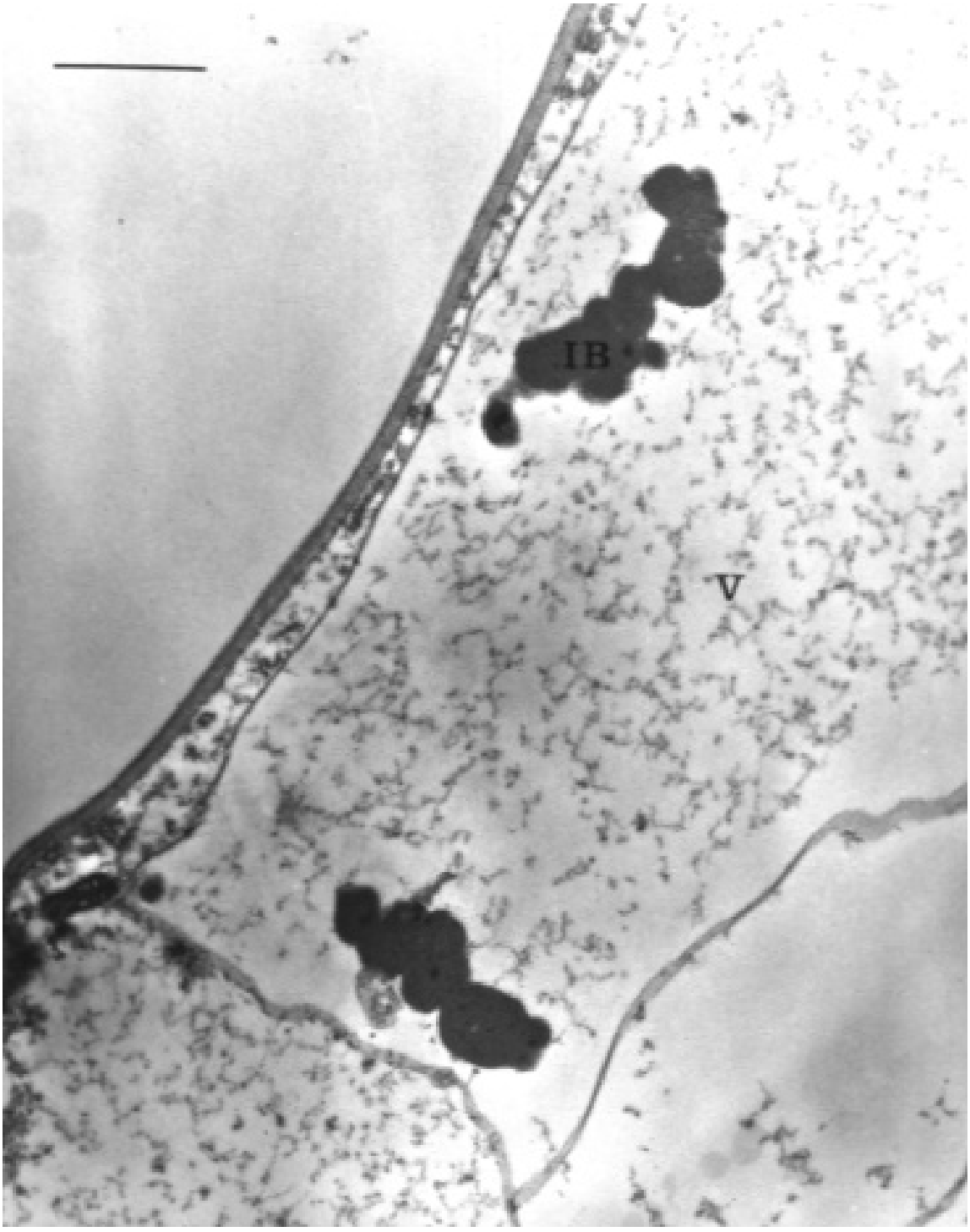


Fig. II.3f. Electron micrograph of a leaf mesophyll cell from a wild-type Xanthi plant 5 d after inoculation with 50 μg/ml TMV strain U1. Cells adjacent to necrotic lesions showed similar electron-dense inclusion bodies within the vacuole as leaf mesophyll cells from

non-inoculated, transgenic plants expressing the full-length *hmg2*-construct. Bar = 0.1 μm. V, vacuole; IB, inclusion body.

II. 3. 3. HMGR activity is lower in leaves exhibiting fewer and smaller lesions

HMGR enzyme activity in the inoculated leaves was assayed to determine if the lesion phenotype in the plants expressing *hmg2* was correlated with higher HMGR activity. The assay measured total HMGR activity comprising endogenous tobacco HMGR activity and HMGR activity resulting from introduction of the tomato transgene.

Other studies have shown that transgene products under the control of the 35S promoter are more highly expressed in young tissues (Cramer *et al.* 1996). HMGR activity was higher in young leaf tissue from the transgenic plants expressing the full-length *hmg2*-constructs than in the wild-type or in the plant expressing the membrane domain of *hmg2*, without the catalytic domain (Table II.1). However, for the TMV-assay, it was necessary to use more mature leaves, because the effectiveness of the HR to TMV is age dependent, and necrotic lesions will not develop in very young leaves (Dr. Sue Tolin, personal communication). Leaves of approximately 15 to 18 cm in length and of similar age were used in the study. In these leaves, the levels of HMGR activity immediately after TMV inoculation were comparably low (about 0.5 nmol/mg/h) in all the plants tested (Table II.1), which is probably due to the $35S^{DE}$ promoter not being expressed as strongly in the older tissue. Five days post inoculation, HMGR activity had increased in all plants, but to variable degrees. In the wild-type (Xanthi), the plant expressing the membrane domain of *hmg2* (SL2-6, with wild-type lesions) and the *hmg2*-expressor that exhibited the large, but restricted and dark-brown lesions (SL7-6), HMGR activity had increased from 5- to 10-fold. In contrast, in the plant 2421-1 exhibiting the small pinpoint-lesions, HMGR activity had increased only about 3 to 4-fold. In various assays on younger tissue from HMG2-expressors HMGR activities ranged from 2 to 15-fold higher than the wild-type.

II. 3. 4. Accumulation of capsidiol and sesquiterpene cyclase requires pathogen-based induction

Accumulation of the tobacco sesquiterpenoid phytoalexin, capsidiol, was measured by gas chromatography to determine if the constitutive expression of *hmg2* resulted in elevated phytoalexin production. Leaves of non- and mock-inoculated plants (both wild-type and *hmg2*-expressors) contained little capsidiol. Five days after inoculation with TMV, the amount of capsidiol in the wild-type leaves had accumulated 4-fold. In contrast, no detectable increase in capsidiol levels were found in the plant 2421.

We were intrigued by the darker brown color of the lesions in *hmg2*-expressing plants, since tobacco cell suspension cultures will also turn brown after treatment with elicitors (Chappell and Nable 1987, Chappell *et al.* 1991). Five days after TMV inoculation, the level of capsidiol (per gram of leaf) was lower in the plant 2421 than in the wild-type, but if high amounts of capsidiol were produced only in the cells surrounding the lesion, this amount would be diluted out by the large number of healthy, nonresponsive cells in the plant 2421 leaf. On the contrary, the much larger number of responsive cells around wild-type lesions, even if they were producing a lower amount of capsidiol per cell, could bring the total amount of capsidiol per leaf higher. To further investigate, if expression of *hmg2* (without elicitation) was also inducing the latter part of

the pathway leading to capsidiol production, we analyzed expression of sesquiterpene cyclase (EAS).

Leaf extracts from mock-inoculated *hmg2*-expressors did not contain any EAS, as determined by SDS-PAGE immunoblot analysis (Fig. II.4). Five days after inoculation with TMV, a polypeptide of the approximate size of EAS (60 kDa) was detected in the soluble protein fraction. This result indicates that the latter part of the isoprenoid pathway, including EAS, was not induced by constitutive expression of *hmg2* in the un-inoculated plants. Other bands with a lesser affinity to the antibody were detected in both the microsomal and the soluble fraction. These may represent other members of the multigene family of cyclases in tobacco (Facchini and Chappell 1992). EAS was similarly induced in the wild-type Xanthi (data not shown).

Table II.2. HMGR activities (nmol/mg/h) in leaves of Xanthi, 2421-1, SL2-6 and SL7-6 assayed 0,3, and 5d post inoculation with TMV strain U1. (nd = not determined).

	0d	3d	5d
Xanthi	0.5	3.5	4.8
2421-1	0.4	2.2	1.5
SL2-6	0.6	nd	3.2
SL7-6	0.6	nd	4.6

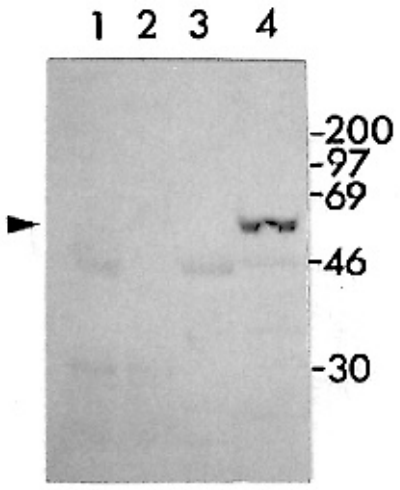


Fig. II. 4. Immunoblot detection of sesquiterpene cyclase (EAS) in microsomal and soluble protein fractions of 2421 five days after mock- or TMV-inoculation. Proteins (approximately 40 μg) were separated by gel electrophoresis, transferred to PVDF membrane and reacted with antibody to tobacco sesquiterpene cyclase. Lane 1: microsomal pellet from a mock-inoculated leaf; lane 2: 100,000 x g supernatant from a mock-inoculated leaf; lane 3: microsomal pellet from a TMV-inoculated leaf; lane 4: 100,000g supernatant from a TMV-inoculated leaf. Arrow points at EAS at approximately 60 kDa.

II. 4. DISCUSSION

In a recent review, Smith (1996) makes the point that although in a number of plant-pathogen interactions the resistant plant cultivar or species has been shown to accumulate higher concentrations of phytoalexins than the susceptible one, other examples show phytoalexins accumulating to higher levels in compatible interactions. Results from the present study suggest that in fact, for an incompatible interaction, the timing and location of the response could prove to be more critical than the overall amount of phytoalexins accumulated.

The increased resistance to TMV observed in the transgenic tobacco plants expressing tomato *hmg2* was not due to constitutive production of capsidiol, since prior to inoculation with TMV, both the wild-type and the *hmg2*-expressors contained the same small amount of capsidiol. Sesquiterpene cyclase, a later enzyme in isoprenoid pathway leading to sesquiterpene phytoalexins, was also not induced in *hmg2*-expressors, consistent with the absence of any significant amounts of capsidiol in the tissue. When excised leaves were challenged with TMV, sesquiterpene cyclase was induced and capsidiol accumulated in the wild-type. In *hmg2*-expressing leaves, sesquiterpene cyclase was induced, but no detectable increase in capsidiol levels were found. The wild-type tobacco developed larger lesions than the *hmg2*-expressing leaves. Logically, if the amount of phytoalexin produced were correlated with the number of cells responding around the infection site, as the lesions grew larger, more cells would have produced capsidiol in the wild-type than in *hmg2*-expressors.

How then does constitutive *hmg2* expression lead to effective lesion limitation in the transgenic plants? A possible explanation is an advantage of speed in capsidiol biosynthesis over the wild-type, gained by the constitutive expression of *hmg2* in the transgenic tissues. If high concentrations of phytoalexins are produced fast enough to contain the pathogen and restrict the spread of the lesion to a few cells, it will become unnecessary for more peripheral cells to activate their defense responses. Even very high concentrations of capsidiol per cell could have gone undetected by our methods, if only the cells immediately around the infection site were producing the phytoalexin. The study of Essenberg *et al.* (1992) supports this view. One day after infiltration of cotton leaves with *Xanthomonas campestris* pv. *malvacearum*, only a few autofluorescent, phytoalexin-producing cells were observed, after which the number of the fluorescent cells increased and clusters started to appear. We plan to test this hypothesis using suspension cultured tobacco cells expressing the same *hmg2*-constructs. In suspension cell culture, all cells will respond to the elicitor (*e.g.* cellulase), and HMGR activity and capsidiol amount in the cells as well as the speed at which capsidiol is produced will reflect the situation in the few cells surrounding the lesion *in planta.* Another possibility is that the smaller lesion size did not result from capsidiol biosynthesis *per se*, but expression of *hmg2* somehow induced other defense mechanisms in the cells. However, the fact that plants expressing the construct without the catalytic domain of HMG2 (SL2) did not show increased resistance to TMV indicates that constitutive HMGR activity, although at a low level, was necessary to restrict the lesions.

Evaluation of the role of phytoalexin production compared to the other inducible defense mechanisms in HR (including deposition of hydroxyproline-rich proteins and lignin in the cell wall, lipid peroxidation reactions, production of pathogenesis-related proteins, chitinases and glucanases, protease inhibitors, and polygalacturonases) is hampered by the fact that several of these responses often occur simultaneously in a given plant-pathogen interaction. Modern molecular biological methods are making it possible to study phytoalexin production by manipulating one specific component of phytoalexin biosynthesis and assessing its impact on disease resistance. We found it especially interesting that non-inoculated tobacco cells expressing tomato *hmg2* exhibited ultrastructural changes similar to cells responding to TMV. Increased cytoplasmic content and activity is typically seen in host cells responding to pathogens (Beckman *et al.* 1991, Benhamou 1995, Mould *et al.* 1991, Shi *et al.* 1991), and is probably indicative of a high metabolic rate in the cells. Our results suggest that expression of *hmg2* triggers a more broad-spectrum response than phytoalexin production alone would account for. However, constitutive expression of *hmg2* does not lead to elevation of all defense-inducible responses since sesquiterpene cyclase levels were not elevated.

In mammalian and yeast cells, overexpression of HMGR leads to accumulation of membrane structures inside the cell called crystalloid ER and karmellae (Chin *et al.* 1982, Wright *et al.* 1988). Expression of tomato *hmg2* in tobacco plants did not result in ordered structures like crystalloid ER or karmellae, which could be due to the low expression level of the plant transgene compared to the 500-fold and 10 to 15-fold increases in mammalian and yeast HMGR activities, respectively (Chin *et al.* 1982, Wright *et al.* 1990). Also, the membrane domain of plant HMGRs is significantly smaller than the corresponding domains of yeast or mammalian HMGR (Denbow *et al.* 1996), which probably influences the spatial architecture of the membrane where HMGR is situated. The two *hmgr* genes that were found to cause this kind of membrane proliferation (mammalian HMGR and yeast HMG1) are both similarly regulated by mevalonate-derived early compounds of the pathway (Hampton *et al.* 1996). On the contrary, expression of yeast HMG2, which is modulated by molecules synthesized late in the pathway, did not cause karmellae, and the amount of HMGR was estimated to be

considerably less than in the strains overexpressing HMG1. Consequently, it will be interesting to determine whether expression of tomato *hmg1*, the sterol-associated isogene in tomato, has any effect on membrane ultrastucture in plant cells. Experiments to address this question are underway in our laboratory.

Based on the dramatic impact of the *hmg2*-transgene on TMV- interactions we were somewhat surprised at the low levels of HMG2 detected by enzyme assays or immunoblots of the tagged protein (data not shown). Re *et al.* (1995) also reported, that expression of *Arabidopsis thaliana hmg1* led to very high levels of HMGR mRNA, but that only modest (3-fold) increases in enzyme activity were detected. It is possible that plant HMGRs require stabilization by protein-protein interactions, and accumulation of HMGR could be limited by the availability of these other components. This could account for the discrepancy of low and variable HMGR activity yet very dramatic resistance in the transgenic plants. Preliminary results from immunolocalization studies of the tagged HMG2 show significant increase in c-*myc*-tagged HMG2 peptides after TMV inoculation even though the constitutive 35S promoter is supposedly not induced by pathogens. (See Chapter III).

The appearance of the electron-dense inclusion bodies (IBs) inside the vacuole seemed to be correlated with HMGR activity, or at least the product of the full-length protein, since they were not observed in plants expressing only the membrane domain of *hmg2* without the catalytic domain. The IBs are somehow associated with defense as they were present in both wild-type and transgenic cells adjacent to necrotic cells in TMV lesions. Palmer and Bender (1995) noted large, spherical particles inside the vacuoles of tomato leaf cells treated with the pseudomonad phytotoxin coronatine. It was suggested that they were protein bodies consisting of chymotrypsin inhibitor, based on the structural similarity of the particles compared to previous reports, and on the large amount of chymotrypsin inhibitor activity in the coronatine-treated tissue. The IBs we observed inside tobacco vacuoles resemble the protein bodies described by Palmer and Bender, but we did not detect significant amounts of chymotrypsin- or chymopapain-inhibitor activities in the transgenic tobacco leaf tissue (results not shown).

The IBs stained heavily with osmium, which suggests that they are of lipophilic nature. Osmiophilic substances in vessel contact cells of cotton (Shi *et al.* 1991) and eggplant (*Solanum melongena*) (Benhamou 1995) parenchyma infected with *Fusarium oxysporum* f.sp. *vasinfectum* and *Verticillium albo-atrum*, respectively, were speculated to contain terpenoids. In our experiments, non-inoculated transgenic plants which contained the IBs did not have elevated capsidiol levels, and sesquiterpene cyclase, the committed branch point enzyme leading to phytoalexin biosynthesis, was not induced. These results suggest that the IBs do not represent accumulated sesquiterpene phytoalexins, or capsidiol in particular.

Many defense-inducible proteins are known to accumulate in the vacuole in response to pathogens and stress. Some of these have been identified as pathogenesis related (PR) proteins, including the vacuolar, basic forms of chitinase and β-glucanase. The vacuolar PR proteins have been found to have more antifungal activity than the secreted, acidic proteins (Sela-Buurlage *et al.* 1993). Tomato plants infected with citrus exocortis viroid are induced to produce a 23-kD pathogenesis-related protein (P23), which accumulates in vacuoles in association with dense inclusion bodies (Rodrigo *et al.* 1993). The purified P23 protein inhibits the growth of several phytopathogenic fungi *in vitro*. Vera *et al.* (1989a, 1989b) reported the presence of two other tomato PR proteins, P1(p14) and P69, in both in vacuolar inclusion bodies and intercellular spaces. The reduction in lesion size and number in the HMG2 transgenic (2421) plants is reminiscent of viral cross-protection or systemic acquired resistance (SAR) responses (reviewed in Ryals *et al.* 1996). During a pathogen- or salicylic acid-induced SAR, both vacuolar and

extracellular forms of proteins accumulate in tissues distal to the site of induction. Based on similarity in phenotype, it would be interesting to determine whether expression of *hmg2* leads to elevated levels of PR proteins associated with SAR.

II. 5. METHODS AND MATERIALS

II. 5. 1. Plants

The wild-type tobacco line used in the study was *Nicotiana tabacum* var. Xanthi nc. Transgenic plants were generated from Xanthi by *Agrobacterium tumefaciens*-mediated transformation (Horsch *et al.* 1978). All experiments were conducted on individual plants started from cuttings of wild-type or T_0-plants and grown for approximately six weeks in the greenhouse.

II. 5. 2. Vector construction

Three constructs consisting of a full-length tomato *hmg2*, a c-*myc*-tagged full-length *hmg2*, and a c-*myc*-tagged membrane domain of *hmg2*, each fused to an enhanced CMV35S promoter, were used to transform Xanthi nc. Tomato *hmg2* sequences encoding the putative membrane domain were generated from a tomato *hmg2* genomic clone, pTH295 (Park *et al.* 1992) by PCR amplification using primers 23 (5'-CTTTGTCGACCATGGACGTTCGCCGGAGAT) and 5 (5'-CGCAAGCTTGTTCATT CCCATTGCATCACCAGT) as described by Denbow *et al.* (1996). The resulting *hmg2*-specific fragment, which contains exon I starting at the translation start site, intron I, and part of exon II, was inserted into the *Sma*I site of pBluescript SK^- to yield plasmid pXY12. A c-*myc* epitope (Evan *et al.* 1985) was incorporated into a *Bgl*II site of pXY12 just downstream of the translational start site. The resulting plasmid, pXY12(c-*myc*), encodes a polypeptide (MYC-HMG2md) that begins MDVRRR**SEQKLISEEDLLG**S... (c-*myc* epitope is bolded). It was demonstrated that the epitope did not interfere with appropriate targeting of MYC-HMG2md to microsomal membranes (Denbow *et al.* 1996). A tagged, full-length sequence encoding HMG2 was generated by combining the tagged 5'-half of the gene from pXY12(c-*myc*) (*Hind*III-*Xba*I) with the 3'-end from plasmid pBSNM6 (Yu 1995) (*Xba*I-*Pst*I) in pBluescriptSK$^-$, resulting in plasmid pSK$^-$(c-*myc*I).

Vectors for plant transformation were constructed as follows. For all constructs we used an enhanced 35S promoter ($35S^{DE}$) consisting of a double enhancer of the CaMV 35S promoter and the 3'-untranslated leader sequence from the tobacco etch virus (Carrington and Freed 1990) excised from pRTL2 provided by Dr. John Mullet (Texas A&M). For pSL2, $35S^{DE}$ was inserted into *Hind*III-site of *A. tumefaciens* binary vector pBIB-HYG (Becker 1990). Prior to introduction into pBIB-HYG, the transcription start site codon in *Nco*I of pRTL2 was removed by Mung Bean exonuclease treatment and addition of a blunt-*Hind*III linker. The tagged membrane domain from pXY12(c-*myc*) was then inserted as a 0.7 kb *Sal*I/*Xba*I fragment downstream of the promoter. For pSL7, the 35S promoter was first inserted into the *Eco*RV site of pSK$^-$(c-*myc*I) to facilitate cloning the promoter and the full-length tagged gene as a single cassette into the *Hind*III-site of pBIB-HYG. Plasmids were transferred into *A. tumefaciens* LBA4404 by the freeze-thaw method (Holsters *et al.* 1978).

II. 5. 3. Analysis of transformed plants

Stable insertion of the constructs into tobacco genome was analyzed by Southern analysis (SL2-6, two inserted copies; SL2-8, one copy; SL7-plants, not determined). The copy numbers were confirmed by germinating seeds from T_0-plants on media containing 50 µg/ml hygromycin, and counting the ratio of resistant versus susceptible seedlings. Products from the c-*myc*-tagged transgenes were detected in western analyses using young leaf tissue (see Chapter III, Fig. III.1).

II. 5. 4. Inoculation and tissue sampling

Leaves of 15 to 18 cm in length from randomized positions were excised at the petioles and inoculated with 50 µg/ml TMV (strain U1). TMV was purified by the method of Asselin and Zaitlin (1978) and provided by Ms. Elizabeth Schwartz and Dr. Sue Tolin (Virginia Polytechnic Institute and State University). The inoculum contained 1% K_2HPO_4 and 1% Celite 545 (Fisher Scientific, Fair Lawn, NJ), and was spread on the top side of the leaves with cotton swabs. Inoculated leaves were rinsed slightly with water, placed in plastic bags on wet paper towels to retain moisture, and incubated under a fluorescent lamp at room temperature for five days. Mock-inoculation was done similarly without TMV.

II. 5. 5. Tissue blots

Leaves were blotted on S&S filter paper no. 410 (Schleicher & Schuell, Keene, NH) by placing a piece of leaf containing several lesions between two strips of filter paper, placing this sandwich between two plexiglass plates, and administering a series of gentle blows with a hammer over the sandwich. Blots were air-dried, after which they were decolorized in 5% Triton-X100 in distilled water at room temperature with slow agitation. Blots were rinsed in KPST (0.02M K_2HPO_4, 0.15M NaCl, 0.05% Tween-20, pH 7.4) and blocked overnight at 4˚C in 5% blocking agent NIP 551 (Amersham, Arlington Heights, IL) in KPS (0.02M K_2HPO_4, 0.15M NaCl). Incubations in the primary antibody, rabbit anti-TMV 102 (a gift from Sue Tolin, VPI&SU; diluted 1:8,000 in KPS) and the secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO; diluted 1:10,000 in KPS) were done for one hour at room temperature. Blots were washed 3x10 min in KPS between incubations and before detection with BCIP and NBT according to manufacturers instructions (Boehringer Mannheim, Indianapolis, IN).

II. 5. 6. HMGR assays and capsidiol determinations

Total HMGR activity from leaves was determined according to Chappell *et al.* (1995).

Capsidiol determinations were performed by Dr. Jingrui Wu according to Chappell and Nable (1987) with the following modifications. Sesquiterpenoids were extracted from 0.5-1g leaves by shaking overnight at room temperature in 50 ml of 10% methanol. Fifty ml of chloroform and 5 ng of internal standard (hexadecane) were added, and the extract was shaken vigorously. After centrifugation for 10 min at 3,000 x g the supernatant was dried by rotary evaporation. The dried material was resuspended in 5 ml of hexane and centrifuged using the top speed of a clinical centrifuge to pellet the debris. Samples were dried under nitrogen gas and resuspended in 1 ml hexane.

Qualitative evaluation of capsidiol was done by thin layer chromatography. Silica gel G plates were activated at 110˚C for 90 min, and cooled down to RT. Fifty microliters of concentrated extract was spotted on the plate, and run in chloroform:methanol (19:1). The plate was stained with vanillin-H_2SO_4 and baked at 90˚C for 10 min.

Quantitation of capsidiol was achieved by gas chromatography (15m x 0.25 mm DB-5 capillary column) with injector temperature at 220˚C, detector temperature at 280˚C, and program settings as follows. Initial temperature was set at 60˚C, followed by 60˚C for 0.5 min. Ramp 1: 60˚C to 100˚C at 10˚C/min. Ramp 2: 100˚C to 180˚C at 5˚C/min. Ramp 3: 180˚C to 280˚C at 10˚C/min, followed by 280˚C for 2 min. Two microliters of the concentrated extract was injected into the system.

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II. 5. 7. SDS PAGE and immunoblot analyses

Microsomal proteins for SDS PAGE analysis were extracted as for HGMR assay. One gram of frozen tissue was homogenized in 6 ml ice-cold 100 mM K-phosphate (pH 7.0), 4 mM $MgCl_2$ and 5mM DTT, and the homogenate was centrifuged at 10,000 x g for 15 min at 4˚C. The supernatant was subsequently centrifuged at 100,000 x g for 60 min, and the pellet was resuspended in 100 mM K-phosphate (pH 7.0), 50 mM DTT. Samples were heated at 60˚C for 10 min in SDS-PAGE sample buffer (Laemmli, 1970), centrifuged for 3 min in a microfuge, and solubilized proteins were analyzed in 7.5% SDS polyacrylamide gels (Laemmli 1970). Proteins were transferred to PolyScreen PVDF membrane (NEN Research Products, Boston, MA) in 192 mM glycine; 25 mM Tris-base; 0.2% (w/v) SDS using Bio-Rad transfer apparatus at 25V overnight. The blot was blocked overnight in 20 mM Tris, pH 7.6; 137 mM NaCl; 1% (w/v) non-fat dry milk and 0.5% (w/v) bovine serum albumin (BSA). Monoclonal mouse anti-tobacco sesquiterpene cyclase (anti- 5-epi-aristolochene synthase, a gift from J. Chappell, Univ. of Kentucky) was used as primary antibody following dilution of 1:5,000 in blocking buffer. Secondary antibody was horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham, Arlington Heights, IL) diluted 1:2,000 in blocking buffer. Incubations were for 1 hour at room temperature followed by 4x15 min washes in 20 mM Tris, ph 7.6; 137 mM NaCl; 0.1% (v/v) Tween-20. ECL chemiluminescent reagents (Amersham) were used for detection following manufacturer's instructions.

II. 5. 8. Tissue processing and electron microscopy

Approximately 1 mm x 2 mm squares of leaf tissue (from approximately 6 weeks-old tobacco plants started from cuttings of T_0 plants) containing single lesions were cut in half, bisecting the lesion, to ensure obtaining thin sections with some necrotic and some healthy green tissue. Leaf pieces from TMV- and mock-inoculated leaves were infiltratrated with 2% paraformaldehyde, 1% glutaraldehyde, 0.1 M sucrose in 0.05 M Na-phosphate buffer (pH 7.2) for 2 h at room temperature with vacuum. Tissue was rinsed with 0.5 M sucrose in 10 mM Na-phosphate buffer (pH 7.2) for 3x10 min, postfixed in 1% OsO_4 in 0.05 M sucrose in 10 mM buffer, and rinsed again 3x10 min in distilled water. Following dehydration in an ethanol series, tissue blocks were embedded in London Resin White acrylic resin (Electron Microscopy Sciences, Ft. Washington, PA) and polymerized at 58-60ºC under vacuum. Thin sections (<100 nm) were cut using a MT6000-XL microtome (Research and Manufacturing Company Inc., Tucson, Arizona) and viewed with Zeiss 10CR transmission electron microscope after staining with uranyl acetate and lead citrate.

II. 6. ACKNOWLEDGMENTS

We thank Dr. Joseph Chappell for generously providing the antibody to tobacco sesquiterpene cyclase, capsidiol standards, protocols for HMGR assay and capsidiol determinations, and helpful discussions. We also thank Dr. Sue Tolin for the anti-TMV antibody, the TMV inoculum, and helpful discussions. This work was supported by grants from Academy of Finland (#7517), Cultural Fund of Finland, Alfred Kordelin's Fund, Thanks to Scandinavia Inc., Kemira Research Fund, and Tiura' s Fund to S. S. L. and by the National Institutes of Health grant #R29-GM39549 and National Science Foundation grant #IBN-9514177 to C. L. C.

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CHAPTER III: CO-LOCALIZATION OF 3-HYDROXY-3-METHYLGLUTARYL CoA REDUCTASE AND SESQUITERPENE CYCLASE IN THE VACUOLES OF TOBACCO LEAF CELLS FOLLOWING INOCULATION WITH TOBACCO MOSAIC VIRUS

The information in this chapter will be incorpotated into a manuscript [Lång, S.S., and Cramer, C.L. (1997)], to be submitted to the Plant Cell.

III. 1. ABSTRACT

Plant cells respond to a pathogen with a repertoire of molecular and cellular changes. In a resistant interaction, the most dramatic response is the hypersensitive response (HR), in which plant cells in the immediate vicinity of the pathogen undergo programmed cell death. Other responses include strengthening of the cell wall, and production of enzymes (such as chitinases and glucanases) and antibiotic compounds (phytoalexins) that are harmful to the pathogen (Bent 1996). These activities often require massive metabolic and cellular rearrangements. In the present study, the defense-inducible isoform of tomato 3-hydroxy-3-methylglutaryl CoA reductase (HMGR, EC 1.1.1.34) HMG2, was used as a molecular membrane marker to trace possible subcellular differentiation during a defense response. HMGR catalyzes an early step in the isoprenoid pathway from hydroxymethylglutaryl CoA to mevalonic acid. In solanaceous plants, one class of endproducts of the pathway are sesquiterpenoid phytoalexins, antibiotic compounds produced by plants in response to pathogens. We are interested in the role of defense-inducible isoforms of HMGR in phytoalexin production and disease resistance. Tomato HMG2 is an integral membrane protein, the membrane domain of which spans the membrane twice leaving the N-terminal and the catalytic C-terminal end on the cytosolic side. The protein was tagged with a c-*myc* epitope to facilitate immunodetection of the defense-specific HMG2 isoform and constitutively expressed in transgenic tobacco (*Nicotiana tabacum*) plants. In leaf mesophyll cells of non-inoculated plants, MYC-HMG2 was localized in the endoplasmic reticulum (ER). Following tobacco mosaic virus (TMV)-inoculation, the localization of MYC-HMG2 had changed to electron-dense inclusion bodies within the vacuole of cells adjacent to necrotic cells in a TMV lesion. Similar bodies were observed in non-transgenic plants in the cells surrounding TMV-lesions. Inclusion bodies were also observed in non-inoculated transgenic plants that expressed the full-length tomato (*Lycopersicon esculentum*) *hmg2*: plants expressing only the tagged membrane domain without the catalytic domain did not contain them, suggesting that HMGR activity was triggering their appearance. However, the inclusion bodies in the non-inoculated plants did not react with anti-c-*myc* antibody, indicating that MYC-HMG2 was only present in the vacuolar bodies after TMV-inoculation. Sesquiterpene cyclase (5-*epi*-aristolochene synthase or EAS), a key enzyme of the isoprenoid pathway controlling the branch point to sesquiterpene phytoalexins, localized to the same inclusion bodies within the vacuoles, suggesting that they may contain a defense-induced, membrane-associated multienzyme complex dedicated to sesquiterpene production. TMV-inoculated leaves contained a much higher amount of MYC-HMG2 than the non-inoculated leaves (as determined from the micrographs), although the expression of the transgene was driven by a constitutive CaMV 35S promoter. This may be due to a stabilizing effect of the other proteins in the putative multienzyme complex. Our results support the hypothesis of the multibranched plant isoprenoid pathway being partly regulated by pathway partitioning. The route and mechanism of relocation of the defense-inducible sesquiterpene biosynthetic complex from the ER to the vacuole is currently unknown.

III. 2. INTRODUCTION

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC 1.1.34) catalyzes the synthesis of mevalonic acid in the isoprenoid biosynthetic pathway. In mammals, this is a rate-limiting step for cholesterol biosynthesis, and the enzyme is feedback regulated by cholesterol. The mammalian HMGR also takes part in the production of other sterols, isoprenoid compounds such as dolichol, steroid hormones, ubiquinone, isopentenyladenine, and prenyl modifications of proteins (Goldstein and Brown 1990).

In plants, the isoprenoid pathway is responsible for a large number of the products of plant secondary metabolism. Over 22,000 isoprenoids (terpenoids/terpenes) identified so far include compounds for photosynthesis (chlorophylls, carotenoids, plastoquinone), respiration (ubiquinone, cytochrome a), membrane architecture (sterols and triterpenoids), regulation of growth and development (gibberellic acids, abscisic acid, brassinosteroids, certain cytokinins), defense against pathogen attack (phytoalexins), and others (Bach 1995, Chappell 1995). Thus it is not surprising that plants contain multigene families of *hmgr*-genes compared to a single *hmgr*-gene in mammals. The specific isoforms are differentially regulated by light, developmental stage of the plant, and environmental stimuli such as wounding and pathogens (reviewed in Bach 1995, Chappell 1995, Stermer *et al.* 1994).

We are interested in the regulatory role of HMGR in plant phytoalexin production and disease resistance. Phytoalexins are small antibiotic compounds produced by the plant in response to pathogens. The major phytoalexins accumulating in solanaceous plants are sesquiterpene isoprenoids, produced by the isoprenoid pathway. A number of reports demonstrate a dramatic switch between sterol versus terpenoid phytoalexin production upon pathogen challenge, correlated with increased HMGR activity. Increased HMGR activity precedes pathogen- or elicitor-induced sesquiterpenoid production both in potato tubers and in tobacco cell suspension cultures (Chappell and Nable 1987, Chappell *et al.* 1991, Ôba *et al.* 1985, Stermer and Bostock 1987, Vögeli and Chappell 1988) while simultaneously, the production of steroid glycoalkaloids and sterols is decreased (Threlfall and Whitehead 1988, Tjamos and Kuc 1982, Vögeli and Chappell 1988). The two key branch point enzymes squalene synthetase and sesquiterpene cyclase utilize farnesyl pyrophosphate to synthesize sterols and sesquiterpene phytoalexins, respectively. The activity of squalene synthetase is increased by wounding of potato (*Solanum tuberosum*) tubers, presumably leading to steroid glycoalkaloid production; but if the discs are then treated with elicitor or pathogen, squalene synthetase activity is suppressed, and sesquiterpenoid phytoalexins accumulate in the tissue (Zook and Kuc 1991). Similarly, treatment of tobacco cell cultures with elicitor supresses squalene synthetase activity and increases sesquiterpene cyclase activity (Vögeli and Chappell 1988).

It has been hypothesized that specific HMGR isoforms and other enzymes later in the pathway might form separate enzyme complexes or channels leading to specific classes of endproducts. (Bach 1995, Chappell 1995, Denbow *et al.* 1996, Stermer *et al.* 1994, Weissenborn *et al.* 1995). In tomato, two specific isoforms of HMGR, HMG1and HMG2, have been connected with the proposed branches of the isoprenoid pathway: HMG1with sterol biosynthesis, and HMG2 with defense (Choi *et al.* 1992, Cramer *et al.* 1993, Gillaspy *et al.* 1993, Narita and Gruissem 1989, Park *et al.* 1992, Weissenborn *et al.* 1995, Yang *et al.* 1991). Tomato *hmg1* is highly expressed in the early stages of rapid cell division and cell expansion during fruit development, when there is an increased need for mevalonate for sterol biosynthesis (Narita and Gruissem 1989, Gillaspy *et al.* 1993). In contrast, *hmg2* is expressed only later during the fruit ripening phase (Gillaspy

et al. 1993), and is highly induced in response to pathogens and wounding (Cramer *et al.* 1993, Park *et al.* 1992, Weissenborn *et al.* 1995), presumably associated with terpenoid phytoalexin production.

In yeast (*Saccharomyces cerevisiae*), the partitioning of the isoprenoid pathway is evident as witnessed by the differential regulation of the two HMGR isoenzymes, HMG1 and HMG2. Yeast HMG1 is feedback regulated by mevalonate-derived compounds early in the pathway, similar to the mammalian HMGR. In contrast, yeast HMG2 appears to be modulated by molecules synthesized late in the pathway (after squalene). This feedback regulation coordinates with "contra-regulation" of the two enzymes by oxygen, which is required for the synthesis of the late products of the pathway, but not for the early ones. (Casey *et al.* 1992, Hampton *et al.* 1996). These studies suggest that parallel pathways leading to early versus late compounds exist in yeast. Analogous independent pathways leading to sterols versus phytoalexins may exist in plants. Pathway partitioning may be mediated by enzyme complexes which channel intermediates and/or by compartmentation within the cell.

The subcellular location of plant HMGRs is not known. Mammalian HMGR is an integral endoplasmic reticulum (ER) glycoprotein, the catalytic domain of which lies on the cytosolic side of the ER membrane (Roitelman *et al.* 1992). *Arabidopsis haliana* HMG1 and tomato HMG1 and HMG2 have been shown to be inserted into microsomal membranes *in vitro* (Enjuto *et al.* 1994, Denbow *et al.* 1996). Compared to the eight transmembrane regions in the mammalian HMGR (Roitelman *et al.* 1992), the membrane domain of plant HMGRs spans the membrane only twice, leaving the N-terminal and catalytic C-terminal end on the cytosolic side (Denbow *et al.* 1996). The membrane spanning regions function as integral signal sequences that are cotranslationally inserted into the membranes without cleavage of a signal peptide.

No antibodies are available that can distinguish between the HMGR isoforms, and thus immunolocalization of the isoforms has not been pursued. A possible location in the ER membrane is supported by the fact that no transit signals to other organelles have been recognized in plant HMGR sequences. Additionally, the N-termini of most plant HMGRs have the consensus sequence MetAspXArgArgArg (where X can be Val, Ile, Leu or Ser), which fulfills the requirements for an RR-type ER retention motif (Schutze *et al.* 1994).

In the present study we set out to determine the subcellular location of the defense-related tomato HMGR isoform, HMG2. Furthermore, we wanted to know if the location of HMG2 changes during pathogen challenge. In the study of Snyder and Nicholson (1990), two pigmented sorghum (*Sorghum bicolor*) phytoalexins were shown to be delivered to fungal infection sites in vesicular inclusions that accumulated beneath the fungal appressoria. As a transmembrane protein, HMG2 has the potential ability to serve as a molecular marker in observing subcellular changes that may be associated with phytoalexin biosynthesis. HMG2 was tagged with a c-*myc*-epitope to facilitate immunodetection of the specific isoform. The chimeric gene was expressed in transgenic tobacco plants and the location of the transgene product was assessed by subcellular fractionation and immunolocalization studies pre- and post-TMV inoculation. Prior to TMV inoculation, MYC-HMG2 was found localized in small clusters associated with the ER. In TMV-inoculated leaves MYC-HMG2 co-localized with sesquiterpene cyclase to the vacuolar inclusion bodies. A possible model for the mechanism of relocation of the sesquiterpene biosynthetic complex from the ER to the vacuole is discussed.

III. 3. RESULTS

III. 3. 1. Expression of c-*myc*-tagged tomato *hmg2* in transgenic tobacco leaves

Tomato *hmg2* gene was tagged with the c-*myc*-epitope to facilitate immunodetection of the specific isozyme HMG2. Two constructs were made: a full-length, c-*myc*-tagged *hmg2* [pSK$^-$(c-*myc*I)] and a tagged membrane domain of HMG2 [pXY12(c-*myc*)]. The resulting chimeric protein contained a 13 aa C-MYC insert six residues downstream of the N-terminal methionine, a placement designed to avoid interference with targeting signals or catalytic activity. It was previously demonstrated that the epitope-tagged HMG2 membrane domain was efficiently targeted to microsomal membranes *in vitro* (Denbow *et al.* 1996). The *hmg2*-constructs were fused to a strong constitutive plant promoter, CaMV 35S^{DE} and introduced into tobacco via *Agrobacterium tumefaciens*-mediated transformation. The resulting transformants were analysed by SDS-PAGE of microsomal proteins of leaf extracts. The full-length, c-*myc*-tagged HMG2 (MYC-HMG2) was detected as a single polypeptide with molecular mass of approximately 66 kDa, which is the predicted size for the chimeric gene product (Fig. III.1a). The tagged membrane domain of HMG2 (MYC-HMG2md) was detected as a polypeptide of approximately 27 kDa, also as predicted by the primary sequence (Fig III.1b).

The transgenic plants grew normally and had a normal phenotype (data not shown). The expression level of the transgene products varied between individual plants as determined from immunoblots. The amount of MYC-HMG2 or MYC-HMG2md never accumulated enough to be detected from crude leaf extracts by immunoblot analysis; the peptides were only detected in microsomal preparations. Cross-reacting proteins were not detected in the supernatant of the microsomal isolation indicating that MYC-HMG2 and MYC-HMG2md are microsomal membrane proteins. The transgene products were not detectable after the plants had flowered, which is probably due to the promoter used in the study. It has been shown that the expression of transgenes driven by the CaMV 35S promoter is higher in meristematic tissue and younger parts of the plants (Cramer *et al.* 1996).

At the cellular level, leaf mesophyll cells expressing the full-length, c-*myc*-tagged *hmg2* exhibited morphological changes compared to the wild-type plants. The cytosol occupied a larger area of the cell volume and the amount of endomembrane increased. Moreover, electron-dense inclusion bodies (IBs) had accumulated inside the vacuole (Fig. III.2). These changes were not observed in the plants expressing MYC-HMG2md, which lacks the catalytic domain of HMG2 (see Chapter II).

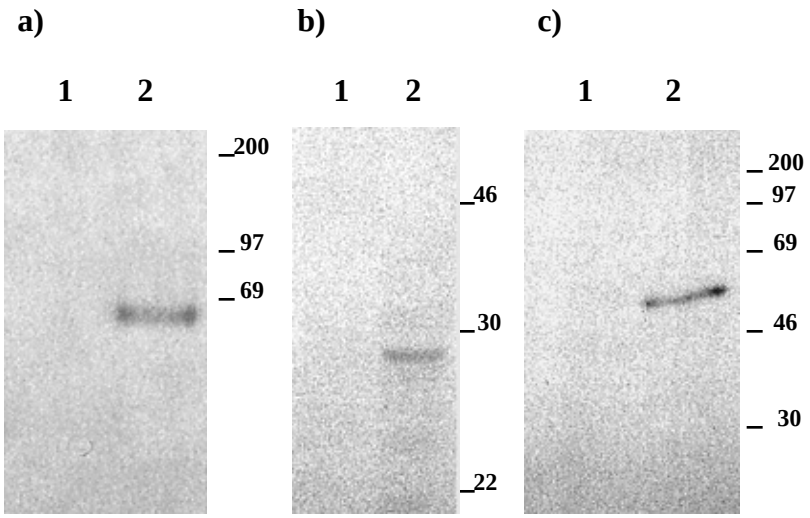

Fig. III.1. SDS-PAGE immunoblot analyses from wild-type and transgenic tobacco plants. Migration of molecular weight standards is as shown (kDa).
a) Microsomal membrane proteins (approximately 40 µg) were separated by gel electrophoresis, transferred to PVDF membrane and reacted with antibody to the c-*myc* epitope. Lane 1, non-transgenic tobacco; lane 2, SL7-6 plant (MYC-HMG2).
b) Microsomal membrane proteins (approximately 40 µg) were separated by gel electrophoresis, transferred to PVDF membrane and reacted with antibody to the c-*myc* epitope. Lane 1, non-transgenic tobacco; lane 2, SL2-6 plant (MYC-HMG2md).
c) Soluble proteins (approximately 40 µg) were separated by gel electrophoresis, transferred to PVDF membrane and reacted with antibody to tobacco sesquiterpene cyclase. Lane 1, 2421 plant immediately after inoculation with TMV; lane 2, 2421 plant 5 d after inoculation with TMV.

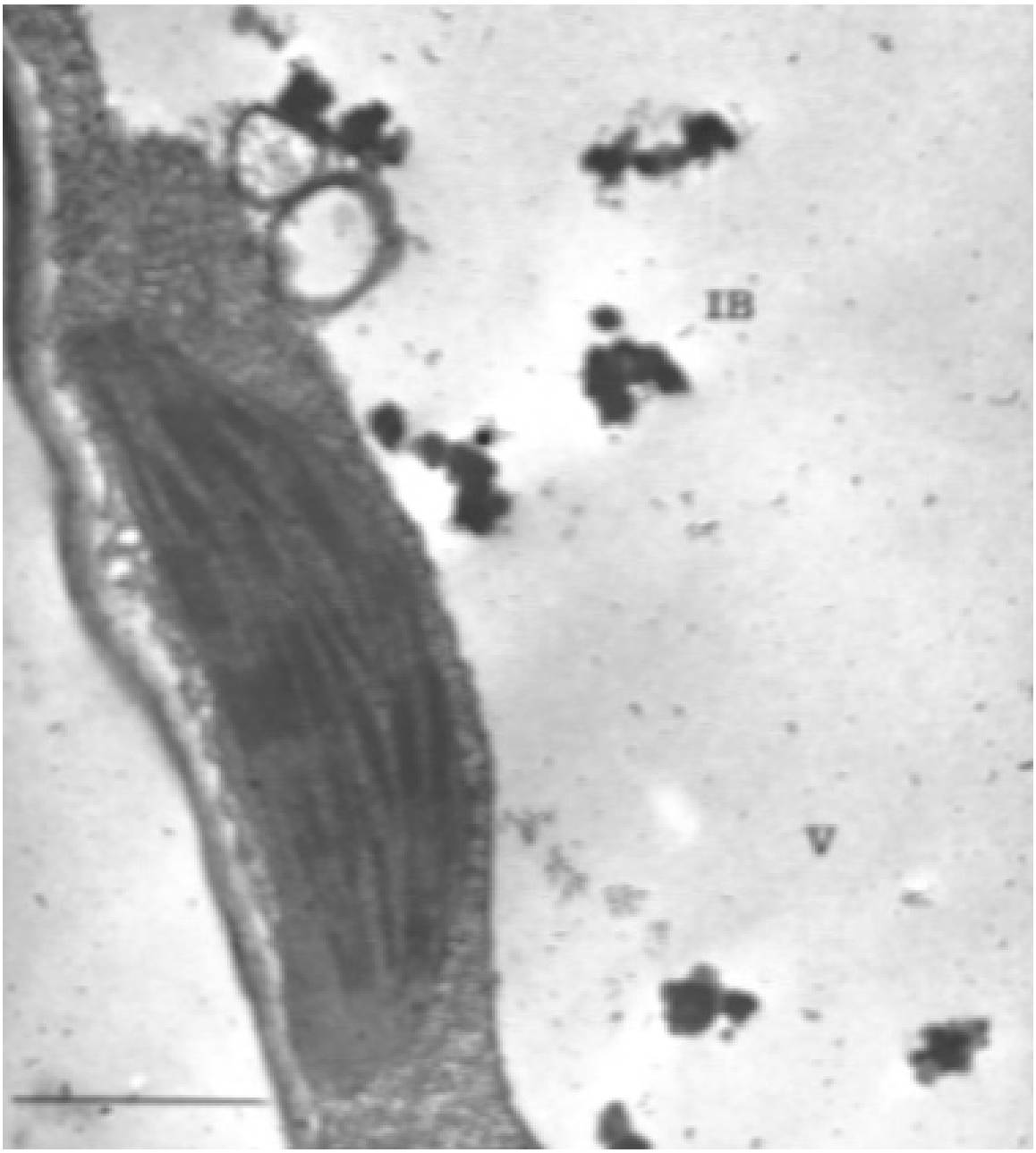

Fig. III.2. Electron micrograph of a leaf mesophyll cell from plant SL7-5 (expresses a c-*myc*-tagged, full-length tomato *hmg2*) showing dense inclusion bodies within the vacuole. Bar = 1 μm. IB, inclusion body.

III. 3. 2. Immunolocalization of MYC-HMG2 in nonstressed cells

To determine the endomembrane compartment(s) of MYC-HMG2 in nonstressed cells, leaf mesophyll cell membranes were fractionated in a continuous (20% to 50%) sucrose gradient and the fractions were analyzed for marker enzyme activities (the Golgi marker latent IDPase, the plasma membrane marker potassium-stimulated ATPase, chlorophyll, and the ER marker NADH-cytochrome-c reductase) and for the presence of MYC-HMG2. The membranes carrying MYC-HMG2 (as determined from SDS-PAGE immunoblot analysis) separated as two peaks among the fractions: a peak coinciding with the endomembrane markers NADH-cytochrome-c reductase (ER) and latent inosine diphosphatase (Golgi) (fractions 13 and 14), and another, broader peak (fractions 8 to 11), which did not coincide with any of the peaks of the marker enzyme activities tested. A portion of membranes carrying MYC-HMG2 may have travelled further down the gradient together with heavier membranes, *i.e.* the gradient may have been overloaded. The ER marker enzyme (NADH-cytochrome-c reductase) activity was also slightly elevated in fractions 8 to 11 (Fig. III.3).

Electron micrographs of immunogold labeled sections of nonstressed cells showed MYC-HMG2 and MYC-HMG2md localized in small clusters, probably associated with the ER (Figs. III.4a,b), and in clusters over small electron-opaque areas either in the cytosol or at the tonoplast, close to the vacuolar inclusion bodies (Figs. III.4c,d). The labeling was very scarce, which is consistent with the low expression levels detected in the plants by immunoblot analysis. A low amount of labeling was also present over the vacuolar inclusion bodies. Sections from non-transgenic plants did not label with anti-c-*myc*-antibody at the dilution used.

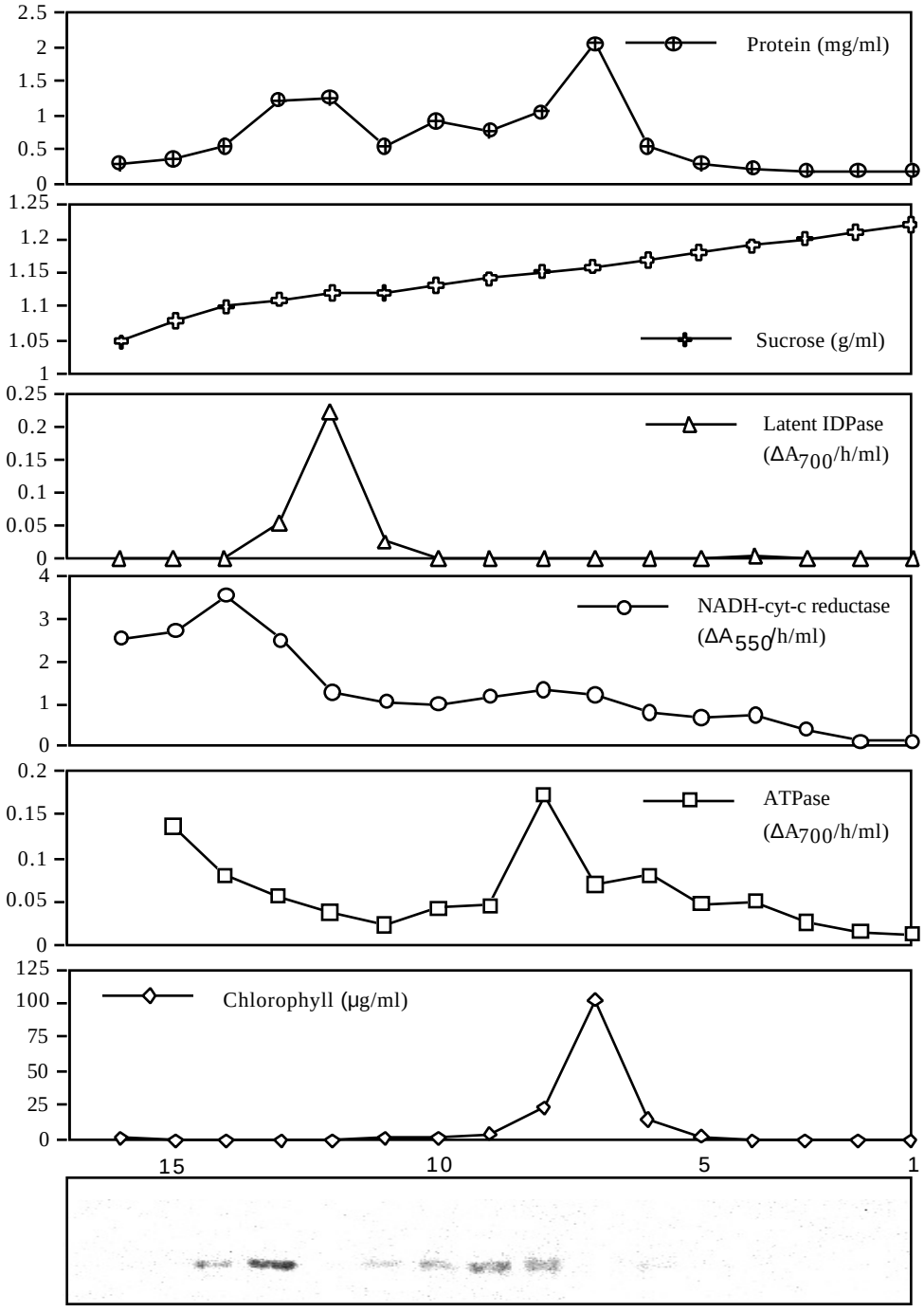

Fig. III.3. Membrane localization of MYC-HMG2 in subcellular fractions. Cell membranes were fractionated on 20 to 50% linear sucrose gradient and fractions were assayed for membrane marker enzyme activities [Golgi marker, latent inosine diphosphatase (latent IDPase); ER-marker, NADH-cyt-c reductase; plasma membrane marker, potassium-stimulated ATPase; and chloroplast marker, chlorophyll] and for the

presence of MYC-HMG2 (bottom panel, an SDS-PAGE immunoblot with anti-c-*myc* from the same fractions). The pattern from the immunoblot matches the pattern of the ER-marker enzyme, NADH-cyt-c reductase. Top panel = protein concentration in the fractions. Second panel = sucrose density in the fractions.

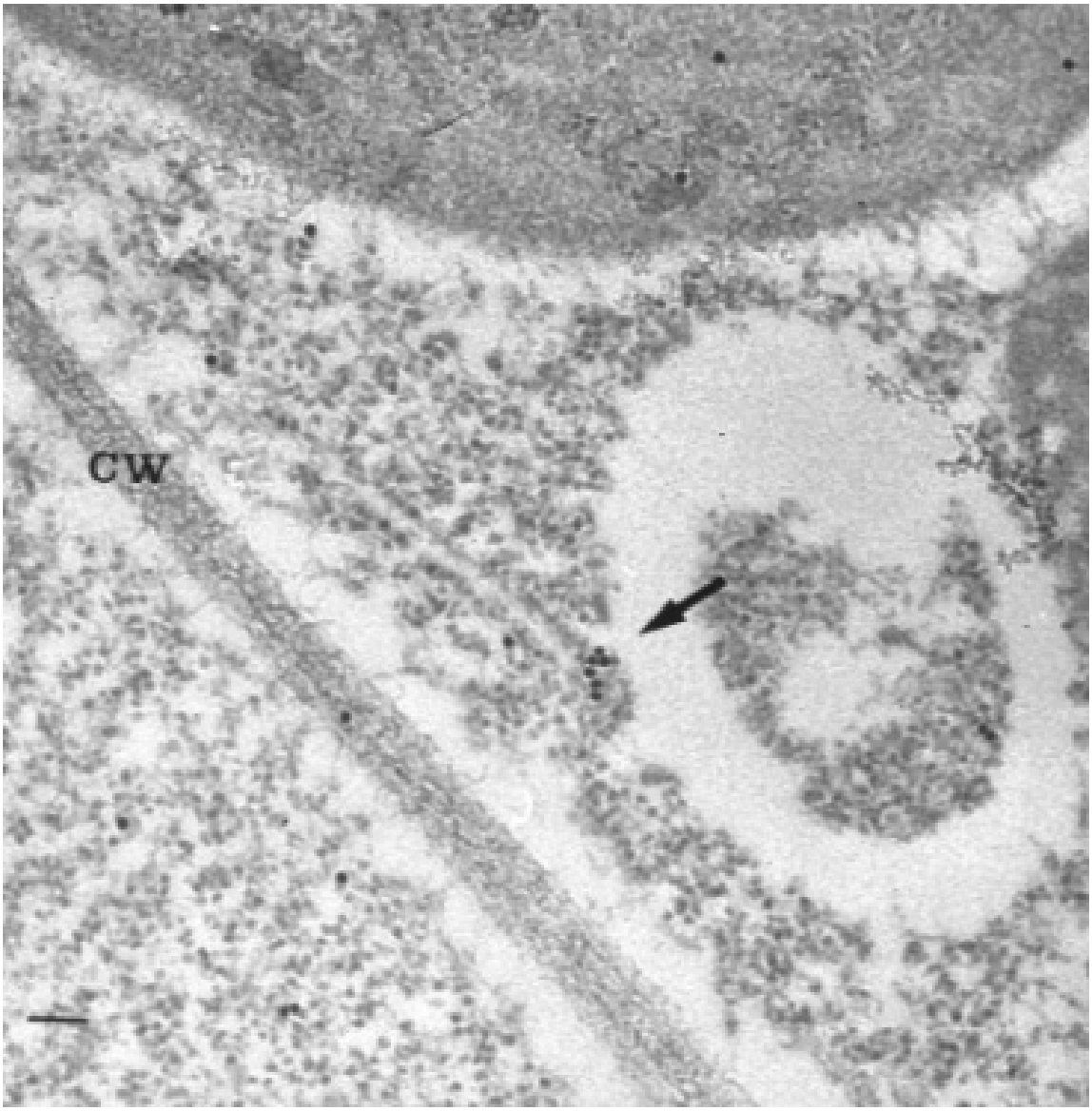

Fig. III.4a. Electron micrograph of a leaf mesophyll cell from plant SL7-6 (expressing a c-*myc*-tagged, full-length tomato *hmg2*) showing immunogold labeling of MYC-HMG2 associated with rER. Bar = 0.1 μm. CW, cell wall.

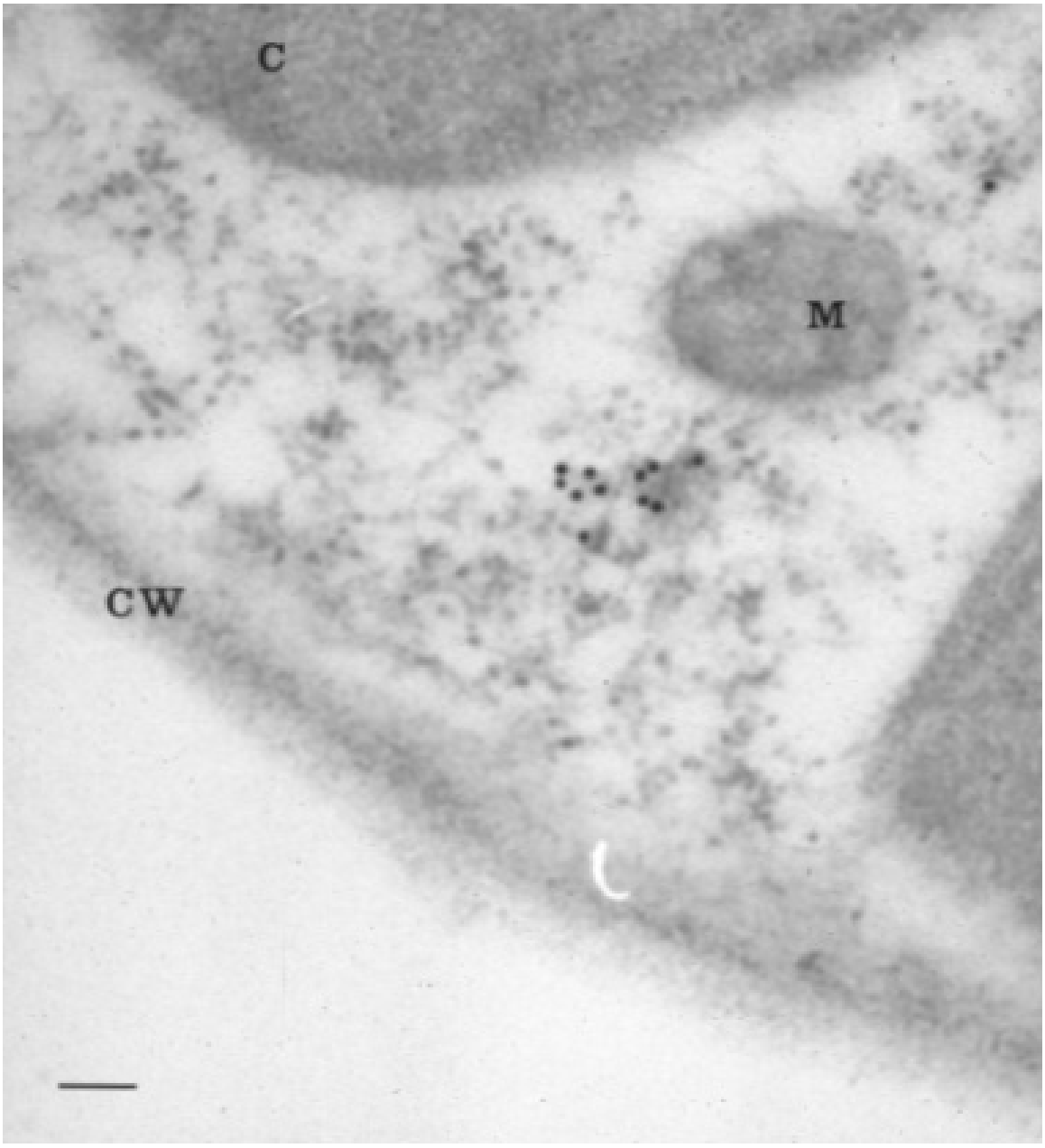

Fig. III.4b. Electron micrograph of a leaf mesophyll cell from plant SL7-6 (expressing a c-*myc*-tagged, full-length tomato *hmg2*) showing immunogold labeling of MYC-HMG2 in small clusters in the cytoplasm. Bar = 0.1 μm. C, chloroplast; CW, cell wall; M, mitochondrion.

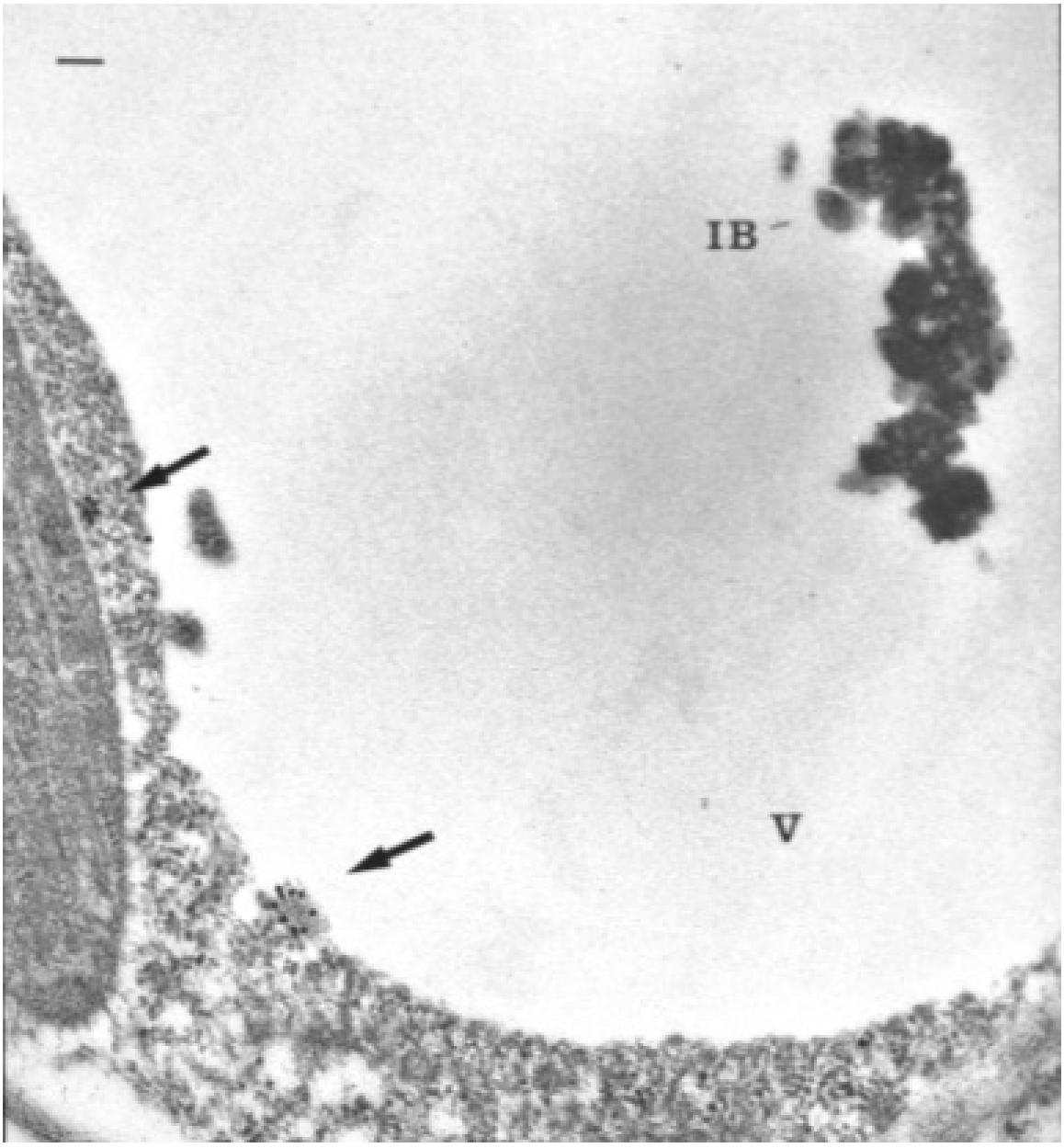

Fig. III.4c. Electron micrograph of a leaf mesophyll cell from plant SL7-5 (expressing a c-*myc*-tagged, full-length tomato *hmg2*) showing immunogold labeling of MYC-HMG2 in small clusters associated with the tonoplast. Note the presence of the vacuolar inclusion bodies in close proximity. Bar = 0.1 µm. IB, inclusion body; V, vacuole.

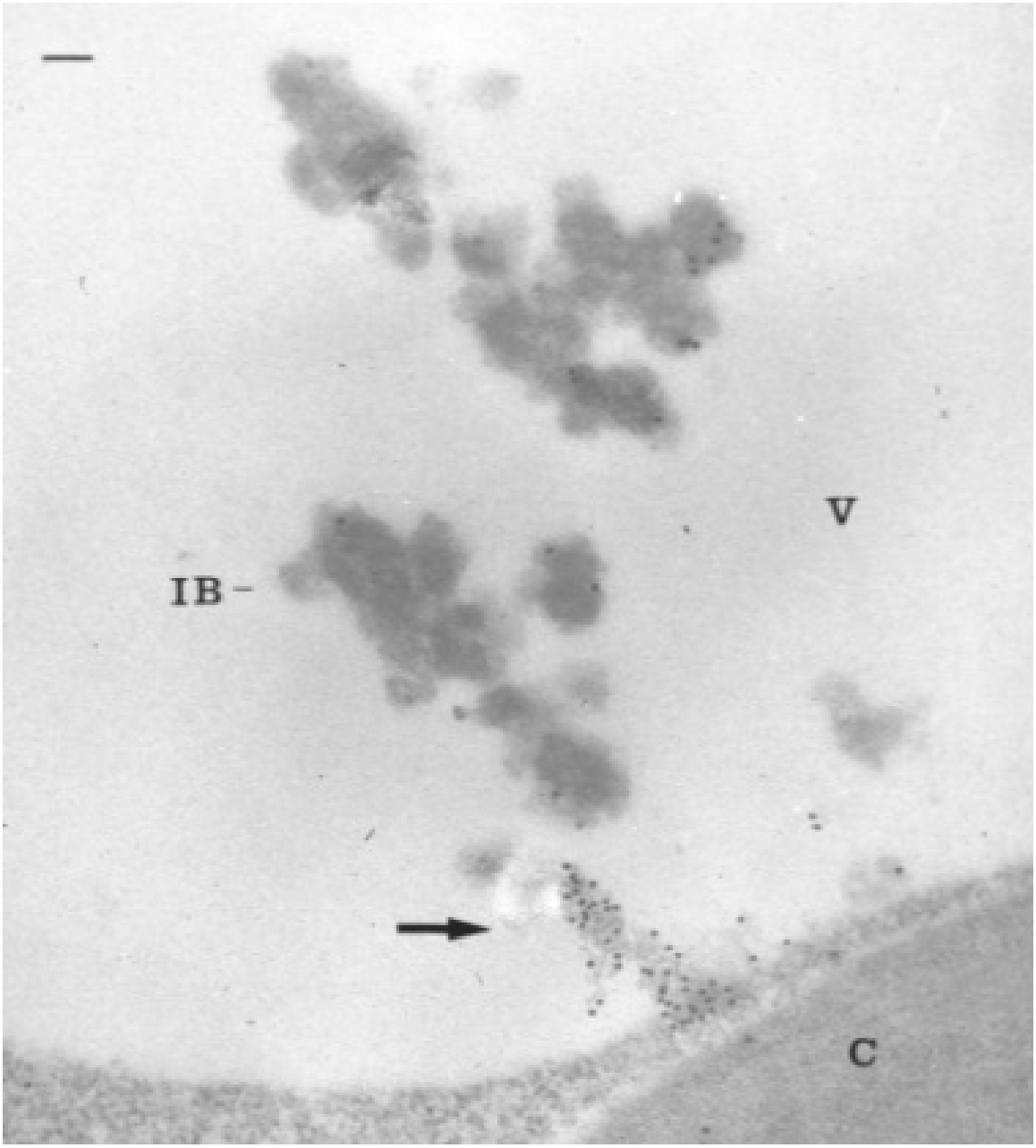

Fig. III.4d. Electron micrograph of a leaf mesophyll cell from SL7-6 (expressing a c-*myc*-tagged, full-length tomato *hmg2*) showing immunogold labeling of MYC-HMG2 in small clusters associated with the tonoplast, close to the vacuolar inclusion bodies. Bar = 0.1 μm. C, chloroplast; IB, inclusion body; V, vacuole.

III. 3. 3. Immunolocalization of MYC-HMG2 after inoculation with TMV

Excised leaves from the transgenic plants (TMV-resistant cultivar) were surface inoculated with TMV strain U1. Five days after inoculation, cells directly at the inoculation site (inside the lesion) were completely overtaken by the virus (Fig. III.5). Cells adjacent to these, and a few cell layers beyond in both the wild-type and the transgenic plants showed morphological changes similar to the ones observed in unchallenged leaves of MYC-HMG2 transgenic plants: increased volume of cytosol, enlarged endomembrane structure, and accumulation of electron-dense material within the vacuole (data not shown). Immuno-electron microscopy revealed a change in the location of MYC-HMG2 and MYC-HMG2md as compared to the non-stressed plants. In the leaves of non-stressed plants, MYC-HMG2 and MYC-HMG2md were found in the cytosol in clusters associated with the ER. In cells adjacent to the TMV-lesion, MYC-HMG2 and MYC-HMG2md localized to the inclusion bodies within the vacuole (Fig. III.6), which now labeled heavily with the gold-conjugated antibody. The gold-particles were not evenly distributed over the inclusion bodies; they seemed aggregated around more dense areas.

III. 3. 4. Sesquiterpene cyclase co-localizes with MYC-HMG2

Detection of HMG2 in the vacuolar inclusion bodies after TMV-inoculation led us to investigate whether tobacco sesquiterpene cyclase, a key branch-point enzyme leading to sesquiterpene phytoalexin production, would co-localize with HMG2. The specificity of anti-sesquiterpene cyclase antibody (anti-5-*epi*-aristolochene synthase or anti-EAS) was tested by SDS-PAGE immunoblot analysis of soluble proteins from leaf extracts. The monoclonal antibody bound to a polypeptide of approximately 60 kDa, the size of EAS (Fig. III.1c). The peptide was found in extracts prepared from leaves 5-days post TMV-inoculation (in both wild type and transgenic plants), but not in extracts from nonstressed plants. In tobacco cell suspension cultures, EAS is similarly induced by cellulase treatment (Facchini and Chappell 1992, Vögeli and Chappell 1990).

When section of samples prepared five days post TMV-inoculation were immunolabeled with anti-tobacco cyclase, cyclase co-localized with MYC-HMG2 to the vacuolar inclusion bodies in both the wild-type and the transgenic plants. (Fig. III. 7). Although the anti-tobacco sesquiterpene cyclase used in the study was a monoclonal antibody, a heavy background labeling was present especially over the chloroplasts in sections of both non-inoculated and TMV-inoculated leaves. This background may have been due to other, constitutively expressed cyclases (tobacco sesquiterpene cyclase belongs to a family of tobacco cyclases containing ~12-15 members). In sections from non-inoculated leaves, however, the cytosol did not carry a significant amount of background label, and even more importantly, the vacuolar inclusion bodies did not react to the antibodies (data not shown). In sections from TMV-inoculated leaves, in addition to the chloroplasts and the inclusion bodies, the cytosol labeled heavily with anti-cyclase (data not shown) consistent with earlier reports of EAS being a defense-inducible, cytosolic enzyme.

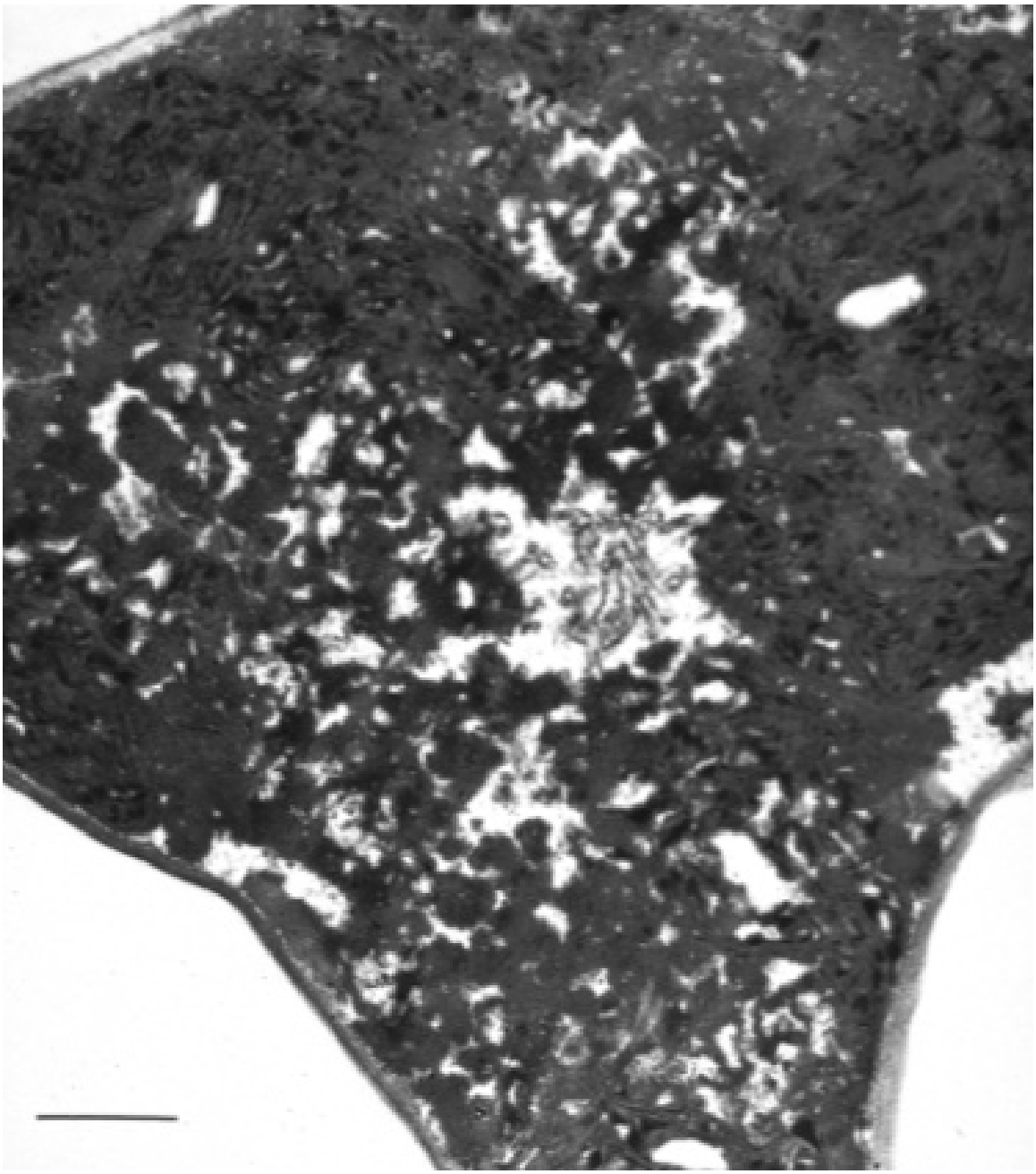

Fig. III.5. A necrotic cell from a TMV lesion. Note total disintegration of the cytosol and organelles and accumulation of spherical protein bodies. TMV-particles can be seen in the middle of the cell. Bar = 1 μm.

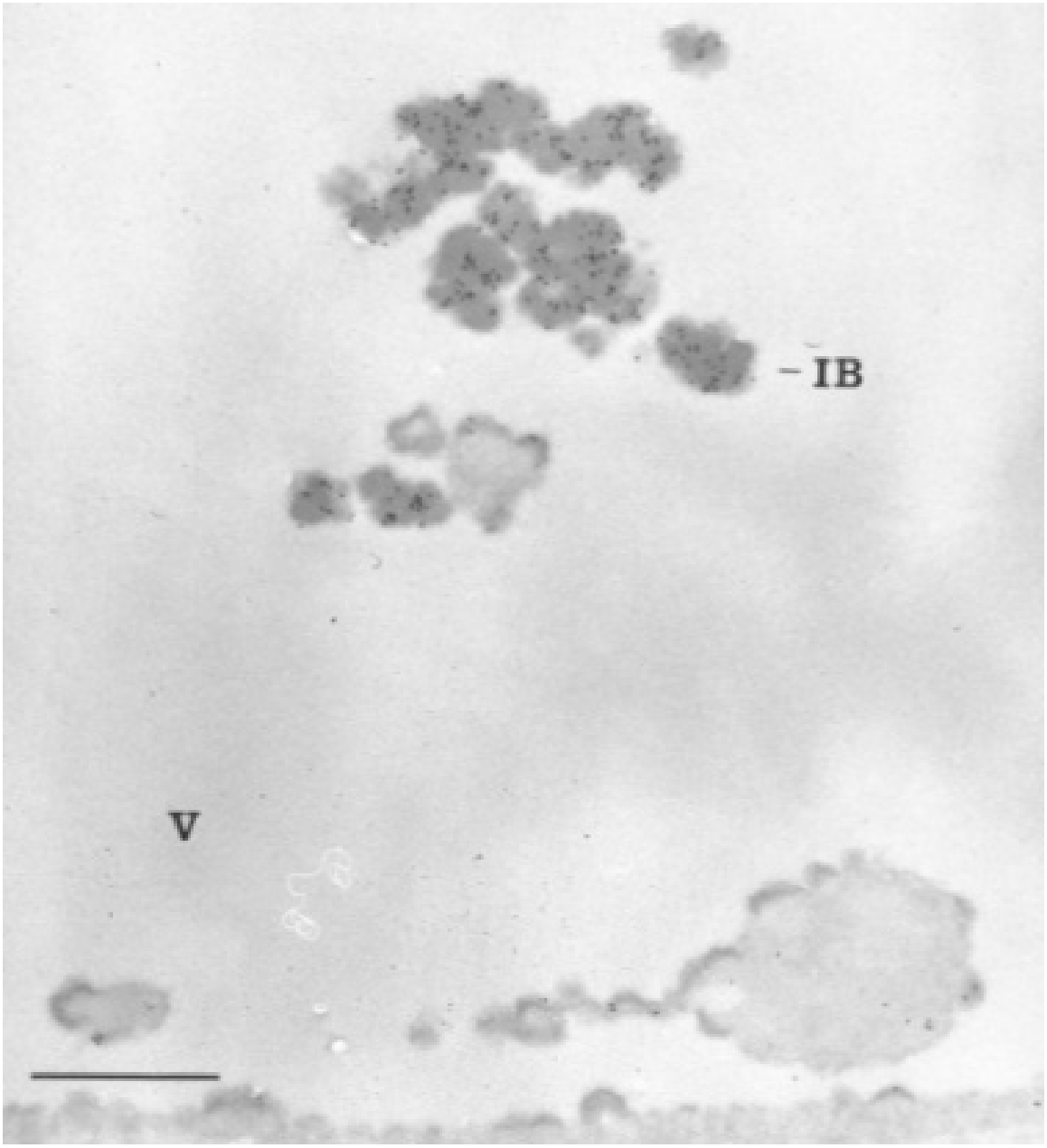

Fig. III.6a. Immunolocalization of MYC-HMG2 to the electron-dense inclusion bodies within the vacuole of a leaf mesophyll cell from plant of SL7-6 (expressing a c-*myc*-tagged, full-length tomato *hmg2*) five days after inoculation with 50 µg/ml TMV-strain U1. Bar = 1 µm. IB, inclusion body; V, vacuole.

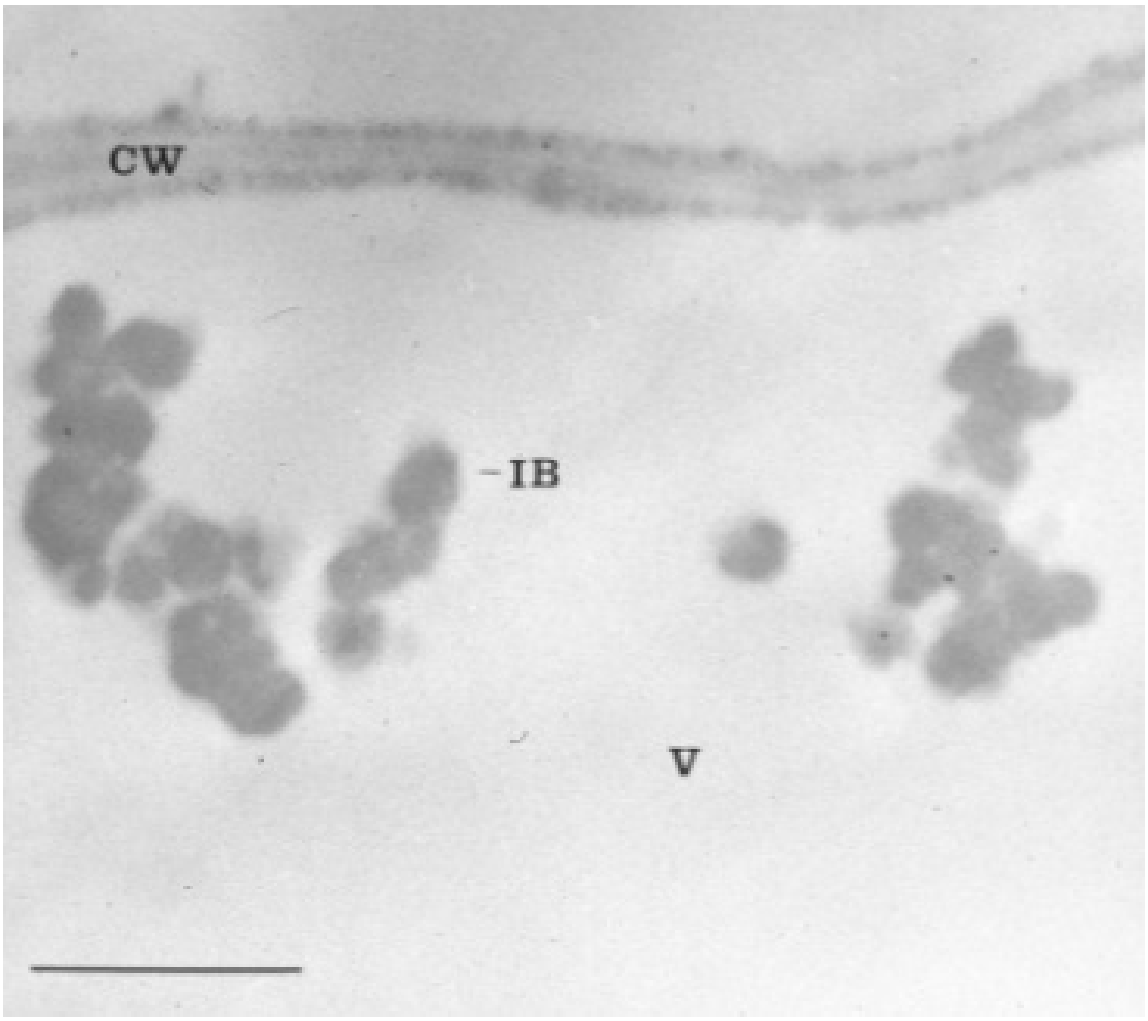

Fig. III.6b. Electron micrograph of a leaf mesophyll cell from plant SL7-6 (expressing a c-*myc*-tagged, full-length tomato *hmg2*) prior to TMV-inoculation. The inclusion bodies within the vacuole do not label with the anti-c-*myc* antibody. Bar = 1 μm. CW, cell wall; IB, inclusion body; V, vacuole.

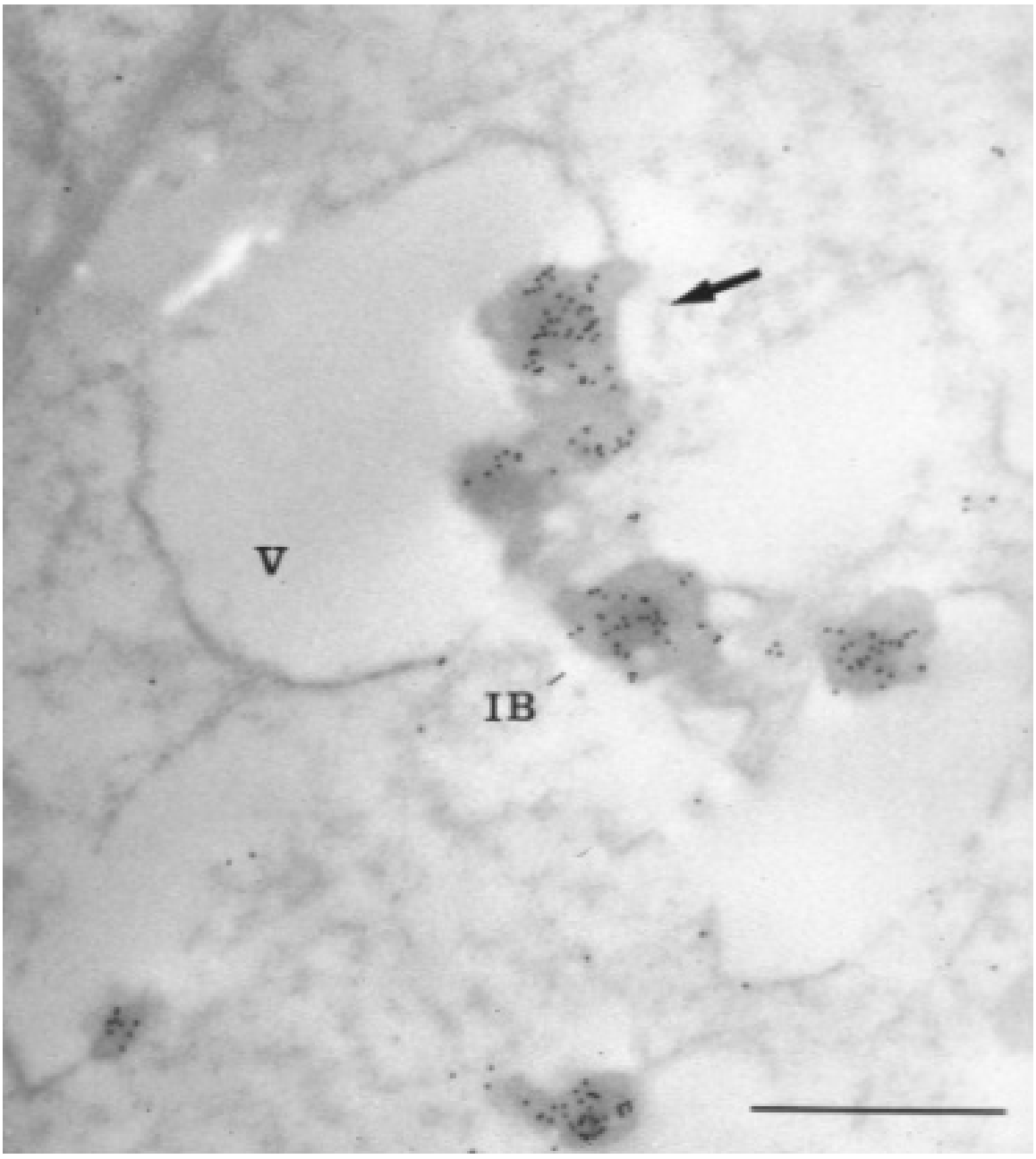

Fig. III.6c. Electron micrograph of a leaf mesophyll cell from plant SL7-6 (expressing a c-*myc*-tagged, full-length tomato *hmg2*) 5 d after inoculation with 50 µg/ml TMV-strain U1 showing immunogold labeling of MYC-HMG2 over inclusion bodies in small vacuoles or the tip of the central vacuole. Note uneven distribution of the gold particles. Bar = 1 µm. IB, inclusion body; V, vacuole.

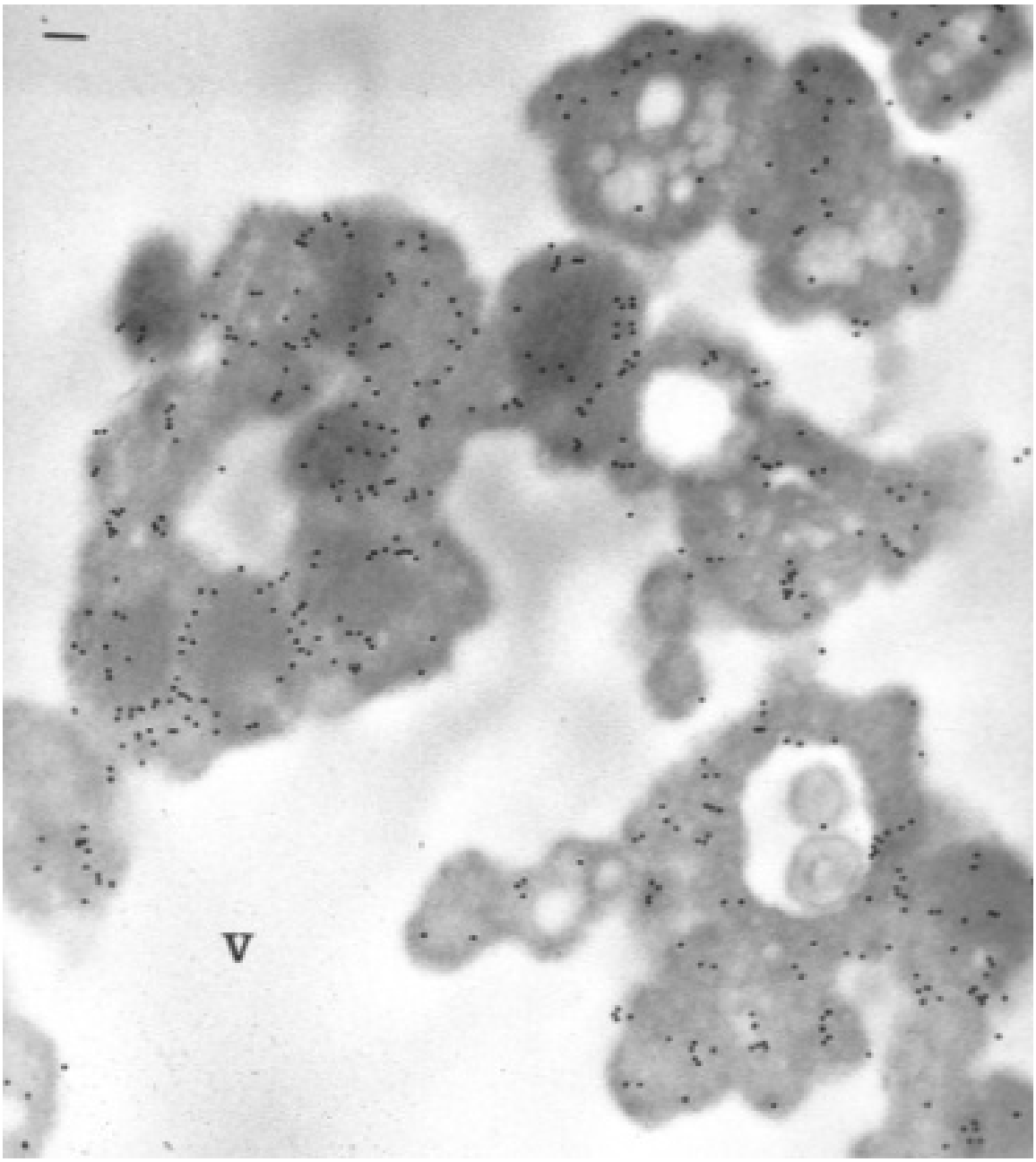

Fig. III.6d. Electron micrograph of a leaf mesophyll cell from plant SL7-6 (expressing a c-*myc*-tagged, full-length tomato *hmg2*) 5 d after inoculation with 50 µg/ml TMV-strain U1 showing immunogold labeling of MYC-HMG2 over vacuolar inclusion bodies. MYC-HMG2 is aggregated around more electron-dense areas in the bodies. Note also presence of vesicles surrounded by a double-membrane associated with the inclusion bodies. Bar = 0.1 µm. V, vacuole.

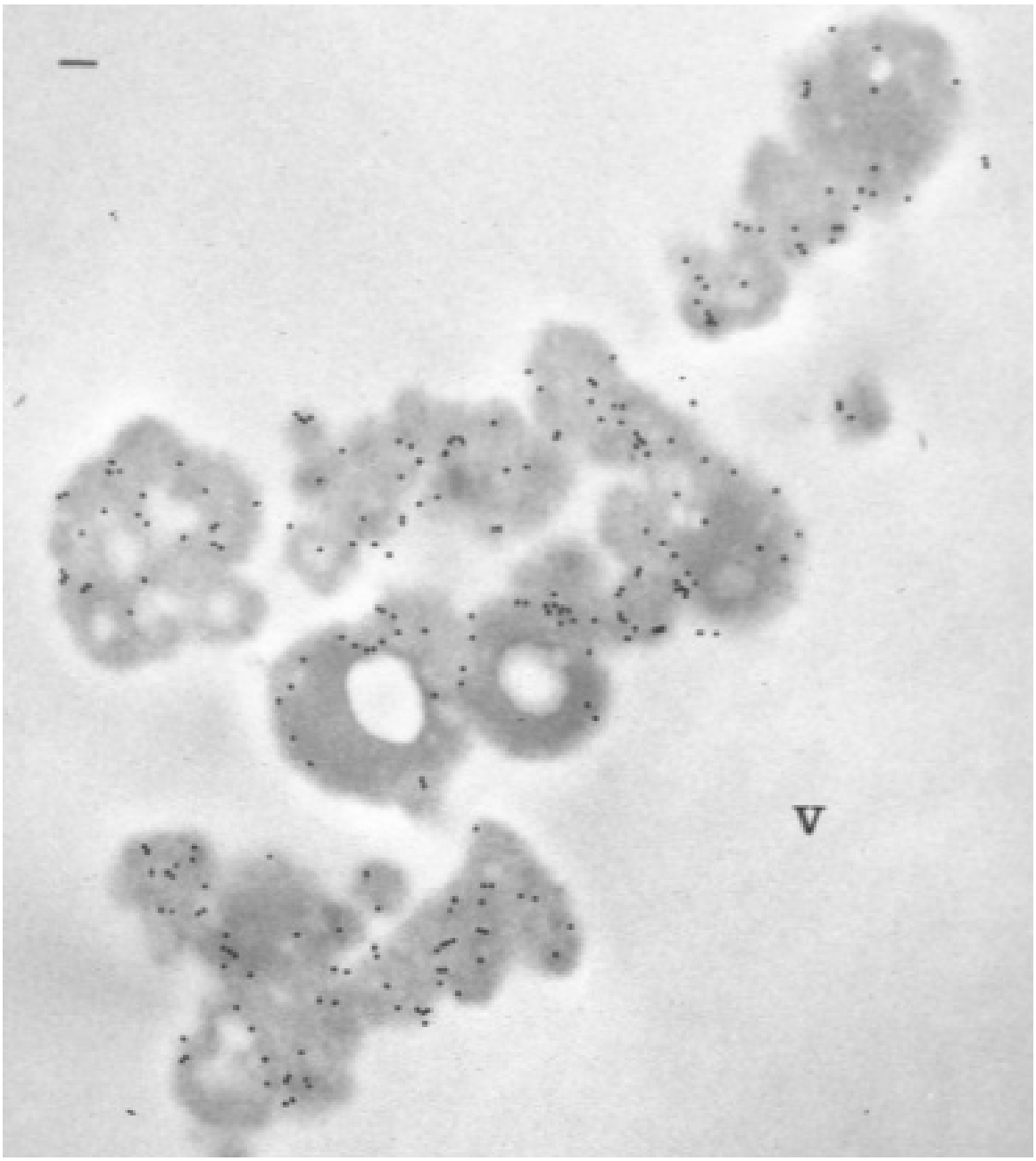

Fig. III.7. Electron micrograph of a leaf mesophyll cell from plant SL7-6 (expressing a c-*myc*-tagged, full-length tomato *hmg2*) 5 d after inoculation with 50 µg/ml TMV-strain U1 showing immunogold labeling of tobacco sesquiterpene cyclase in the vacuolar inclusion bodies. Bar = 0.1 µm. V, vacuole.

III. 4. DISCUSSION

Constitutive expression of tomato *hmg2* in transgenic tobacco has been shown to enhance resistance to TMV and *Erwinia carotovora* subsp. *carotovora* (*Ecc*). The plants were more resistant to rotting by *Ecc* and developed fewer and smaller lesions in resistant interactions with TMV (Yu *et al.* 1997, Yu 1995). Unexpectedly, the enhanced resistance did not result from constitutive production and accumulation of capsidiol, the tobacco sesquiterpene phytoalexin (Chapter II). The wild-type tobacco developed larger lesions and accumulated more capsidiol per gram of tissue than the transgenic plants. More likely, enhanced resistance may have resulted from constitutive expression of *hmg2* supplying the transgenic plants with an advantage of speed in capsidiol biosynthesis, with HMG2, the presumed rate-limiting step already present in the tissues. If high concentrations of phytoalexins were produced fast enough to contain the pathogen and restrict the spread of the lesion to a few cells it would become unnecessary for more peripheral cells to activate their defense responses. (See Chapter II).

Expression of *hmg2* was also found to induce ultrastructural changes in the transgenic plants. The changes (increased volume of the cytosol and accumulation of inclusion bodies within the vacuole) were indicative of increased metabolic activity, and similar characteristics were observed in wild-type tobacco cells adjacent to TMV-lesions (Chapter II). In the present study, we found that in response to TMV-inoculation, a c-*myc*-tagged HMG2 encoded by a modified tomato *hmg2* accumulated in the vacuolar inclusion bodies of transgenic tobacco leaf cells. Sesquiterpene cyclase, a key branch point enzyme downstream HMGR leading to sesquiterpene phytoalexin biosynthesis, co-localized to the same inclusion bodies.

A number of defense-inducible proteins, *i.e.*, the vacuolar forms of pathogenesis related (PR) proteins, are known to accumulate in the vacuole in response to pathogens. These include the vacuolar, basic forms of chitinase and β-glucanase. The vacuolar PR proteins have more antifungal activity than their acidic counterparts, which are secreted from the plant cell (Sela-Buurlage *et al.* 1993). A 23-kD pathogenesis-related protein (P23) is induced in tomato plants when inoculated with citrus exocortis viroid and accumulates in vacuoles in association with dense inclusion bodies (Rodrigo *et al.* 1993). The purified P23 protein inhibits the growth of several phytopathogenic fungi *in vitro*. Two other tomato PR proteins, P1(p14) and P69, are present in both in vacuolar inclusion bodies and intercellular spaces (Vera *et al.* 1989a, 1989b). Palmer and Bender (1995) reported large, spherical particles inside vacuoles of tomato leaf cells treated with the pseudomonad phytotoxin coronatine. The inclusions in coronatine-treated tissues were concluded to contain chymotrypsin inhibitor, based on the high correlation with chymotrypsin inhibitor activity in the tissues. Notably, the inclusion bodies in tomato cells were also observed only in viroid-infected or coronatine- or methyl jasmonate-treated cells and not in healthy tissues. The vacuolar inclusion bodies we observed are possibly protein bodies containing a number of different PR-proteins since they were visible in the vacuoles when neither MYC-HMG2 nor sesquiterpene cyclase were present in the vacuole. It is also interesting to note that the inclusion bodies had areas of different grades of electron-density possibly suggesting that they could contain different compartments. Accumulation of several PR proteins in the vacuole would be consistent with constitutive expression of *hmg2* triggering part of the cells' defense responses. Expression of a truncated HMG2 lacking the catalytic domain did not lead to formation of inclusion bodies, suggesting that the catalytic domain (and HMGR activity) is required for activating the observed subcellular changes.

The vacuolar and extracellular PR proteins are envisioned to work synergistically by increasing the level of resistance and to have their major effect after signifigant

cellular decompartmentalization has already occurred (Hammond-Kosack and Jones 1996) (See Fig. III.5). Logically, phytoalexin release should likewise be controlled until the cell has effectively committed to cell death, since the compounds are deleterious to the plant cell as well as to the pathogen. Therefore, it would be advantageous to have them produced in a contained environment, such as the vacuole, so that the cytosol could maintain function and the cell could recover if pathogen invasion did not occur.

In a remarkable study by Freytag *et al.* (1994), cytoplasmic rearrangements preceding wall apposition and hypersensitive cell death in potato/*Phytophthora infestans* interactions were followed utilizing video microscopy techniques. Major rearrangements of the cytosol and rapid apposition of autofluorescent material and callose took place directly under the attempted fungal penetration site. If the invasion was stopped at this stage, hypersensitive cell death did not occur, the cytosol restored its normal activity, and the cell survived. The only difference between the incompatible and compatible interaction was a slightly larger number of cells that responded to fungal attack. The constitutive thickening and enlargement of the cytosol and the appearance of the vacuolar inclusion bodies in the *hmg2*-expressing plants suggests that they may have been in a constitutive state of alert, and that the enhanced resistance to TMV may have resulted from activity of a number of other defense mechanisms, in addition to the cell's readiness to produce capsidiol on demand.

The localization of MYC-HMG2 in the ER of transgenic plants is consistent with the mammalian and yeast HMGRs being integral membrane proteins of the ER (Roitelman *et al.* 1992, Wright *et al.* 1988). However, it has to be taken into consideration that we did not study the location of the endogenous tobacco HMG2, but of an introduced chimeric tomato HMG2, which was constitutively expressed under the control of the CaMV 35S promoter. The pathogen-inducible isoforms of HMGR are not constitutively expressed, and especially since after TMV-inoculation MYC-HMG2 was relocated to the vacuole, it is questionable whether the small clusters observed in the cytosol represent a true location of HMG2 in the nontransgenic plant. They may rather be a temporary compartment or a vehicle from the site of HMG2 synthesis (ER) to the vacuole. In the non-stressed plant, other proteins possibly needed for transportation of MYC-HMG2 to the vacuole could have been absent due to lack of appropriate defense-related signals activating their synthesis. The amount of MYC-HMG2 in the vacuolar inclusion bodies after TMV-inoculation was dramatically greater compared to the amount in the small clusters observed in the cytosol prior to inoculation.

The vacuolar localization of MYC-HMG2 fits well the hypothesis of specific HMGR isoforms participating in distinct metabolons leading to different endproduct classes of the isoprenoid pathway. Determining the subcellular location of the constitutively expressed tomato HMGR isoform, HMG1, may assist in answering this question. Tomato HMG1 is associated with sterol biosynthesis, and is thus similar in function to the mammalian HMGR. If, like the mammalian HMGR, HMG1 were an integral ER membrane protein, and HMG2 resided in the vacuole with other defense-inducible enzymes of phytoalexin biosynthesis, then the isoprenoid intermediates would be efficiently sequestered in independent pathways.

The following evidence supports the vacuolar localization of a membrane associated multienzyme complex containing HMG2 and sesquiterpene cyclase in cells undergoing a pathogen triggered defense response. The defense-specific electron-dense inclusion bodies were only observed within the vacuole (this study, Palmer and Bender 1995, Rodrigo *et al.* 1993, Vera *et al.* 1989a, 1989b) and no analogous structures were seen in the cytosol or in the vacuoles of cells from healthy tissues with one exception. In the HMG2-overexpressing cells, the inclusion bodies were observed in the absence of pathogen challenge. However, neither antibodies to MYC-HMG2 nor to sesquiterpene

cyclase reacted with the vacuolar IBs prior to TMV-inoculation (see Fig. III.6). Following TMV-inoculation, immunolocalization showed high levels of both MYC-HMG2 and sesquiterpene cyclase associated with the vacuolar IBs in cells adjacent to necrotic cells in TMV-lesions. Furthermore, cells neighboring the TMV-induced lesions contained a much higher amount of MYC-HMG2 than cells of non-inoculated leaves (as determined from the micrographs), although the expression of the transgene was driven by a constitutive CaMV 35S promoter. This may be due to a stabilizing effect of the other proteins in the putative defense-related multienzyme complex.

The route and mechanism of relocation of the sesquiterpene biosynthetic complex into the vacuole is currently unknown. There is evidence for two different types of vacuoles in plant cells, and three different mechanisms of targeting proteins to the vacuole (reviewed by Okita 1996). Griffing and Fowke (1995) found vacuoles with and without peroxidase activity in the same cell. Immunolabeling studies with aleurain, a barley vacuolar cysteine proteinase, showed labeling in small vacuoles in imbibed barley aleurone cells, but not in the morphologically distinct protein storage vacuoles (Holwerda *et al.* 1990). Two morphologically distinct vacuole types are also evident during the differentiation of cotton seed trichomes (Wilkins and Tiwari 1995). In developing pea cotyledons, two integral tonoplast proteins, α-TIP (Tonoplast Intrinsic Protein) and γ-TIP, localized specifically to tubular membranes enclosing protein bodies, and to the tonoplast of vegetative vacuoles, respectively (Hoh *et al.* 1995).

According to Okita (1996), proteins utilizing the Golgi-dependent pathway into the vacuoles may enter the protein storage vacuole utilizing smooth transport vesicles and the classically defined acidic vacuole utilizing clathrin-coated vesicles. There is also evidence of a third, Golgi-independent pathway to the vacuole. Levanony *et al.* (1992) proposed that wheat storage protein bodies are formed in the ER, released into the cytoplasm and transported to the vacuole by a process resembling autophagy. The wheat homolog of Binding Protein (BiP), an ER marker protein, was present within the protein bodies in the cytoplasm as well as inside the vacuoles. Another novel mechanism of transport to the vacuole was demonstrated by Herman and Lamb (1992), who observed endosome-like multivesicular bodies which may have been involved in internalizing cell wall materials for degradation in the vacuole. The transport of TIP to the tonoplast may also be independent of Golgi, since neither monensin nor brefeldin A blocked its arrival to the tonoplast (Gomez and Chrispeels 1993). Monensin inhibits correct sorting in the *trans*-Golgi network by disrupting the proton gradient across the membrane; brefeldin A prevents the anterograde vesicle transport between the ER and the Golgi.

We did not observe significant labelling of MYC-HMG2 or sesquiterpene cyclase at the plasmalemma suggesting that delivery of these proteins to the vacuole does not follow the endocytosis-like pathway suggested by Herman and Lamb (1992). Support for direct delivery of wheat storage proteins from ER localized protein bodies to the vacuole was provided by co-localization of BiP to the vacuolar protein bodies (Levanony *et al.* 1992), and analogous studies will be undertaken in our tobacco system. Tomato HMG2 contains a putative Asn-linked glycosylation site in the short segment within the ER lumen (Denbow *et al.* 1996), and analysis of this glycan may provide insight as to whether HMG2 traverses the Golgi prior to being delivered to the vacuole.

Transport of membrane proteins within the endomembrane system generally involves the directional blebbing off of vesicles from one compartment and subsequent fusion to the target membranes (reviewed in Chrispeels 1991, Bednarek and Raikhel 1992). If delivery of HMG2 as a membrane protein to the vacuole were mediated by direct fusion of transport vesicles with the tonoplast we would expect to see MYC-HMG2 localized to the tonoplast (see Fig. III.8, model A). According to this model, both the catalytic domain and the N-terminal c-*myc*-epitope are predicted to remain on the

cytosolic side of the vesicle with any associated cytosolic enzymes such as sesquiterpene cyclase. We have not observed significant amounts of labelling of either HMG2 or sesquiterpene cyclase at the tonoplast suggesting that delivery through simple vesicular fusion does not occur.

Our data are most consistent with an invagination and uptake of the entire ER-membrane associated complex into the vacuole. According to this model (see Fig. III.8, model B) the HMG2/sesquiterpene cyclase complex would be surrounded by a tonoplast-derived membrane. Observation of membranes in close association with the inclusion bodies is difficult to observe because of the highly electron-dense nature of the IBs. Use of antibodies directed to tonoplast-localized proteins *e.g.* TIPs (Hoh *et al.* 1995) or vacuolar H^+-ATPases (Herman *et al.* 1994) may be the most direct approach to test this model. In addition, time course analyses using three-dimensional imaging systems (fluorescent confocal microscopy) may be an effective means of delineating the process of HMG2 relocalization to vacuoles.

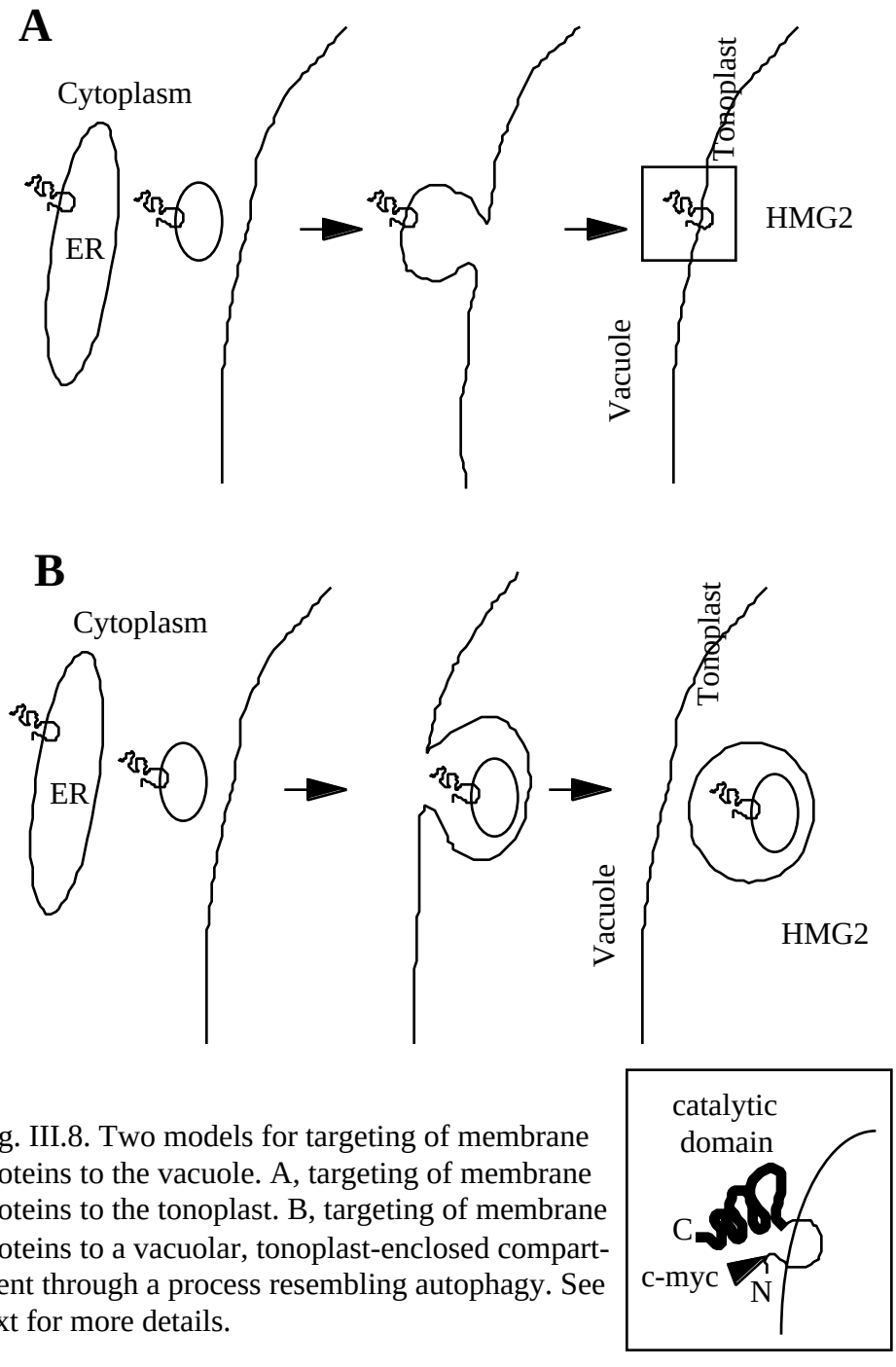

Fig. III.8. Two models for targeting of membrane proteins to the vacuole. A, targeting of membrane proteins to the tonoplast. B, targeting of membrane proteins to a vacuolar, tonoplast-enclosed compartment through a process resembling autophagy. See text for more details.

III. 5. METHODS AND MATERIALS

III. 5. 1. Vector construction and plant materials

Vectors used for tobacco transformation were described earlier in Chapter II. *Nicotiana tabacum* var. Xanthi grown under greenhouse conditions was used in all experiments. Transgenic plants were generated from Xanthi by *A. tumefaciens*-mediated transformation (Horsch *et al.* 1978).

III. 5. 2. Immunoblot analysis

Microsomal membranes for SDS-PAGE were isolated by grinding 1g leaf tissue with mortar and pestle in 3 ml homogenization buffer (100 mM K-phosphate pH 7.0; 4 mM $MgCl_2$; 5 mM DTT) together with "Complete protease inhibitor cocktail" (Boehringer Mannheim Biochemicals, Indianapolis, IN) on ice. The homogenate was centrifuged at 12,000 x g for 15 min at 4˚C, and the supernatant was subsequently centrifuged at 100,000 x g for 1h at 4˚C. The pellet was resuspended in buffer conatianing 100 mM K-phosphate, pH 7.4; 50 mM DTT; 0.1% Triton X-100; 0.1% sarcosyl and 1 mM EDTA. Protein concentrations were determined with a Coomassie kit (Pierce, Rockford, IL), which employs the method of Bradford (1976). Samples (40 µg of protein) were heated at 60˚C for 10 min in SDS-PAGE sample buffer, and analyzed in 7.5% SDS polyacrylamide gels (Laemmli, 1970). Proteins were transferred to PolyScreen PVDF membrane (NEN Research Products, Boston, MA) in 192 mM glycine; 25 mM Tris-base; 0.2% (w/v) SDS using a Bio-Rad transfer apparatus at 25V overnight. Blots were blocked overnight in 20 mM Tris pH 7.6; 137 mM NaCl; 1% (w/v) non-fat dry milk and 0.5% (w/v) bovine serum albumin (BSA). Monoclonal mouse anti-c-*myc* (Invitrogen, Carlsbad, CA) and monoclonal mouse anti-tobacco sesquiterpene cyclase (anti- 5-*epi*-aristolochene synthase, a gift from J. Chappell, University of Kentucky) were used as primary antibodies diluted 1:5,000 in blocking buffer. Secondary antibody was horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham, Arlington Heights, IL) diluted 1:2,000 in blocking buffer. Incubations were for 1 h at room temperature followed by 4x15 min washes in 20 mM Tris, pH 7.6; 137 mM NaCl; 0.1% (v/v) Tween-20. ECL chemiluminescent reagents (Amersham) were used for detection.

For immunoblot analysis of fractions from sucrose gradients, 250 µL of each fraction was diluted to <10% sucrose with TE (10mM Tris; 1mM EDTA, pH 8.0) and centrifuged at 150,000 x g at 4˚C for 1h, the pellet was resuspended in SDS-PAGE loading buffer and separated in 7.5% SDS polyacrylamide gel (Laemmli, 1970). Transfer onto membrane and subsequent antibody and detection steps were as above.

III. 5. 3. Subcellular fractionation

Leaf tissue was homogenized as for microsomal membrane preparations, and the homogenate was filtered through Miracloth (Calbiochem, La Jolla, CA) and centrifuged at 1,000 x g for 10 min at 4˚C. The supernatant was subsequently centrifuged at 150,000 x g for 1h at 4˚ and the pellet was resuspended in 100 mM K-phosphate; 50 mM DTT. Membranes were loaded on a linear (20% to 50%) sucrose gradient in TE (10mM Tris; 1mM EDTA, pH 8.0) and centrifuged at 150,000 x g in SW41Ti swinging bucket rotor (Beckman) at 4˚C for 16h. Approximately 0.7 mL fractions were collected from the bottom of the tube. The fractions were assayed for protein concentration using the Coomassie kit (Pierce, Rockford, IL) and sucrose concentration using a refractrometer.

The amount of chlorophyll in the fractions was calculated from the formula (A_{645} x 20.2) + (A_{663} x 8.02), measured after (1:6) extraction with 80% acetone (personal communication, Dr. Ruth Alscher). Marker enzyme activities were assayed according to Briskin *et al.* (1987) except KCN was used instead of NaCN in NADH-cytochrome-c reductase assay.

III. 5. 4. NADH-cytochrome-c reductase activity

Aliquots of 50 µl of each fraction were added to 800 µL reaction buffer (50mM sodium phosphate, pH 7.5; 0.2 mM NADH; 1.7 mM KCN; 30 µM cytochrome c) and the reduction of cytochrome c was measured by the change of optical density at 550 nm.

III. 5. 5. Potassium-stimulated ATPase activity

Aliquots of 50 µl of each fraction were added to 800 µl reaction buffer (30 mM Tris-MES, pH 6.5; 3 mM ATP; 3 mM $MgSO_4$; 1 mM ammonium molybdate; 50 mM KCl, when present) and incubated at 38°C for 1h. The ATP substrate was present as the Tris salt converted from the sodium salt by treatment with Dowex 50-W exchange resin (H^+ form) (Hodges and Leonard, 1974). Potassium stimulation represents the difference in activity between assays performed in the absence and presence of KCl. The released inorganic phosphate was determined by adding 0.87 ml stopping reagent [0.42% (w/v) ammonium molybdate in 1N H_2SO_4 : 10% (w/v) ascorbic acid (6:7)] to the assay tube and determining A_{700} after 20 min incubation at room temperature.

III. 5. 6. Latent inosine diphosphatase (IDPase) activity

Aliquots of 50 µl of each fraction were added to 800 µl reaction buffer [30 mM Tris-MES, pH 7.5; 3 mM inosine diphosphate (sodium salt); 3 mM $MgSO_4$; 50 mM KCl] and incubated at 38°C for 1h. Released inorganic phosphate was determined as above. Latent IDPase activity represents the difference in activity observed upon six days of storage at 2-4°C.

III. 5. 7. Immuno-electron microscopy

Approximately 1 mm x 2 mm squares of leaf tissue (from approximately 6 weeks-old tobacco plants started from cuttings of T_0 plants) containing single lesions were cut in half, bisecting the lesion, to ensure obtaining thin sections with some necrotic and some healthy green tissue. Tissue was fixed according to Schroder *et al.* (1993), omitting postfixation in OsO_4 of samples that were to be immunolabelled. Following dehydration in ethanol series, tissue blocks were embedded in London Resin White acrylic resin (Electron Microscopy Sciences, Ft. Washington, PA) and polymerized at 58-60ºC under vacuum. Thin sections (<100 nm) were cut using a MT6000-XL microtome (Research and Manufacturing Company Inc., Tucson, Arizona) and viewed with Zeiss 10CR transmission electron microscope after immunolabeling and/or staining with uranyl acetate and lead citrate.

Sections were blocked in 2% BSA in PBS/0.1% Tween (171 mM NaCl, 3 mM KCl, 13 mM Na_2PO_4, 2 mM KH_2PO_4, 0.1% Tween-20) 2x30 min before incubation in the primary antibody. All antibodies were diluted in the blocking buffer. Primary antibodies were a monoclonal mouse anti-c-*myc* diluted 1:50 and a monoclonal mouse anti-EAS diluted 1:50. Rabbit anti-mouse IgG (Sigma), diluted 1:100, was used as a

bridge between the primary and the secondary antibody. Secondary antibody was goat anti-mouse IgG conjugated to either 15 nm or 6 nm gold 1:100 (Electron Microscopy Sciences, Ft. Washington, PA). Incubations were done at room temperature for 1 h, and sections were washed 4x5 min in PBS/0.5% Tween (171 mM NaCl, 3 mM KCl, 13 mM Na_2PO_4, 2 mM KH2PO4, 0.5% Tween-20) and blocked 2x15 min between incubations, and washed 4x5 min in PBS/0.5% Tween and 3x5 min in distilled water after the secondary antibody. Double immunostaining was done by staining for c-*myc* first, with 15 nm gold, after which the procedure was repeated with anti-EAS as a primary antibody, and 6 nm gold as a secondary antibody.

III. 6. ACKNOWLEDGEMENTS

We thank Joseph Chappell for generously providing the anti-sesquiterpene cyclase antibody, and Dr. Brian Storrie for help with immunolocalization studies. This work was supported by Academy of Finland grant #7517 and grants from Cultural Fund of Finland, Alfred Kordelin's Fund, Thanks to Scandinavia Inc., Kemira Research Fund, and Tiura' s Fund to S. S. L. and by the National Institutes of Health grant #R29-GM39549 and National Science Foundation grant #IBN-9514177 to C. L. C

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CHAPTER IV: CONCLUSIONS AND FUTURE DIRECTIONS

IV. 1. CONCLUSIONS AND FUTURE DIRECTIONS

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC 1.1.1.34) catalyzes an early step in the plant isoprenoid pathway from HMGCoA to mevalonic acid. In mammalians, this is the rate-limiting step for cholesterol biosynthesis, and the pathway is feedback regulated by sterols. In plants, which contain multiple *hmgr* isogenes compared to a single gene in mammals, the isoprenoid pathway and its regulation is more complex. Certain important plant isoprenoid endproducts (such as chlorophylls, quinones and carotenoids) are synthesized in plastids, and others (such as ubiquinone) in mitochodria, while sesquiterpene and sterol biosynthesis occurs in the cytosol. The expression of *hmgr* isoforms is regulated by the developmental stage of the plant, by wounding and pathogens, and by light. The isoforms are also known to be regulated post-translationally by protein phosphorylation, degradation, and possibly feedback inhibition by isoprenoid endproducts.

The mammalian HMGR is an integral membrane protein of the endoplasmic reticulum (ER). The location of the plant HMGR isoforms is still controversial. Although HMGR activity has been localized to plastids and mitochondria, none of the cloned *hmgr* genes contain transit signal sequences for targeting into these organelles. In fact, most of them contain an RR- ER-retention motif in the N-terminal end of the protein. Tomato (*Lycopersicon esculentum*) HMG1 and HMG2 and *Arabidopsis thaliana* HMG1 have also shown to be inserted into microsomal membranes *in vitro*. These facts suggest that all the known isoforms of HMGR may be located in the ER, even though there may may other, unknown isoforms in the plastids and mitochondria.

Pathway partitioning has been suggested to play a role in the differential regulation of the multiple cytosolic isoforms of HMGR. In yeast, HMG1 and HMG2 are differentially regulated by early and late products of the isoprenoid pathway, as well as by oxygen, which is required for the synthesis of the late products, but not for the early ones. In plants, several studies have shown that during a defense response, the pathway can be shunted from constitutive sterol biosynthesis to production of defense compounds, sesquiterpene phytoalexins.

We used the defense-inducible HMGR isoform of tomato, HMG2, as a tool to study subcellular differentiation in plant disease resistance. Constitutive expression of tomato *hmg2* in transgenic tobacco results in plants that are more resistant to tobacco mosaic virus (TMV) and the soft-rot bacterium *Erwinina carotovora* subsp. *carotovora* (Yu, 1995), suggesting that phytoalexin production is an important component of the hypersensitive response. HMG2 is a good candidate for a molecular marker protein for studying subcellular differentiation during a defense response because it is a membrane protein, and the gene is induced by pathogens.

In ultrastructural studies, expression of tomato *hmg2* in transgenic tobacco did not lead to formation of orderly stacked ER-membranes, as observed in yeast and hamster cells overexpressing *hmgr*. Instead, other ultrastructural changes compared to the wild-type tobacco cells included enlargement of the endomembrane structure and accumulation of electron-dense inclusion bodies within the vacuole. Similar characters were seen in wild-type cells adjacent to necrotic cells in a TMV-lesion, indicating that expression of *hmg2* was inducing defense-related morphological changes in the absence of a pathogen.

The fact that HMG2-mediated resistance to TMV did not result from constitutive capsidiol (the tobacco phytoalexin) production led us to speculate about the mechanism of the enhanced resistance observed in the transgenic plants. Perhaps the ready

availability of HMG2 in the constitutively expressing transgenic plants was giving them an advantage of speed in phytoalexin production compared to the wild-type plants. Consistent with this hypothesis, capsidiol amounts in the *hmg2*-expressors did not increase significantly in response to TMV-inoculation, whereas in the wild-type, capsidiol accumulated in a 4-fold amount. Logically, if the cells surrounding the initial infection site were able to respond more quickly and restrict the size of the lesion, less cells per leaf would be required to produce capsidiol. This hypothesis could be tested in a tobacco cell suspension culture system. If capsidiol is actually produced in high amounts in the few cells surrounding the lesions, cell cultures of the *hmg2*-expressors, when elicited with fungal cell wall fragments or cellulase, should produce capsidiol faster and in higher amounts than the wild-type, since in cell culture practically all cells are elicited. It is also possible that other sesquiterpene phytoalexins than capsidiol may have accumulated in the TMV-inoculated leaves of the overexpressors, although in wild-type tobacco cell suspension cultures capsidiol is the only sesquiterpene phytoalexin that has been observed to accumulate after elicitor-treatment (Chappell and Nable, 1987).

The HMG2-mediated enhanced resistance to TMV resembles systemic acquired resistance (SAR), where, if the plants are challenged again after the initial infection, the new lesions are fewer and smaller than the ones on the initially infected leaf. It would be interesting to determine whether the ultrastructural changes we observed in the wild-type are localized to the cells in near vicinity of the lesions, or if they can be found also in non-inoculated leaves of the same plant, suggesting an association with SAR. Another way to compare HMG2-mediated enhanced resistance to TMV with SAR would be to determine if the expression of pathogenesis-related (PR) proteins that are known to be expressed in SAR is elevated in the *hmg2*-expressors. Recently, Shirasu *et al.* (1997) showed that physiological concentrations of salicylic acid (SA), if administered to soybean cell suspension cultures together with an avirulent strain of *Pseudomonas syringae* pv *glycinea*, markedly enhanced the induction of defense gene transcripts, H_2O_2 accumulation, and hypersensitive cell death. The synergistic effect of SA was potent, rapid, and insensitive to the protein synthesis inhibitor cycloheximide. The authors concluded that SA stimulated an agonist-dependent gain-control operating at an early step in the signal pathway. These results support the possibility that in *hmg2*-expressing cells, a part of the defense responses could be alerted, but hypersensitive cell death would not occur without recognition of a pathogen. Several studies have shown earlier, that in the absence of a pathogen, high concentrations of SA and a long preincubation time are often required to induce SAR or markers such as PR proteins (Shirasu *et al.* 1997).

The immunolocalization of MYC-HMG2 in transgenic tobacco plants before and after TMV-inoculation also supports this hypothesis. In non-inoculated cells, MYC-HMG2 localized in the ER and in small clusters in the cytosol and associated with the tonoplast. Post TMV-inoculation, MYC-HMG2 localized in the vacuolar inclusion bodies. The inclusion bodies were present in the non-inoculated plants expressing the full-length *hmg2*, but they did not label with anti-c-*myc* antibody, indicating that transport of MYC-HMG2 from the cytosol to the vacuole was dependent on recognition of the pathogen.

Tobacco sesquiterpene cyclase, a soluble enzyme further downstream from HMGR in the sesquiterpene biosynthetic pathway, co-localized with MYC-HMG2 in the same vacuolar inclusion bodies, supporting the hypothesis of pathway partitioning and involvement of a multienzyme complex in sesquiterpene phytoalexin biosynthesis. The obvious next experiments would be to determine the subcellular localization of the sterol biosynthetic isoform, HMG1, by using an analogous tagging strategy, and squalene synthetase, if antibodies to this enzyme are available. Our expectation is that these enzymes will localize differently from the defense-inducible isoform, HMG2, and

sesquiterpene cyclase, *e.g.* their localization probably will not change in response to a pathogen. It will also be interesting to see, whether the localization of HMG1 and squalene synthetase suggests the involvement of a distinct multienzyme complex for the sterol branch.

We also need to pay attention to the other possible channels of isoprenoid synthesis, that may be able to utilize the mevalonic acid synthesized by expression of the tomato *hmg2* transgene. Mevalonate (or other intermediates, *e.g.* IPP) may be used for sterol biosynthesis or transported to the chloropasts for synthesis carotenoids, phytylPP and other plastidic isoprenoids. This may especially be the case in the transgenic plants prior to TMV-inoculation, when MYC-HMG2 was still localized in the cytoplasm. Experiments are underway in collaboration with Dr. David Orcutt (VPI&SU) to determine the amount of sterols produced in the tomato *hmg2*-expressing plants, and it would also be interesting to quantitate the amount of chlorophyll and carotenoids in the wild-type and the transgenic plants, taking care to standardize for leaf age and growth conditions. Carotenoid biosynthesis usually correlates with chlorophyll synthesis (Dr. Ruth Alscher, VPI&SU, personal communication).

The mechanism of transport of HMG2 and tobacco cyclase into the vacuole is another interesting area to expand into from this study. There are numerous questions waiting to be answered. For example, are HMG2 and cyclase transported to the vacuole separately, or together? If a multienzyme complex exists, is it assembled in the vacuole or in the cytosol, prior to transport? Does transport involve trafficking through the Golgi, or is it an autophagy-resembling process directly from the ER to the vacuole? To answer some of these questions, we may be able to take advantage of the putative N-glycosylation site located in the small stretch of HMG2 peptide protruding into the ER lumen side of the membrane. If the site is glycosylated, analysis of the glycans may help in determining whether the protein is processed through the Golgi. Immunofluorescence microscopy may also be a helpful tool in delineating the transport process.

Another interesting question is simply why were HMG2 and cyclase transported to the vacuole? In tobacco cell suspension cultures elicited with fungal cell wall fragments or cellulase, capsidiol is secreted out of the cells. Localization of HMG2 and cyclase in the vacuole of TMV-inoculated cells suggests that production of sesquiterpene phytoalexins in response to TMV occurred within the vacuole. Perhaps there are differences in the cells' response to different classes of pathogens, depending on their invasion strategy. Fungi and bacteria attack the cell from the apoplast, and there are a number of studies showing cytoplasmic activity and deposition of compounds directly under the attempted infection site. In a study by Snyder and Nicholson (1990), phytoalexins were delivered to the invading fungal appressorium in vesicular inclusions that accumulated until the cell collapsed. TMV moves from one cell to another symplastically, through the plasmodesmata. Maybe a better strategy to defend against TMV instead of secreting the phytoalexins would be to sequester them inside the vacuoles of cells surrounding the initially infected cells. Upon infection, the phytoalexins could then be released into the cytoplasm in an efficient manner leading to the death of the infected cell.

IV. 2. LITERATURE CITED

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