

**FUNCTIONAL ANALYSES OF TOMATO 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE (HMGR) GENES IN TRANSGENIC PLANTS ENGINEERED FOR ALTERED HMGR EXPRESSION**

by

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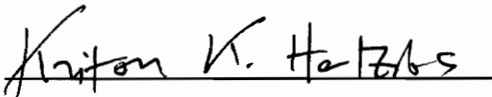
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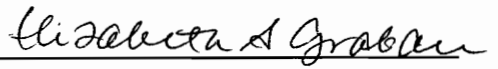
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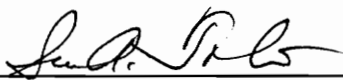
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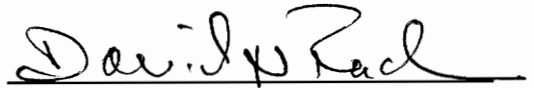
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**ABSTRACT**

3-Hydroxy-3-methylglutaryl CoA reductase (HMGR, EC 1.1.1.34) mediates the first regulatory step (HMG-CoA reduction to mevalonate) in isoprenoid biosynthesis. The tomato genome contains at least four differentially regulated *hmg* isogenes encoding HMGR. Functions of tomato *hmg2* in defense responses were studied by promoter analyses of *hmg2*:GUS gene fusions, overexpression of *hmg2* cDNA, and antisense inhibition of *hmg1* and *hmg2* in transgenic plants. Activity of the *hmg2* promoter is developmentally regulated showing expression in seedling cotyledons and hypocotyls, in trichomes, and in reproductive tissues including pollen, stigmas, ovules, petals and mature seeds. *hmg2*:GUS activity is rapidly induced by wounding or in response to pathogenic viruses or bacteria. *hmg2*:GUS expression is localized to tissue surrounding lesions generated through interactions with either TMV or the bacterial pathogen, *Erwinia carotovora* subsp. *carotovora* (Ecc). Tomato *hmg2* cDNA was cloned by PCR, expressed

in *E. coli* to confirm its HMGR activity, inserted behind the double enhanced CaMV 35S promoter, and engineered into tobacco. Southern and northern analyses confirmed transformation and message expression. Enzyme activity was enhanced compared to nontransformed plants. Selected transgenic plants were significantly reduced for *Ecc* tissue maceration. The size of necrotic lesions induced by TMV was also significantly reduced compared to the nontransformed or vector controls. Thus, genetic manipulation of the rate-limiting step in a major defense pathway provides a novel strategy for enhancing disease resistance. We also generated transgenic tobacco and tomato containing antisense constructs for tomato *hmg1* and *hmg2* to study their effect on disease resistance. Full-length *hmg2* and 5' regions of *hmg1* or *hmg2* were inserted in the antisense orientation behind a 35S promoter. Tomato expressing the full-length *hmg2* antisense showed lower HMGR enzyme activity and were more susceptible to soft rot by *Ecc* than control plants. In contrast, expression of either antisense *hmg1* or antisense *hmg2* in the heterologous tobacco system resulted in plants with enhanced resistance to *Ecc* and reduced TMV lesion sizes. These results may indicate that antisense inhibition is non-specifically exerted on isogenes other than the defense-specific HMGR gene.

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## **Dedication**

This dissertation is dedicated to my wife, Shaoli Wang.

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## **Chapter 1. Literature review**

## **Current understanding of plant disease resistance**

Resistance of plants to pathogens is conferred by a combination of physical and chemical barriers, some of which are preformed and some induced. The physical changes are those that directly affect the properties of the plant cell wall and are designed to strengthen the wall and inhibit access of the pathogen to the plant cells. Physical changes include the accumulation of hydroxyproline-rich glycoproteins and cross-linking of cell wall proteins, lignification and suberization, the deposition of callose, and the accumulation of phenolic compounds (Keen 1993). The major biochemical changes are the biosynthesis and accumulation of phytoalexins, (low molecular weight compounds that are toxic to bacteria and fungi), the accumulation of proteinase inhibitors (Ryan 1990), the release of oligosaccharide elicitors of plant origin, and the accumulation of pathogenesis-related proteins (PR proteins), such as chitinases and glucanases. Breakdown of race-specific disease resistance is primarily associated with a failure of the plant to recognize the pathogen rather than an absence of resistance mechanisms (Keen 1992). Expression of active defense in plants involves a complex of very different biochemical mechanisms which are coordinately induced (Cramer *et al.* 1985).

### **Activation of plant defense-related genes**

The genes encoding PR proteins and those identified by differential library screening have been termed defense response genes (Keen 1993). These genes include those encoding phytoalexin biosynthetic enzymes, those encoding plant cell wall proteins, those encoding enzymes lytic to bacteria or fungi, and other functionally unknown proteins. The synthesis of PR proteins is often correlated with disease resistance with

necrotizing pathogens (Keen 1993). It would be very efficient if plants only synthesized defensive gene products in the presence of pathogens. The majority of plant defense response genes described in the literature are induced at mRNA levels. Induction of defense response genes encoding phenylalanine ammonia lyase (PAL), chalcone isomerase, chalcone synthase, chitinase,  $\beta$ 1,3-glucanase and other genes are coordinately induced (Cramer *et al.* 1985). The influence of environmental stimuli on the synthesis of plant-defense related factors is well documented (Lamb *et al.* 1989). Defense responses induced by different pathogens appears to involve many common elements, suggesting regulatory overlap in the signal transduction pathways (Collinge and Slusarenko 1987).

The research described in this dissertation addresses the regulation of phytoalexin biosynthetic enzymes of tomato and tobacco involved in production of sesquiterpenoid phytoalexins. Because defense-related genes and the products they encode are regulated by diverse pathogenic agents and are differentially activated during compatible (host susceptible) and incompatible (host resistant) interactions, we chose to use two model interactions. The tobacco mosaic virus represents an interaction with well defined genetic control mediating either the hypersensitive resistance (HR) response or a systemic infection visualized as a mosaic pattern of chlorosis in susceptible hosts. We have also utilized interactions with tobacco and the broad host-range bacterial pathogen, *Erwinia caratovora* subsp. *caratovora* (Ecc). There are no well-defined resistance genes for this pathogen although under appropriate environmental conditions, general host defenses, including terpenoid phytoalexin accumulation (Yang *et al.* 1991), lead to maceration limitation and host survival. The biology of host-erwinias interaction in soft rot disease is well characterized and reviewed in (Barras *et al.* 1994)

## **Tobacco mosaic virus**

Tobacco mosaic virus disease occurs in all countries where tobacco is grown. It accounts for about 1% of the annual loss of the tobacco crop in U.S.A. (Lucas 1975). Tobacco plants respond to infection with TMV in two ways: they produce a systemic mosaic pattern in the susceptible cultivars such as NC-95 or local necrotic lesions in resistant cultivars such as Xanthi nc. Necrotic lesions are formed at the site of infection as the result of a hypersensitive reaction of resistant cultivars carrying the resistance gene, the N gene (Whitham *et al.* 1994). Necrotic lesions are produced by the rapid death of a group of infected cells. The formation of a local lesion serves to confine the infecting pathogen to a small area and slow the rate of virus movement (Lucas 1975). Hypersensitive response is one of the most efficient natural mechanisms of defense induced by the infection. The cells surrounding the necrotic area undergo metabolic changes which are involved in plant defense and resistance. These changes are known to include the production of an array of plant defense compounds including hydrolases, pathogenesis-related proteins, phytoalexins, and protease inhibitors (Fritig *et al.* 1987). Final lesion size may be related to the rates of virus replication and spread, and the effectiveness of the resistance mechanism.

## **Systemic acquired resistance (SAR) and salicylic acid**

Studies of the interaction of tobacco with TMV led to the discovery of systemic acquired resistance (SAR) or induced resistance, the phenomenon that an initial inoculation by a necrotizing pathogen can result in broad, systemic immunity to subsequent (bacterial, fungal and viral) infections (Lawton *et al.* 1993). To induce resistance there must first be a necrotic reaction to a pathogen. The necrosis triggers the release of a signal molecular that



translocates to various parts of the plant where its perception results in the expression of a set of genes responsible for the maintenance of the resistant state. There is a considerable amount of evidence implicating salicylic acid (SA) as a signal molecule involved in triggering resistance. Perhaps the most definitive demonstration of the key role of SA utilized transgenic plants expressing salicylate hydroxylase (*nahG*), an enzyme that degrades SA. Plants that expressed high levels of transgene mRNA are not capable of inducing SAR, whereas plants expressing moderate levels of *nahG* mRNA give moderate levels of resistance and transformants with undetectable levels of the mRNA responded normally (Lawton *et al.* 1993).

The current model of SAR is that the salicylic acid induced by a pathogen translocates throughout the plant and is recognized by a specific receptor. The receptor transduces the signal, resulting in the coordinate expression of a set of SAR genes. The encoded SAR proteins (such as hydrolases) accumulate to high levels systemically. The pathogen protection is provided by certain groups of proteins acting on particular pathogens (Lawton *et al.* 1993). A catalase was found to be the SA receptor and signal transduction involves free radicals (Chen *et al.* 1993; Cote *et al.* 1991; Yalpani *et al.* 1991).

### **Genetic engineering of disease resistant plants**

Currently available genetic engineering tools include the transfer of genes into higher plants, the controlled expression of foreign genes, the directed targeting of proteins into subcellular organelles, and the inhibition of expression via antisense RNA approaches (Lamb *et al.* 1992). By taking advantage of these techniques transgenic plants have been created where enzymes involved in disease resistance were modulated via antisense

mediated inhibition or ectopic expression of foreign genes. Transgenic plants were subsequently analyzed with respect to molecular, biochemical and physiological parameters.

Most strategies to obtain disease-resistant plants through genetic engineering are presently focused on introducing genes which encode potential antimicrobial PR proteins with chitinase or 1,3- $\beta$ -glucanase activities or other antimicrobial proteins. The constitutive overexpression of just one type of defense gene holds the risk of selecting variants of pathogens that might regain pathogenicity on such engineered plants (Lamb *et al.* 1992). The first example of a plant genetically engineered for enhanced fungal resistance was reported by Broglie *et al.* (1991). They demonstrated that incorporation of a bean chitinase (*chiA*) in transgenic tobacco leads to an increased resistance against *Rhizoctonia solani*. (Broglie *et al.* 1991). *ChiA* gene was expressed under the control of the 35S-promoter, *ChiA*-mRNA and ChiA-protein were detected in leaves, stems and roots of transgenic tobacco plants. Plants containing higher levels of the bean polypeptide displayed a greater survival rate in *R. solani* infested soil.

Several other strategies have also proved effective in engineering host resistance. The expression of the ribosome inhibiting protein (RIP) gene under the control of the wound- and pathogen inducible promoter *wun1* led to an accumulation of RIP-protein in wounded leaves, stems, and roots of transgenic tobacco plants. Further analysis of transgenic tobacco plants expressing the RIP gene under the control of the constitutive 35S-promoter revealed high levels of RIP protein in leaves, stems and roots. Preliminary data indicated that these plants exhibit a degree of tolerance to infection that is higher even than in the *wun1*-RIP transgenic plants (Logemann *et al.* 1993). Genetic manipulation of

phytoalexin synthesis is more difficult to engineer. However, one strategy involved introduction of a grapevine stilbene synthase into tobacco (Hain *et al.* 1993). This resulted in production of a novel stilbene phytoalexin, resveratrol, and enhanced disease resistance of transgenic tobacco towards grey mould (*Botrytis cinerea*) (Hain *et al.* 1993). A number of plant virus nucleic acid sequences, including those encoding virus coat proteins, have been utilized in the development of virus-resistant plants. Transgenic plants expressing virus capsid protein genes developed the strongest virus resistance compared to plants expressing antisense RNAs or satellite RNAs (for review, see Beachy *et al.* 1990).

### **Plant disease resistance genes**

In plant-pathogen interactions, there are two types of defense responses: specific and general resistance. Specific resistance is mediated by the gene-for-gene interaction (the genetic interaction between the pathogen avirulence gene and the plant resistance gene). Gene-for-gene hypothesis was proposed in early 1940's (Flor 1942). Recognition functions are provided by dominant alleles of genes in the plant (resistance, or R-genes) which are speculated to interact, either directly or indirectly with either the direct or indirect product of a single pathogen gene (avirulence, or *avr* genes). For each gene conditioning race-specific resistance in the host plant, there is a corresponding gene conditioning avirulence in a race of the pathogen. Gene-for-gene complementarity occurs most frequently in plant-pathogen interactions involving obligate and biotrophic pathogens which are highly specialized and have a narrow host range.

Plant disease resistance genes function in highly specific pathogen recognition pathways. The genetic dissection of disease resistance is the first step in elucidating the

signal transduction pathways required for expression of plant disease resistance (Jones *et al.* 1994b; Martin *et al.* 1994). Recently, significant progress has been made in understanding molecular function of plant disease resistance genes. Four plant disease resistance genes were cloned within the last two years. The first cloned disease resistance gene was the tomato Pto gene (Martin *et al.* 1993). This gene confers resistance to *Pseudomonas syringae* pv. tomato (Pst), the causal agent of bacterial speck, and is at the single semi-dominant locus, Pto. The Pto locus interacts in a gene-for-gene fashion with a cloned avirulence gene, avrPto, from the pathogen. The Pto resistance gene encodes a putative serine-threonine protein kinase, suggesting a role for the gene product of Pto in signal transduction (Martin *et al.* 1993). RPS2 is a resistance gene in *Arabidopsis thaliana* that confers resistance to *Pseudomonas syringae* strains expressing the avirulence gene avrRpt2. This gene was isolated by the use of a positional cloning strategy (Bent *et al.* 1994). The derived amino acid sequence of RPS2 contains leucine-rich repeat, membrane-spanning, leucine zipper, and P loop domains. The function of the RPS2 gene product in defense signal transduction is postulated to involve nucleotide triphosphate binding (P loop) and protein-protein interactions, and may also involve the reception of an elicitor produced by the avirulent pathogen (Bent *et al.* 1994; Mindrinos *et al.* 1994). The tomato Cf-9 gene confers resistance to infection by races of the fungus *Cladosporium fulvum* that carry the avirulence gene Avr9. The Cf-9 gene was isolated by transposon tagging with the maize transposable element "Dissociation". The DNA sequence of Cf-9 encodes a putative membrane-anchored extracytoplasmic glycoprotein. The predicted protein shows homology to the receptor domain of several receptor-like protein kinases in *Arabidopsis*, to antifungal polygalacturonase-inhibiting proteins in plants, and to other members of the leucine-rich repeat family of proteins. This structure is consistent with that of a receptor that could bind Avr9 peptide and activate plant defense (Jones *et al.* 1994a).

Most relevant for the research of this dissertation, the tobacco N-gene which mediates resistance to TMV has recently been cloned (Whitham *et al.* 1994). The resistance N-gene was isolated by transposon tagging using the maize *Activator* transposon.. The product of the resistance gene N is similar to Toll and the interleukin-1 receptor. Thus the N-gene product may function as a receptor in the recognition signal transduction pathway. Unlike most receptors, the N-gene product appears to be cytosolic rather than on the cell surface. However, this is consistent with TMV interaction since the cytosol is the site of initial uncoating and initiation of viral functions in pathogenicity .

The products of plant disease resistance genes are postulated to recognize invading pathogens and rapidly trigger host defense responses. Generally, the R gene (N gene) products are either receptors or signal transducers. Once the signal was transduced to the nucleus, an array of genes, i.e. defense response genes, would be activated. Among these genes are phytoalexin biosynthesis genes such as genes for sesquiterpene cyclase and HMGR genes (Chappell 1995; Chappell *et al.* 1991).

### **Phytoalexins**

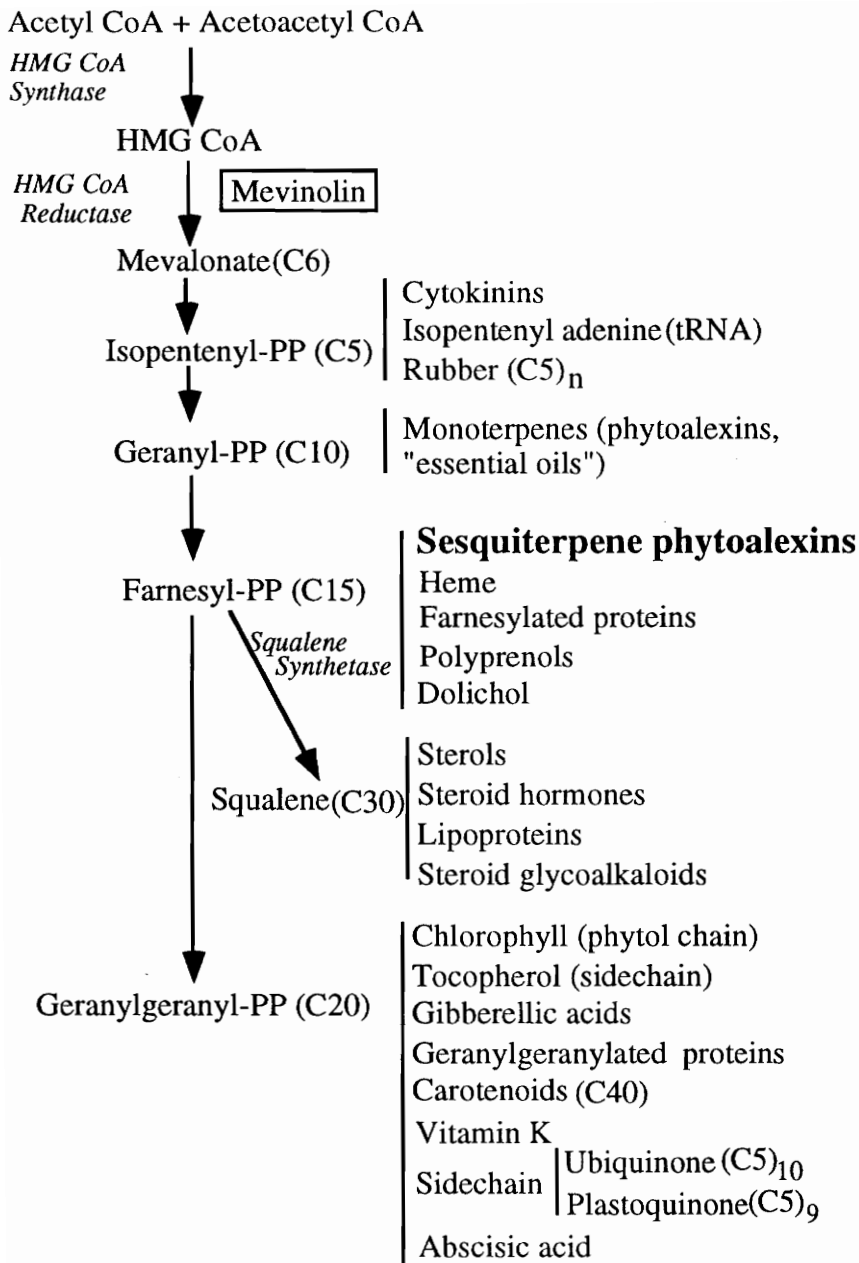
Phytoalexins are low molecular weight compounds synthesized by and accumulated in plants upon induction by pathogens (Paxton 1981). They have anti-microbial activity, an important mechanisms for defending against fungal and bacterial pathogens. There are three major classes of phytoalexins: isoflavonoids, terpenoids, and polyacetylenes. Formation of a particular group of these compounds appears to be associated with certain taxonomic divisions. The *Leguminosae* produce isoflavonoids, *Solanaceae* produce terpenoids, and the *Compositae* produce polyacetylenes (Jadhav *et al.* 1991). Among these phytoalexins,

biosynthetic pathways for isoflavonoids are best understood. The synthesis of isoprenoid (terpenoid) phytoalexins is less studied at the biochemical and molecular levels. Rishitin and phytuberin (sesquiterpenoid phytoalexins from potato) are phytotoxic, causing the death of plant protoplasts and isolated cells. Rishitin affected membrane permeability by increasing fluidity and permitting an increased passage of low molecular-weight compounds through the membrane (Lyon 1989). The major sesquiterpenoid phytoalexins in tobacco are capsidiol and debneyol. Although both have fungicidal function, they were induced when tobacco was infected by viruses (Bailey *et al.* 1975; Burden *et al.* 1985). The relationship between sesquiterpenoid phytoalexin biosynthesis and plant resistance to viruses is unknown.

### **Biological functions of isoprenoids in plants**

The great biosynthetic potentiality of plants is fully illustrated by the synthesis of isoprenoids. Isoprenoids are molecules with varying numbers of carbon atoms which are derived from 5-carbon isoprene units. These isoprene units are condensed into ring compounds commonly containing carbon atom numbers of 10 (the monoterpenoids), 15 (the sesquiterpenoids), 20 (the diterpenoids), or 30 (the triterpenoids) (Figure 1.1). It has been estimated that plants synthesize over 10,000 isoprenoids and that they comprise the largest group of plant products (Goodwin and Mercer 1983). Some of these isoprenoids are essential to primary metabolism while most of the isoprenoids have functions yet to be discovered and are termed secondary metabolites (many of these are economically important plant secondary products).

As in other organisms, isoprenoids in plants function in membrane structure



**Figure 1.1.** The schematic pathway of isoprenoid biosynthesis highlighting important classes of isoprenoid products. Enzymes are italicized. Mevinolin (boxed) is a competitive inhibitor of HMG-CoA reductase.

(sterols), protein prenylation, protein translocation, and electron transport (ubiquinone, heme A). On the other hand, plants have evolved a diverse array of unique functions for isoprenoid compounds. Plants use isoprenoids in such critical functions as photosynthesis (carotenoids, chlorophylls, and plastoquinones), disease resistance, and control of growth, differentiation and reproduction (growth regulators: gibberellins, cytokinins and abscisic acid). Allelopathic roles are often ascribed to mono- and sesquiterpenes. Individual groups of plants may have isoprenoid attractants for insect pollination and phytoalexin antibiotics for defense against fungal or bacterial infection (Goodwin and Mercer 1983). Isoprenoid phytoalexins are the major phytoalexin class of solanaceous species and several important gramineous species such as rice (Nelson *et al.* 1994; Weissenborn *et al.* 1995).

### **3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR)**

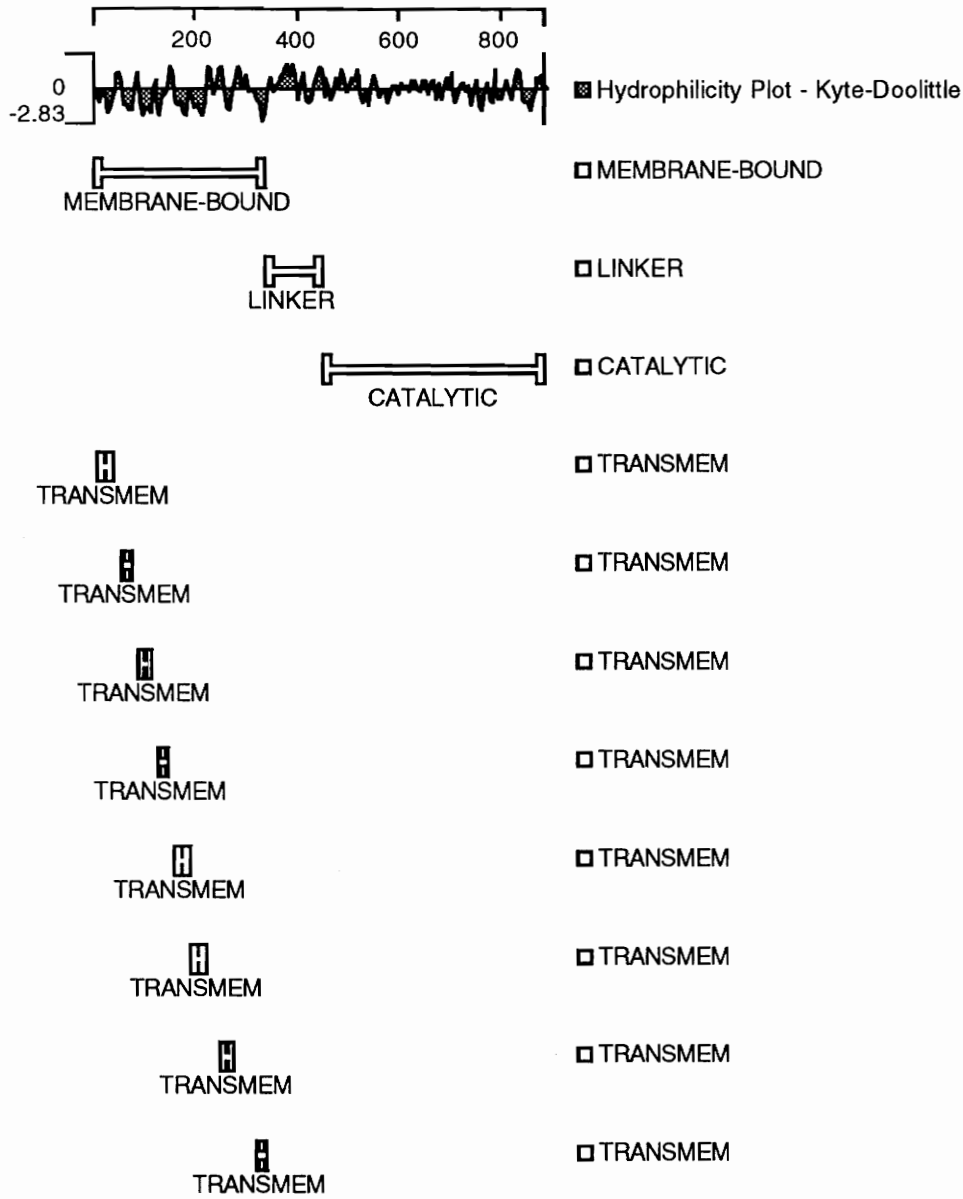
The diverse plant isoprenoid compounds are end products of multi-branched pathways that diverge from the central pathway. In the central pathway, illustrated in Figure 1.1, acetyl-CoAs are converted to mevalonate, (mevalonic acid), the specific precursor of all isoprenoid compounds present in eukaryotic organisms (Goodwin and Mercer 1983). The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is the mevalonate:NADP<sup>+</sup> oxidoreductase (EC 1.1.1.34) which catalyzes the two-step reduction of HMG-CoA to mevalonate with 2 NADPH as the reductant (Durr and Rudney 1960). The catalytic activity of HMGR is (S)-3-hydroxy-3-methylglutaryl-CoA + 2 NADPH = (R)-mevalonate + CoA + 2 NADP(+). In eukaryotic organisms, HMGR is an integral membrane protein, generally present as a glycoprotein (Basson *et al.* 1988; Liscum *et al.* 1985). Structurally HMGR consists of 3 domains: an N-terminal region that contains a variable number of transmembrane segments [7 - 8 in mammals (Roitelman *et al.* 1992),

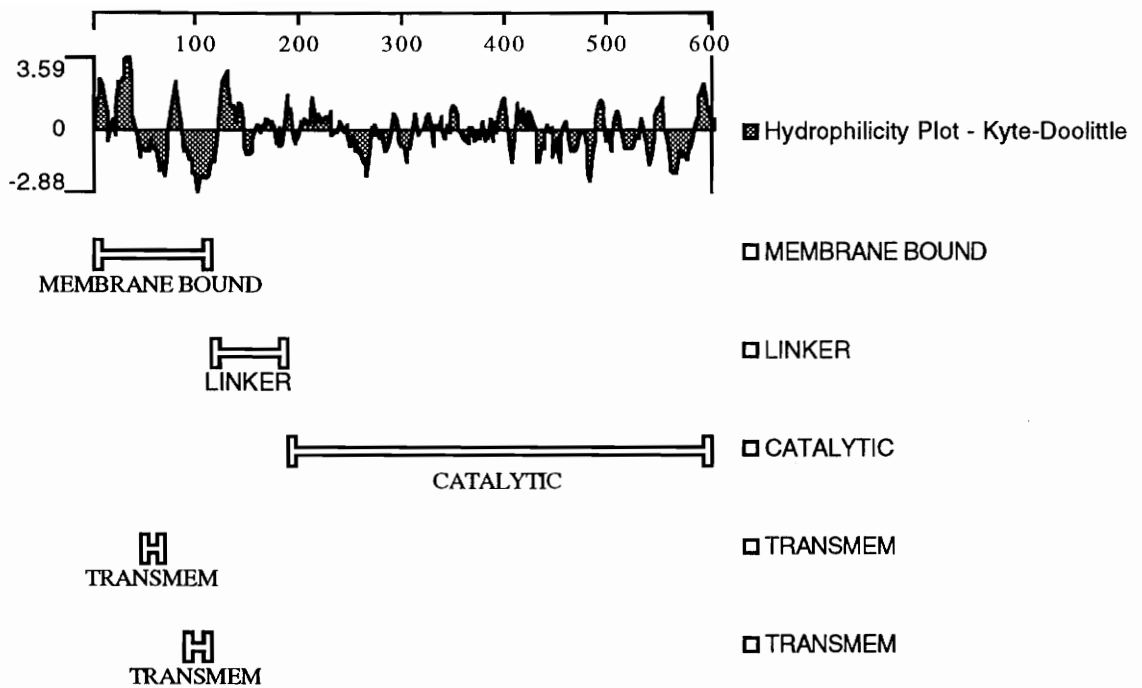


insects (Gertler *et al.* 1988), and fungi (Basson *et al.* 1988); 2 in plants (Caelles *et al.* 1989; Denbow *et al.* 1995; Enjuto *et al.* 1994)], a linker region, and a C-terminal catalytic domain of approximately 400 amino acid residues (Figures 2 and 3). Using the MacPattern program, a software for protein function analysis (Fuchs 1994), to analyze known HMGRs, we can detect two HMGR prosite signatures, PS00318 & PS00066. PROSITE is a compilation of sites and patterns (or motifs) found in protein sequences which can be used to determine the function of proteins (Bairoch 1992). MacPattern contains a prosite index which currently has 1021 entries. Two conserved regions have been selected as signature patterns for HMG-CoA reductases. The first one is located in the center of the catalytic domain, while the second one is a glycine-rich region located in the C-terminal section of that domain. These HMGR signatures are found in all the known HMGRs (from plants, animals, fungi and bacteria). PS00066 is HMGR consensus signature pattern 1: [RKH]-x(6)-D-x-M-G-x-N-x-[LIVM] and PS00318 is HMGR signature pattern 2: [LIVM]-G-x-[LIVM]-G-G-[AG]-T.

In mammalian systems, HMGR is found to be the key regulatory enzyme catalyzing the principal rate limiting step in the biosynthetic pathway of cholesterol and other isoprenoid compounds (Goldstein and Brown 1990). It was found that multiple control mechanisms are imposed upon HMGR ensuring an adequate supply of intermediates and products. The regulation occurs at both the transcriptional and the post-transcriptional levels. HMGR was found to be regulated by sterol and non-sterol isoprenoids at the levels of transcription (Liscum *et al.* 1983; Osborne *et al.* 1985), translation (Nakanishi *et al.* 1988) and enzyme stability (Chun and Simoni 1992; Nakanishi *et al.* 1988). A reversible phosphorylation and dephosphorylation cycle was suggested as one of the control mechanism which may be mediated by a cascade of kinases (Beg *et al.* 1987). It was

**Figure 1.2.** Golden hamster HMGR protein analysis using DNASTAR Protein Analysis software. The average hydrophobicity of each amino acid was calculated over a window of 9 amino acids and plotted as a function of amino acid position. Eight transmembrane span regions were found in the membrane-bound domain (Roitelman *et al.* 1992).





**Figure 1.3.** Tomato HMG2 protein analysis using DNASTAR Lasergene Protein Analysis software. The average hydrophobicity of each amino acid was calculated over a window of 9 amino acids and plotted as a function of amino acid position. Like other plant HMGRs, only 2 transmembrane span regions were predicted in the membrane-bound domain instead of 7 to 8 spans in yeast and mammalian HMGRs.

shown that the first membrane-spanning domain of the syrian hamster HMGR includes a signal sequence for targeting the enzyme to the endoplasmic reticulum (Olender and Simoni 1992). A deletion of the central region of the membrane domain leads to an increased level of proteolytic degradation of the mutated enzyme (Chun and Simoni 1992). Several of the transmembrane spans have been linked to the rapid degradation of mammalian HMGR triggered by elevated cholesterol or low-density lipoproteins (Chun and Simoni 1992; Jingami *et al.* 1987).

It is generally accepted that HMGR is one of the rate-limiting enzymes in isoprenoid biosynthesis in plants (Bach 1987; Chye *et al.* 1992; Stermer *et al.* 1991). In comparison with animal systems, the plant isoprenoid pathway is far more complex, involving multiple isozymes, multiple subcellular locations (Gray 1987), and the production of thousands of diverse isoprenoid compounds. It is anticipated that this complexity will be reflected in differential regulation of specific HMGR isozymes during development or stresses associated with the production of specific isoprenoid compounds. In the past, most HMGR studies in plants have been concerned with HMGR enzyme activity at specific developmental stages or under different environmental conditions. Plant HMGR activity has been shown to be regulated by different physiological and environmental stimuli such as phytohormones, light, pathogen attack, feedback mechanisms, and endogenous factors (Bach *et al.* 1991a; Bach *et al.* 1991b; Stermer *et al.* 1994). The possible compartmentation of HMGR isozymes in the plant cells has also been investigated. Plant HMGRs are integral membrane proteins subcellularly located in endoplasmic reticulum and also in mitochondrial and plastid membranes (Brooker and Russell 1975). One of the pea HMGR isozymes was localized to the envelope membranes of etioplasts, the first report of the suborganelle localization of this key enzyme in isoprenoid synthesis (Russell 1985; Wilson and Russell

1992). The enzyme was purified 156 fold from isolated envelope membranes. Recently, it was shown that HMGR activity profiles correlate with isoprenoid biosynthesis profiles, and plant growth and development can be arrested by mevinolin [a competitive HMGR inhibitor, commercial name is LOVASTATIN made by Merck, N.J. from *Aspergillus terreus* (Gray 1987)]. Activation of HMGR precedes and is necessary for the wound- and elicitor-induced accumulation of sesquiterpenoid phytoalexins and steroid glycoalkaloids in Solanaceous plants (Chappell *et al.* 1991; Choi *et al.* 1992; Stermer and Bostock 1987; Yang *et al.* 1991). Classical biochemical approaches have generated enough data on plant isoprenoid biosynthesis pathway to facilitate the further molecular biological investigation of this complex pathway and its key regulatory enzymes.

### **Molecular biology of HMGR**

The genes encoding HMGRs (*hmg* genes) have been cloned and characterized from the genomes of *Pseudomonas* (Beach and Rodwell 1989), *Haloferax volcanii* (Lam and Doolittle 1992), yeast (Basson *et al.* 1988), *Phycomyces blakesleeanus* and *Gibberella fujikuroi* (Corrochano and Avalos 1992), *Absidia glauca*, *Mucor mucedo*, *Parasitella parasitica*, and *Blakeslea trispora* (Burmester and Czempinski 1994), *Dictyostelium* (De Lozanne 1993), fruit fly (*Drosophila*) (Gertler *et al.* 1988), schistosomes (blood fluke) (Rajkovic *et al.* 1989), cockroach (Martinez-Gonzalez *et al.* 1993), sea urchin (Woodward *et al.* 1988), frog (*Xenopus laevis*) (Chen and Shapiro 1990), Chinese hamster (Chin *et al.* 1984), mouse (Helmberg *et al.* 1990), golden hamster (Skalnik and Simoni 1985) and human (Luskey and Stevens 1985). Mammalian systems have a single *hmg* gene. Yeast, *Dictyostelium* and most fungal species (Burmester and Czempinski 1994) have two *hmg* genes. Hydropathy analyses of all of the above mentioned HMGRs predict seven to eight

trans-membrane regions in the N-terminus except those from bacteria. [The *Pseudomonas* HMGR (EC 1.1.1.88) is a soluble, hydrophilic protein that lacks the hydrophobic domain which function as a membrane anchor (Beach and Rodwell 1989)]. Recently, a more detailed analysis of the membrane domain of the hamster HMGR definitively demonstrated the presence of 8 transmembrane spans and localization of both the C- and N-termini to the cytosolic face (Olender and Simoni 1992; Roitelman *et al.* 1992) (Figure 1.2). The membrane-bound domain of HMGR plays a critical role in the regulated degradation of the enzyme in the ER in response to sterols (Liscum *et al.* 1985).

### **Plant HMGR (*hmg*) genes**

Much less is known about HMGR regulation in plants compared to our understanding of the regulation of HMGR in mammalian systems. Intense interest has developed in studying HMGR as a primary control point for isoprenoid metabolism in plants, and great advances have been achieved since the molecular biology studies on HMGR were initiated in 1989. The first *hmg* genes were cloned from *Arabidopsis* (Caelles *et al.* 1989; Learned and Fink 1989) and tomato (Narita and Gruissem 1989). Since then progress was made in the following plant *hmg* gene studies: rubber tree (Chye *et al.* 1992; Chye *et al.* 1991), tomato (Park *et al.* 1992), potato (Bhattacharyya *et al.* 1991; Choi *et al.* 1992; Roxby *et al.* 1991; Stermer *et al.* 1991), tobacco (Genschik *et al.* 1992), pea (Monfar *et al.* 1990), radish (Ferrer *et al.* 1990; Vollack *et al.* 1994), wheat (Aoyagi *et al.* 1993), rice (Nelson *et al.* 1994), maize (Moore 1995), *Camptotheca* (Burnett *et al.* 1993; Maldonado-Mendoza *et al.* 1992), *Catharanthus* (Maldonado-Mendoza *et al.* 1992), *Artemisia annua* (Kang *et al.* 1995) and soybean (Yu *et al.* 1995).

The initial cloning of plant *hmg* genes was based on hybridization with yeast or hamster sequences. Most subsequent cloning strategies utilized the more closely related plant *hmg* sequences such as *Arabidopsis hmg1* (Caelles *et al.* 1989; Learned and Fink 1989) sequence and tomato *hmg2* (Park *et al.* 1992) sequence. Molecular biological studies have demonstrated that plant HMGRs are generally encoded by small gene families whose members exhibit complex developmental and environmental regulation. *Arabidopsis thaliana* (Caelles *et al.* 1989; Enjuto *et al.* 1994) has two *hmg* genes. Pea HMGR is encoded by a multigene family of at least five members (Monfar *et al.* 1990). The rubber tree (*Hevea brasiliensis*) has three *hmg* genes (Chye *et al.* 1992). The tomato genome contains four *hmg* isogenes, three of which have been isolated (Narita *et al.* 1991; Park *et al.* 1992). At least three *hmg* genes are present in potato (Choi *et al.* 1992; Yang *et al.* 1991). Four genes are detected in wheat (Aoyagi *et al.* 1993). More than two *hmg* genes occur in tobacco (Genschik *et al.* 1992; Yu & Cramer, unpublished data). Two genes were cloned from both *Artemisia annua* (Kang *et al.* 1995) and radish (Vollack *et al.* 1994). A small *hmg* gene family was also detected in rice (Nelson *et al.* 1994). Most of these *hmg* genes have been cloned and sequenced directly confirming the occurrence of multiple *hmg* genes encoding HMGR isozymes as a general feature of higher plants. The contrast of a single *hmg* gene in animal systems versus multiple *hmg* isogene family in plants is quite clear. However, the biological significance of the multiple plant *hmg* isogenes is currently unknown. It might reflect the enormous complexity of the isoprenoid pathway in plants. The operation of this pathway, in which HMGR is the key enzyme, is central to the synthesis of a wide range of isoprenoids required in different parts of the plant during different stages of growth and development.

Based on the hydropathy profile of the proteins, all of the plant HMGRs have two



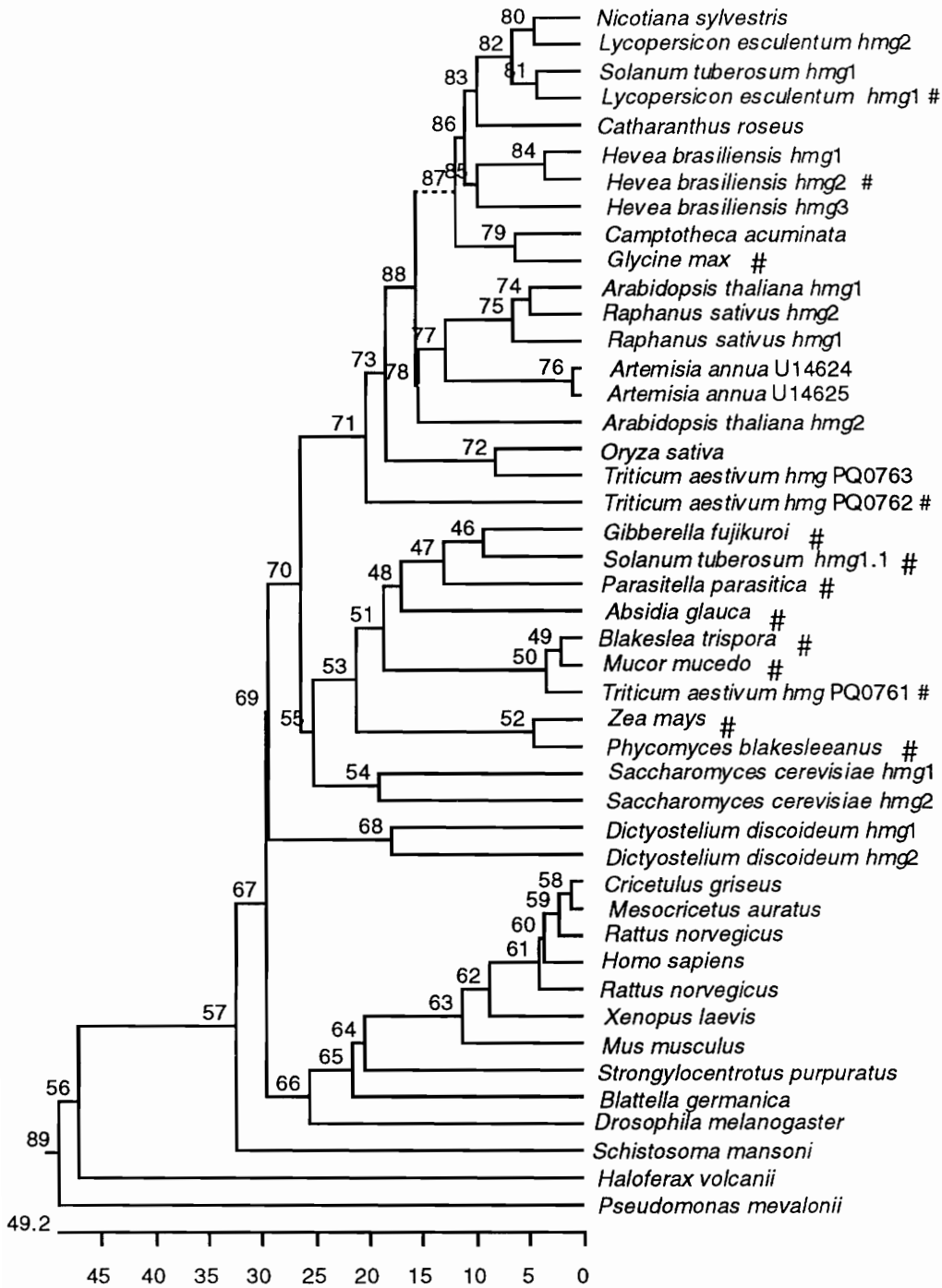
putative membrane-spanning regions in the N-terminal membrane domain in contrast to the enzyme from non-plant eukaryotes (mammals, insects, fungi) which have 7 to 8 transmembrane spans (Figure 1.2 and 1.3). In contrast to the catalytic domain which is highly conserved among plant HMGRs (81% to 97% amino acid identity in the carboxy-terminal 400 amino acid residues), the amino-terminal domain is quite divergent (54% to 85% identity over approximately 200 amino acid residues). However, within the amino-terminal domain, the short segments which actually span the membrane are highly conserved among plant HMGRs. The "linker region" (regions in between the amino-terminal transmembrane domains and the carboxy-terminal catalytic domains) seems only conserved with respect to the presence of different versions of a "PEST" sequence described for proteins having rapid turnover rates (Chye *et al.* 1992; Monfar *et al.* 1990).

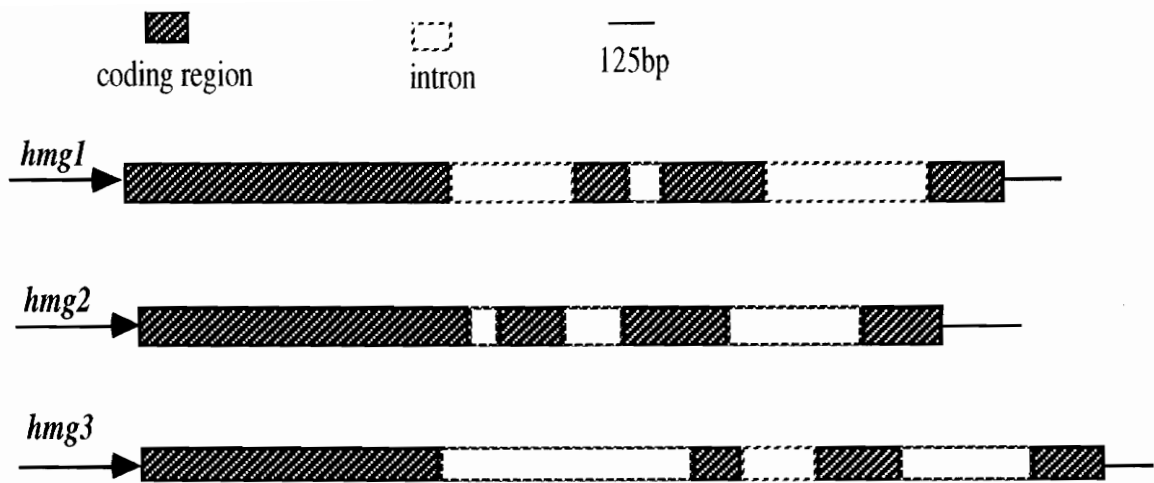
*Arabidopsis hmg1* is used here as an example to illustrate the structure of plant HMGRs (based on GenBank Accession: P14891). It is an endoplasmic reticulum located transmembrane protein. The deduced protein has 592 amino acid residues and has a calculated molecular mass of 63,597. Three domains were assigned: the membrane-bound domain amino acid residue one to 117; linker domain amino acid residue 118 to 171 and the catalytic domain amino acid residue 172 to 592. Within the membrane domain the first potential transmembrane region is amino acid residue 47 to 69 and the second potential transmembrane region is amino acid residue 83 to 117. The PEST sequence is located between amino acid residue 158 and 176, (PNPEPIVTESLPEEDEEIV) (Nelson *et al.* 1994).

Using tomato *hmg2* protein sequence as the query for the homology BLAST search program (Altschul *et al.* 1990), the results produced 42 high-scoring segment pairs. The

protein sequences for these 42 HMGRs were retrieved from the GenBank. A multiple alignment analysis was conducted with the HMGR sequences for soybean (Yu *et al.* 1995), maize (Moore 1995) and *Pseudomonas* (Beach and Rodwell 1989) and the retrieved 42 HMGRs [total 45 HMGR sequences from 34 different organisms (bacteria, fungi, plants, insects and animals)]. The partial results are shown in Figure 1.4. Plant HMGRs were clearly clustered indicating their close relationships based on analysis by DNASTAR Multialign clustal method. The yeast, insect, and mammalian HMGRs are remotely related to plant HMGRs. Bacterial HMGRs are related to each other as illustrated by HMGRs from *Pseudomonas mevalonii* (Beach and Rodwell 1989) and the archaebacterium *Haloferax volcanii* (Lam and Doolittle 1992). Twenty three of the total 45 known HMGRs were plant HMGRs which belong to thirteen different plant species. About a third of the 23 plant HMGRs were characterized at both genomic and cDNA level establishing the intron/exon structure. All of the plant *hmg* genes characterized have four exons with the three introns localized to similar positions (Figure 1.5). Direct confirmation of HMGR activity was demonstrated for only five (*Arabidopsis hmg1* and *hmg2*, radish *hmg1* and *hmg2*, tomato *hmg2*) of the 22 plant HMGR sequences by either yeast complementation of auxotroph mutants (Learned and Fink 1989; Vollack *et al.* 1994) or demonstration of HMGR activity of plant genes expressed in *E. coli* (Enjuto *et al.* 1994; Ferrer *et al.* 1990; Chapter 3).

**Figure 1.4.** Analysis of relationships of 45 HMGR protein sequences from 34 different organisms using Lasergene Multialign clustal method with PAM 250 residue weight table. Phylogenetic tree generated from the Multialign analysis is shown. # sequences represent partial coding sequences, generally within the catalytic domain.





**Figure 1.5.** Gene structures of tomato *hmg1*, *hmg2* and *hmg3* based on data from Park *et al* (1992) and Narita *et al.* (1991).

## HMGR in the *Solanaceae* and their function in defense

In the *Solanaceae*, HMGR is the first major rate limiting point in the production of sesquiterpenoid phytoalexins associated with disease resistance (Brooks and Watson 1991; Park *et al.* 1992; Stermer and Bostock 1987; Stermer *et al.* 1991). Accumulation of these compounds is more rapid and greater in incompatible interactions than in compatible interactions, supporting their role in disease resistance (Kuc 1982). Another class of terpenoid-derived compounds, the steroid glycoalkaloids are found or produced around sites of injury and also appear to be important in plant defense because of their toxicity to microbes as well as animals (Shih and Kuc 1973).

Progress has been made in understanding the regulatory role of HMGR in the biosynthesis of sesquiterpenoid phytoalexins. Yang *et al.* (1991) demonstrated that potato genes encoding HMGR were expressed in response to pathogen, elicitor, and wounding. One isogene of the *hmg* family is pathogen-activated and is distinct from isogene(s) that are wound activated. Defense-related increases in HMGR activity are due to mRNA level increases (Yang *et al.* 1991). The critical role of HMGR regulation in disease interactions was clearly demonstrated in potato:*Erwinia caratovora* interactions by Yang *et al.* (1991). Potato tubers in which HMGR activity was blocked by mevinolin, a specific inhibitor, were significantly more susceptible to soft rot; those pre-induced by elicitor treatment were more resistant. Other experiments further confirmed that specific HMGR isozymes encoded by specific *hmg* isogenes are important for different isoprenoid biosynthetic branches (Choi *et al.* 1992; Stermer *et al.* 1991).

Experiments with tobacco suspension culture also indicate HMGR is a critical

enzyme for regulation of phytoalexin biosynthesis. The sesquiterpenoid phytoalexins in tobacco are capsidiol (an extracellular sesquiterpene) and debneyol (Bailey *et al.* 1975). Treatment of tobacco cell suspension with fungal water-soluble cell wall fractions resulted in the induction of capsidiol production (Chappell *et al.* 1987). HMGR is transiently induced following elicitor treatment. HMGR activity had a sharp increase at 3 hours, peaking by 4 hours, and decreasing after 5 hours (Chappell *et al.* 1991). When suspension cultures were challenged with a fungal elicitor, squalene synthetase (Figure 1.1), the first committed enzyme for sterol biosynthesis, was suppressed. On the other hand, sesquiterpene cyclase, the first branch-pathway enzyme directly involved in the synthesis of sesquiterpenoid phytoalexins, was induced coordinately with HMGR (Vogeli and Chappell 1988). This suggests that defense responses involve not only activation of enzymes for phytoalexin synthesis but suppression of potentially competing isoprenoid branch pathways.

### **Tomato HMGR**

The tomato (*Lycopersicon esculentum*) genome contains at least four genes encoding HMGR isozymes which appear to be differentially regulated during development and defense responses (Cramer *et al.* 1993; Dean *et al.* 1991; Narita *et al.* 1991; Park 1990). Three of these genes (*hmg1*, *hmg2*, *hmg3*) have been analyzed at the molecular level (Figure 1.5). They are differentially expressed during fruit development, seedling growth, wounding, and defense responses. In tomato seedlings, *hmg* mRNA decreases transiently when dark-grown seedlings are transferred to continuous light (Dean *et al.* 1991). Both HMGR activity and *hmg1* mRNA levels are high during early fruit development when cells are undergoing division and expansion, but decrease as the green

fruit reach their mature size (Gillaspy *et al.* 1993). This suggests tomato HMG1 encoded by *hmg1* is important in sterol biosynthesis based on its correlation with rapid cell division. Ripening fruit have very low levels of reductase activity and mRNA, even though large amounts of the carotenoid lycopene are synthesized during this period. Inhibition of HMGR with mevinolin, a potent competitive inhibitor of HMGR, disrupts early tomato fruit development (Narita and Gruitsem 1989). Application of high levels of mevinolin to ripening fruit disks leads to loss of both photosynthetic carotenoids and *de novo* lycopene synthesis. These results suggest that tomato HMGR isozymes play a direct role in lycopene accumulation during chloroplast to chromoplast conversion (Narita *et al.* 1991). However, the specific HMGR isozyme involved in carotenogenesis has not been identified. The *hmg* mRNA that was rapidly induced in response to fungal-elicitor treatment or wounding was derived from the tomato *hmg2* isogene suggesting that *hmg2* functions as a defense-related isogene in tomato (Cramer *et al.* 1991; Park 1990). In our laboratory, tomato *hmg2* isogene was cloned, sequenced, and characterized with respect to expression and gene regulation. The tomato *hmg2* gene consists of 4 exons and 3 introns encoding 601 amino acid residues of the mature HMGR protein (Figure 1.3 and Figure 1.5). *hmg2* appears to be specifically associated with plant defense responses and is activated in response to wounding (Cramer *et al.* 1991; Park 1990). The broad expression of tomato *hmg1* suggests that it may encode a housekeeping form of HMGR, whereas inducibility of *hmg2* by diverse stresses might reflect its specialized role in the synthesis of specific defense isoprenoids.

### **Significance and objectives of this dissertation research**

The major role of sesquiterpenoid phytoalexins in defense responses of solanaceous plants, the excellent genetics studies with tomato (Rick 1984; Tanksley *et al.* 1987) and the



relative ease of genetically manipulating tomato through *Agrobacterium*-mediated transformation system lead us to choose tomato as a model system to study the plant *hmg* isogene family, gene structure, differential expression and function with an emphasis on the role of HMGR in plant disease resistance. It is of theoretical and practical importance to understand the function of different tomato *hmg* isogenes in development and in response to biotic and abiotic stresses. We are particularly interested in determining the role of HMGR in disease resistance. Based on previous results from Yang *et al.* (1991) and Park (1990), it is very likely that tomato *hmg2* is specific for defense functions. The research described in this dissertation was designed to directly test this hypothesis using a variety of approach facilitated by the efficiency of genetic engineering in tomato and tobacco. Transgenic plants altered for *hmg2* expression were used to assess tissue-specific expression of tomato *hmg2* and responses to two model pathogens, tobacco mosaic virus (TMV) and *Erwinia carotovora* subsp. *carotovora* (*Ecc*). TMV-tobacco interactions are well-studied, and involve genetically defined host-viral strain interactions leading to a classic HR-type resistance or the systemic mosaic disease phenotype. The TMV interaction also permits testing *hmg2* relations to the SAR response triggered by TMV. In contrast, *Ecc* is a broad host range bacterial pathogen for which no resistance genes have been identified and gene-for-gene type interactions are not evident (Barras *et al.* 1994). Based on Yang *et al.* (1991), sesquiterpenoid phytoalexins and HMGR activity are determinants important in the disease resistance to *Ecc*.

The overall organization of the research presented in this dissertation is as follows. Results in Chapter 2 utilize tomato *hmg2*:GUS reporter gene fusions to confirm the association of tomato HMG2 with wound-induction and defense specific to a broad array of pathogens. Based on this information and its presumed position as a major rate-limiting

step in phytoalexin production, we predict that genetic manipulation of the expression of tomato *hmg2* by either overexpression or antisense inhibition should impact disease resistance. Results in Chapter 3 (overexpression) and Chapter 4 (antisense inhibition) indicated that indeed engineered *hmg2* overexpressors were more disease resistant while antisense inhibition of tomato *hmg2* in the native tomato genetic system resulted in more disease susceptible plants.

The overall goal of this dissertation research was to study the factors involved in the environmentally and developmentally regulated *hmg2* isogene expression, assess the impact of altered *hmg2* expression on disease resistance and define a novel strategy for enhancing disease resistance. Specific objectives of the proposed research were:

- 1) to analyze tomato *hmg2* expression pattern by promoter:GUS reporter gene analysis.
- 2) to generate a full-length *hmg2* cDNA clone and demonstrate its ability to produce a functionally active HMGR by expressing the generated *hmg2* cDNA in *E. coli*.
- 3) to genetically engineer tobacco and tomato by overexpressing *hmg2* or using antisense *hmg1* and *hmg2* constructs to assess their impact on disease resistance.

The results from this research are quite significant. Overexpression of the defense specific tomato *hmg2* in a heterologous tobacco system yields tobacco plants significantly more resistant to two pathogens (virus and bacterium). Antisense *hmg2* tomato plants were more susceptible to pathogens. Expression of tomato *hmg1* or *hmg2* as antisense constructs in the heterologous tobacco system, while difficult to interpret, also suggested that *hmg*-transgenes impact overall defense responses. Engineering of important

solanaceous crops such as potato and tomato with *hmg2* constructs could contribute to the generation of better cultivars showing broad-spectrum increased resistance to multiple and diverse pathogens.

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**Chapter 2. Defense-related and tissue-specific expression of the tomato *hmg2* (3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR)) promoter in transgenic plants**

## Summary

3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR, EC 1.1.1.34) catalyzes the conversion of HMG-CoA to mevalonate, which is considered the first regulatory step in the biosynthesis of isoprenoids including sterols and phytoalexins. The tomato (*Lycopersicon esculentum*) genome contains at least four genes (*hmg*) encoding HMGR isozymes which appear to be differentially regulated during development and defense responses. In this study we report the expression pattern of tomato *hmg2* using the *hmg2* promoter fused to a  $\beta$ -glucuronidase (GUS) reporter gene. A 2.3 kb 5' upstream region of tomato *hmg2* was fused with the GUS coding sequence and transferred into tomato and tobacco via *Agrobacterium*-mediated transformation. Regulation of the tomato *hmg2* promoter is developmentally regulated and is induced in response to pathogen attack. *hmg2* is expressed in seedling cotyledons and hypocotyls, trichomes, and a variety of reproductive tissues including pollen, stigmas, ovules, sepals, petals and mature seeds of unstressed plants. *hmg2*:GUS activity was rapidly induced by wounding or in response to pathogenic viruses or bacteria. *hmg2*:GUS expression was localized to tissue surrounding lesions generated through interactions with either tobacco mosaic virus or the bacterial pathogen, *Erwinia carotovora* subsp. *carotovora*. The expression pattern of *hmg2* is therefore consistent with a central role in disease resistance and other stress responses.

## Introduction

Almost all biochemical and physiological processes in plant cells require the involvement of isoprenoids. Important isoprenoids include growth regulators (gibberellins, cytokinins and abscisic acid), phytoalexins, phytosterols, photosynthetic apparatus components (carotenoids, plastoquinone, and phytol chain of chlorophylls), mitochondrial electron transfer chain components (ubiquinone and heme of cytochrome oxidase), prenylproteins, terpenoid indole alkaloids and natural rubber (Bach *et al.* 1990). Mevalonic acid (mevalonate) serves as the precursor of all isoprenoid compounds. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) (EC 1.1.1.34) is the first key regulatory enzyme in this pathway catalyzing the reduction of HMG-CoA to mevalonate (Chapter 1, Figure 1.1). In vertebrates, HMGR is the rate-limiting enzyme in the biosynthesis of cholesterol (Goldstein and Brown 1990). Because of its critical role in isoprenoid biosynthesis, it is important to understand the function and regulation of plant HMGR.

The regulation of *hmg* gene expression has been extensively studied in mammalian systems due to its critical role in cholesterol biosynthesis (Goldstein and Brown 1990). *hmg* genes from yeast (*Saccharomyces cerevisiae*; Basson *et al.* 1988), *Dictyostelium* (De Lozanne 1993), fruit fly (*Drosophila*; Gertler *et al.* 1988), schistosomes (blood fluke; Rajkovic *et al.* 1989), cockroach (Martinez-Gonzalez *et al.* 1993), sea urchin (Woodward *et al.* 1988), frog (*Xenopus laevis*; Chen and Shapiro 1990), Chinese hamster (Chin *et al.* 1984), mouse (Helmberg *et al.* 1990), golden hamster (Skalnik and Simoni 1985), and human (Luskey and Stevens 1985) genomes have been cloned and characterized. Mammalian systems have one *hmg* gene. Yeast, *Dictyostelium*, and most fungal species (Burmester and Czempinski 1994) have two *hmg* genes. All of the above mentioned

HMGR presumably have 7 or 8 trans-membrane spans in the N-terminus (Olender and Simoni 1992; Roitelman *et al.* 1992). The C-terminus where the catalytic domain resides (about two-thirds of the polypeptide chain), shares a great deal of sequence homology among species (Roitelman *et al.* 1992).

HMGR has been considered a primary control point for isoprenoid metabolism in plants. Biochemical studies indicate that plant HMGR plays an important role in growth and development, in disease resistance, and in rubber production. HMGR activity has been correlated with isoprenoid biosynthesis (Bach *et al.* 1990). Plant growth and development can be arrested by mevinolin, a potent competitive inhibitor of HMGR (Bach *et al.* 1990). Activation of HMGR precedes and is necessary for the wound- and elicitor-induced accumulation of sesquiterpenoid phytoalexins and steroid glycoalkaloids in Solanaceous plants (Chappell *et al.* 1991; Choi *et al.* 1992; Stermer and Bostock 1987; Yang *et al.* 1991). The first *hmg* genes were cloned from *Arabidopsis* (Caelles *et al.* 1989; Campos *et al.* 1991; Learned and Fink 1989; Monfar *et al.* 1990) and tomato (Narita and Gruissem 1989; Park 1990; Park *et al.* 1992). Since then *hmg* gene studies have appeared in the following plants: rubber tree (Chye *et al.* 1992; Chye *et al.* 1991), potato (Bhattacharyya *et al.* 1991; Choi *et al.* 1992; Roxby *et al.* 1991; Stermer *et al.* 1991), tobacco (Genschik *et al.* 1992), pea (Monfar *et al.* 1990), radish (Ferrer *et al.* 1990; Vollack *et al.* 1994), wheat (Aoyagi *et al.* 1993), rice (Nelson *et al.* 1994), maize (Moore 1995), *Camptotheca* (Burnett *et al.* 1993; Maldonado-Mendoza *et al.* 1992); *Catharanthus* (Maldonado-Mendoza *et al.* 1992), *Artemisia annua* (Kang *et al.* 1995) and soybean (Yu *et al.* 1995). In all of the plants investigated, the cloned *hmg* genes share a high degree of amino acid sequence homology in the carboxy-terminal catalytic domain. Although many plant *hmg* genes have been cloned (twenty one plant HMGR protein sequences are available from the GenBank as

of April 1995), the expression pattern of individual *hmg* genes has been studied only in a limited number of plants (Burnett *et al.* 1993; Enjuto *et al.* 1995).

In the *Solanaceae*, HMGR also directs production of sesquiterpenoid phytoalexins associated with disease resistance (Jadhav *et al.* 1991; Park 1990; Stermer and Bostock 1987). Accumulation of these phytoalexins is more rapid and greater in incompatible interactions than in compatible interactions, supporting their role in disease resistance (Kuc 1982). Yang *et al.* (1991) and Choi *et al.* (1992) demonstrated that potato *hmg* isogenes were activated differentially by wounding or pathogen challenge. An *hmg* gene-specific probe was used to demonstrate that one isogene of the *hmg* family is pathogen-activated and is distinct from isogene(s) that is rapidly activated in response to wounding of potato tubers (Yang *et al.* 1991). Other experiments have confirmed that HMGR isozymes encoded by specific *hmg* isogenes are responsible for different isoprenoid biosynthetic branches (Choi *et al.* 1992). In potato tuber, while *hmg1*, 2, 3 are all induced by wounding, pathogen inoculation or elicitor treatment induces *hmg2* and *hmg3* while *hmg1* gene activity is suppressed (Choi *et al.* 1992).

The tomato *hmg* multigene family is composed of at least four genes. Three of them (*hmg1*, *hmg2*, *hmg3*) have been analyzed. They are differentially expressed during fruit development, seedling growth, wounding, and defense responses (Cramer *et al.* 1993; Dean *et al.* 1991; Gillaspay *et al.* 1993; Narita *et al.* 1991; Narita and Gruijssem 1989; Weissenborn *et al.* 1995). Both HMGR activity and *hmg1* mRNA levels are elevated during early fruit development when cells are undergoing division and expansion suggesting a role in phytosterol biosynthesis (Gillaspay *et al.* 1993). In order to study the role of HMGR in sesquiterpenoid phytoalexin biosynthesis and disease resistance, we have

cloned and characterized the tomato *hmg2* gene from a tomato (cv. VFNT cherry) genomic library (Park *et al.* 1992). This gene is different from the tomato *hmg1* isolated by Narita and Grissem (1989) from a cDNA library from immature fruit RNA. Northern analysis showed activation of *hmg2* by wounding and elicitor treatment suggesting that *hmg2* is associated with plant defense responses (Park 1990). *hmg2* mRNAs are not detected in rapidly dividing tissues, or in tomato cells or fruit during carotenogenesis (Park, Denbow and Cramer, unpublished results). Thus, tomato *hmg2* is unlikely to function in sterol biosynthesis or carotenoid pigment formation. These results suggest that distinct *hmg* isogenes are involved in these developmental responses.

To further analyze the expression pattern of *hmg2* and factors regulating *hmg2* gene expression, we have generated transgenic tomato and tobacco plants containing the tomato *hmg2* promoter fused with the  $\beta$ -glucuronidase (GUS) reporter gene. Promoter:GUS fusions provide a powerful tool for analysis of tissue specificity which in turn provides insights into potential functional roles. Analogous approaches have been used in studying *Arabidopsis hmg2* (Enjuto *et al.* 1995) expression and *Camptotheca acuminata hmg* expression (Burnett *et al.* 1993). *Arabidopsis hmg2* is expressed primarily in floral tissues and is not wound-inducible. The *Camptotheca acuminata hmg* was studied primarily for its potential role in synthesis of the anti-cancer monoterpene indole alkaloid camptothecin (CPT). The *C. acuminata hmg* is induced by wounding but is suppressed by methyl jasmonate treatment (Burnett *et al.* 1993). In this report we study the expression of *hmg2* during plant development and defense responses. Our analysis showed very limited general developmental expression of tomato *hmg2* consistent with its involvement in production of highly specialized isoprenoids. In addition, results from *hmg2*:GUS-expressing plants have demonstrated tomato *hmg2* involvement in plant reactions to pathogenic agents such

as TMV and *Erwinia carotovora* subsp. *carotovora*. These results indicate that *hmg2* is indeed a defense related *hmg* isogene and represents a novel type of *hmg* expression (Weissenborn *et al.* 1995).

## Results

### *Generation of hmg2 promoter:GUS constructs and transformation into tomato and tobacco plants*

The tomato isogene *hmg2* has been most strongly identified with defense responses (Weissenborn *et al.* 1995). In order to monitor *hmg2*-specific gene expression, DNA constructs fusing the *hmg2* promoter to the GUS reporter gene were generated and used to transform tobacco and tomato. A 2.3 kb *EcoRI-BglII* fragment of the *hmg2* promoter region from the tomato genomic clone pTH295 (Park *et al.* 1992) was ligated to the GUS reporter gene (*uidA*) in the binary vector pRK290 to yield plasmid pSLJ 330.1 (Figure 2.1; provided by J. Jones, Sainsbury Lab, Norwich, UK). The *hmg2* promoter fragment contained both the *hmg2* transcription and translation start sites (Park *et al.* 1992) and fusion with the *uidA* from pRK290 provided an additional seven amino acids at the N-terminus of GUS from *hmg2* start codon ATG to the *BglII* site (MDVRRRS). The plasmid, pSLJ330.1, was mobilized into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating (Herrera-Estrella and Simpson 1988). This bacterium was then used to transform tobacco (*Nicotiana tabacum* cvs. Xanthi nc and NC-95) and tomato (*Lycopersicon esculentum* cvs. Gardener and Vendor) by leaf disc co-cultivation (Burow *et al.* 1990; McCormick *et al.* 1986). Seven transgenic tomato plants (generated by Dr. Weissenborn) and twenty transgenic tobacco plants (fifteen Xanthi nc derived and five NC-

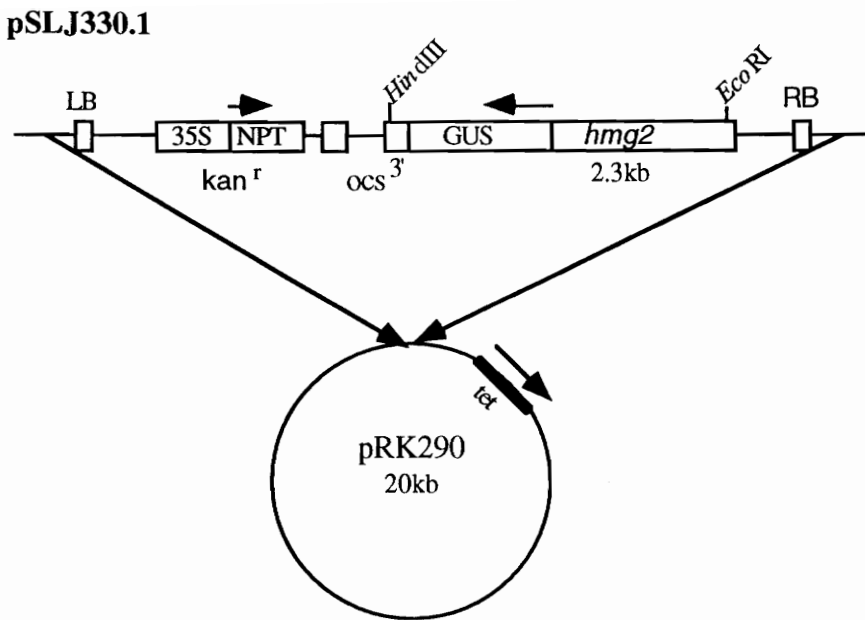


95 derived independent transformants) were independently isolated. Most of these transgenic plants contained more than one copy of the transgene based on Southern hybridization and/or R1 generation seedling segregation analyses.

Four representative Xanthi nc tobacco lines (001, 002, 007 and 008), two NC-95 lines (101, 104), one tomato Gardener line (G330.1 B) and one tomato Vendor line (V330.1) were used in the histochemical analysis. Plant 002 had two head to head copies of the transgene inserted at one locus based on Southern analysis (see Appendix Figure A.1) and seedling segregation analysis. Although variations in GUS staining intensity were observed among different transformants, the qualitative staining pattern which reflected the *hmg2* expression pattern was consistent. Transgenic tobacco plants expressing a 35S:GUS construct were also developed to serve as controls for the histochemical analysis described below.

#### *Tissue specificity of hmg2 expression in unstressed plants*

To study the tissue-specific expression pattern of *hmg2*, different types of tissue from unstressed transgenic tobacco and tomato lines at representative developmental stages were stained histochemically for GUS activity using the chromogenic substrate, 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). In tobacco seedlings, *hmg2*:GUS was strongly expressed in the cotyledons and to a lesser extent in hypocotyl tissues, but was generally not expressed in the roots, although occasional faint staining was observed at the root tip (Figure 2.2 A). Analyses of six different transformants from the two tobacco cultivars, Xanthi nc and NC-95, showed similar *hmg2*:GUS expression in young seedlings. In contrast, 35S:GUS control seedlings showed strong GUS activity throughout the seedling



**Figure 2.1.** pSLJ 330.1 construction. The chimeric gene construct pSLJ330.1 was constructed by inserting a 2.3 kb fragment of the tomato *hmg2* promoter upstream of the *uidA* (GUS) gene from binary vector pRK290. The *hmg2* fragment contained the *hmg2* start codon and sequences encoding seven additional amino acids and was fused in frame with the *uidA* coding sequence. The insert is approximately 10 kb in size [between left border (LB) and right border (RB)]. 35S is the CaMV 35S promoter; NPT is the neomycin phosphotransferase II (kanamycin resistance) gene; tet is the tetracycline resistance gene. OCS is the octopine synthase terminator (Herrera-Estrella and Simpson 1988).

tissues indicating constitutive expression (Figure 2.2B). Four independent transformants using the 35S promoter gave similar results. The expression of 35S:GUS in cotyledons was lower as compared with *hmg2*:GUS while in the root portion 35S:GUS was much higher than the *hmg2*:GUS. The expression pattern in tomato seedlings was the same as in the tobacco seedlings.

Expression of *hmg2* was observed in young vegetative tissues as well as in seedlings and floral tissues. Figure 2.2C shows a leaflet from a tomato plant cv. Gardener where *hmg2*:GUS is expressed throughout the leaflet with highest activity in the vascular system. It was also very strongly expressed in response to wounding as demonstrated by staining adjacent to the two pipet tip wounds on the lower sides of the leaflet. Some trichomes on leaf, stem and floral tissues showed intense blue staining. However, staining patterns of trichomes was not uniform suggesting extreme sensitivity of trichomes to touch/wounding.

*hmg2* was active in a variety of floral organs and its pattern of expression varied somewhat in tomato versus tobacco. *hmg2*:GUS expression was observed around the edges of tomato flower petals (Figure 2.2D), in trichomes on tobacco flower sepals (Figure 2.2E). Different patterns of tissue-specific *hmg2*:GUS expression were observed between tomato and tobacco anthers. Thus *hmg2*:GUS was not expressed in tobacco anther (Figure 2.2F) while it was strongly expressed in tomato anthers (Figure 2.2G). Both tobacco (Figure 2.2F) and tomato showed *hmg2*:GUS expression in the stigma of the pistils. Cross-sections of ovaries in both tomato and tobacco (Figure 2.2H) showed very strong expression in ovules. Staining could also be seen at the edge of the ovary wall, however, we could not rule out the possibility of wound-induced expression during the cutting.

*hmg2* expression was also observed in pollen; roughly about 50% of pollen grains from *hmg2*:GUS tobacco plant 002 were stained blue as one would expect from segregating of gametes (Weissenborn and Cramer, personal communication). Expression of *hmg2* was not found in tobacco anthers, the midrib of carpels, the outside of the ovary, the filaments of stamen, or seed coats. Analysis of histochemically stained tissues that required dissection or significant manipulation of tissues were hindered by strong *hmg2* expression triggered by wounding (see below). Thus, it is possible that additional tissue-specific patterns exist, especially in floral and fruit tissues where penetration of substrate often requires tissue dissection. Additional studies using rapid tissue fixation techniques may facilitate detection of developmental patterns in the absence of wound responses.

#### *Defense-specific expression of tomato hmg2 promoter:GUS fusion*

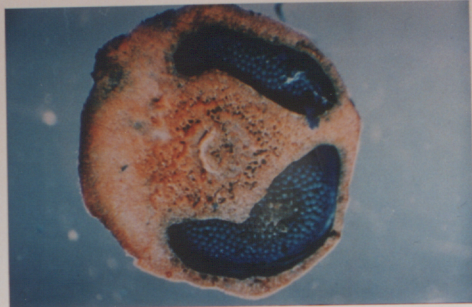
Previous mRNA expression data suggested that *hmg2* is associated with defense-related functions based on induction by wounding and elicitor treatment (Park 1990). To further understand how *hmg2* responds to different pathogen challenges, we studied the responses of *hmg2*:GUS transgenic plants to wounding and to infection by several pathogens.

*hmg2* promoter was very responsive to various types of wounding. We observed the blue staining at the cut edge of leaf, stem, leaf petiole, root, anther (Figure 2.3A) and ovary. Figure 2.3A shows a typical wound induced expression. After cutting one end of a tobacco anther, the originally non-expressing anther tissues shown in Figure 2.2F became highly induced. Figure 2.2C shows expression around pipet tip wounds on a tomato leaflet. Strong wound-induction was observed in response to both excision wounding

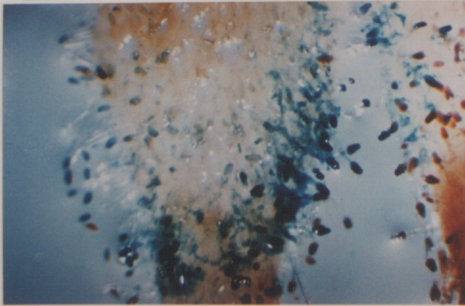
**Figure 2.2.** Tissue specific expression of *hmg2*:GUS. Tissues from transgenic tobacco and tomato plants containing tomato *hmg2*:GUS or 35S:GUS (as controls) were analyzed histochemically using X-Gluc. A. R1 seedlings of Xanthi nc line 002 containing *hmg2*:GUS seven days postimbibition. B. R1 seedling of Xanthi nc tobacco containing 35S:GUS. C. Young tomato leaflet with two pipet tip wounds on either side of a leaflet. D. Petals of tomato flower with *hmg2*:GUS. E. Sepal of tobacco flower with *hmg2*:GUS showing expression in the trichomes. F. Stamen and pistil of tobacco flower with *hmg2*:GUS. G. Stamen of tomato flower with *hmg2*:GUS. H. Ovary cross-section from tobacco flower.



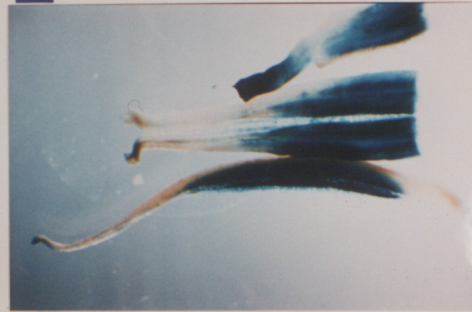
F



H



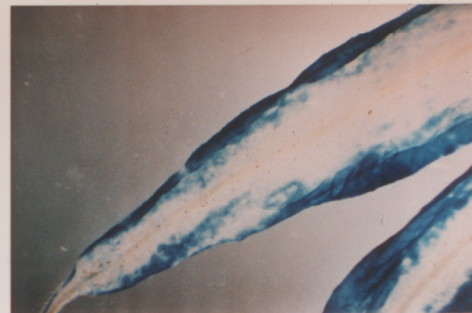
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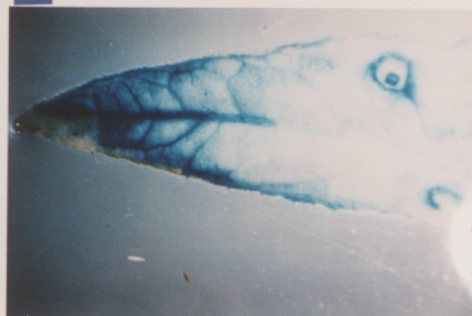
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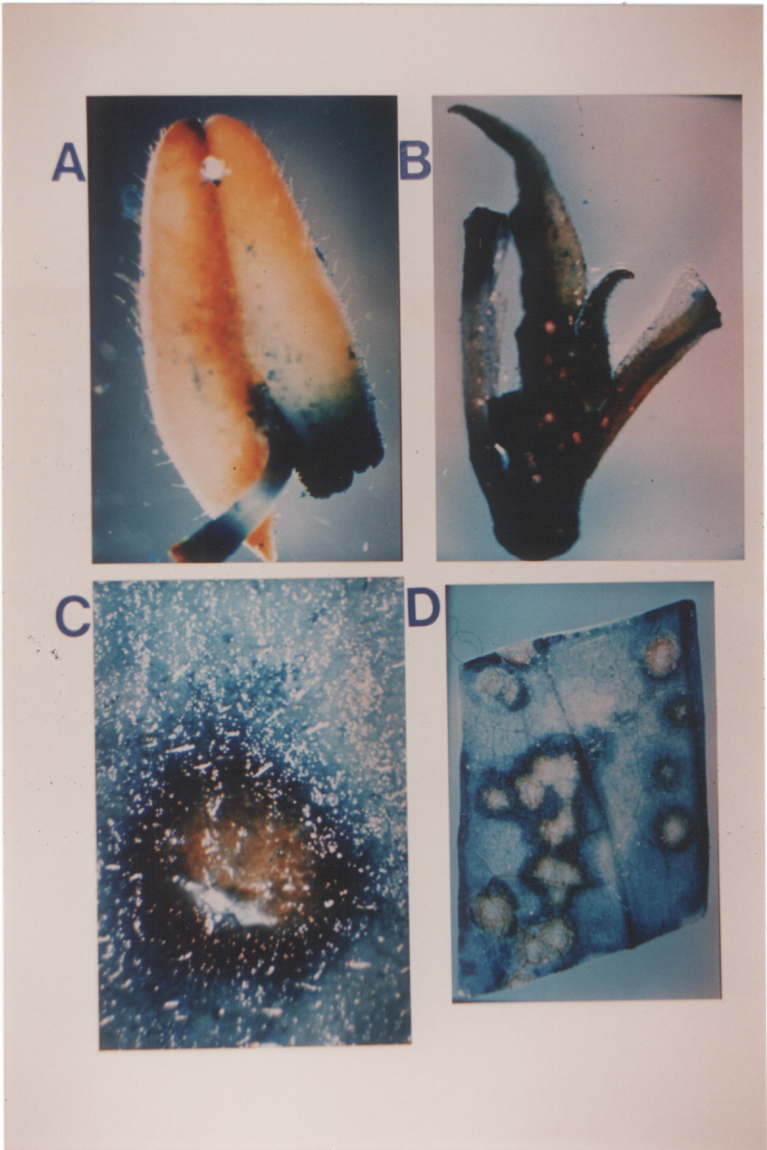
(*e.g.*, razor blade slice) and compression (*e.g.*, forceps press injury). Therefore, we can conclude that *hmg2* is a strong wound responding isogene.

Expression pattern of *hmg2*:GUS in response to bacterial pathogen was also tested. Tobacco leaf petioles and stem sections were incubated with *Erwinia carotovora* subsp. *carotovora* (*Ecc*), a broad-host range soft-rot bacterium for 16 hours. Maceration was observed at the ends of petioles and stem sections. *hmg2*:GUS expression was also observed at the same places suggesting that *Ecc* infection activated *hmg2* expression (Figure 2.3B). When a piece of detached tobacco leaf was inoculated with 5  $\mu$ l of *Ecc* strain EC14 ( $A_{600nm} = 1.0$ ; ca.  $4 \times 10^6$  cfu), gently wounded by pressing with a pipet tip and then incubated at 28°C for about 36 hours, a transparent lesion gradually appeared corresponding to the maceration. *hmg2*:GUS expression was observed in the tissue directly surrounding the disease lesion (Figure 2.3C). The GUS activity was significantly greater and more tightly localized (*i.e.* at lesion border) around the *Ecc* inoculations than in the mock inoculated controls.

Tobacco cultivar Xanthi nc is resistant to TMV and generates a necrotic lesion (hypersensitive reaction) in response to TMV inoculation. It was shown that *hmg2*:GUS was highly expressed around the lesion (Figure 2.3D) and that the area of *hmg2*:GUS expression on TMV infected NC-95 leaves corresponded to the mosaic pattern caused by the virus (more or less a lighter green area, Weissenborn and Cramer, personal communication). Taken together, *hmg2* expression was induced by wounding, viral, and bacterial challenges.

**Figure 2.3.** *hmg2*:GUS expression is activated by wounding and pathogen inoculation. A. Wound-inducible expression as seen in a tobacco anther wounded at one end with razor blade. B. Defense response visualized by X-Gluc staining of tobacco leaf petioles and stem inoculated with 300  $\mu$ l of *Erwinia carotovora* subsp. *carotovora* (*Ecc*) strain EC14 ( $A_{600\text{nm}} = 1.0$ ; ca.  $2 \times 10^8$  cfu) for 16 hours. C. Detached tobacco leaf inoculated with 5  $\mu$ l of *Ecc* strain EC14 ( $A_{600\text{nm}} = 1.0$ ; ca.  $4 \times 10^6$  cfu) and incubated for 36 hours. One fully developed *Ecc* lesion about two millimeter in diameter is shown. D. *hmg2*:GUS expression around the necrotic lesions of TMV on a *hmg2*:GUS leaf. 50  $\mu$ g/ml purified TMV strain U1 was inoculated onto the leaf. After four days, one to two  $\text{cm}^2$  leaf pieces were stained for GUS activity. TMV lesion sizes are around one to two millimeter in diameters.





## Discussion

### *hmg2 shows a novel pattern of tissue-specific expression*

Recent molecular studies have yielded significant new information on mechanisms involved in plant-microbe interactions, pathogen recognition, and defense gene activation and have provided novel strategies for engineering enhanced disease resistance. In this study, we have analyzed the defense-related regulation of tomato *hmg2*, considered the rate limiting enzyme mediating production of isoprenoid defense compounds.

In *Camptotheca acuminata*, a Chinese tree that produces the anti-cancer monoterpenoid indole alkaloid camptothecin (CPT), *hmg1* is highly expressed in young seedlings but not in vegetative organs of older plants (Burnett *et al.* 1993). In vegetative tissues of *Camptotheca*, GUS staining was localized to the epidermis of young leaves and stems, particularly in glandular trichomes. Roots showed intense staining in the cortical tissues in the elongation zone and light staining in the cortex of mature roots. *hmg1*:GUS expression was also observed in sepals, petals, pistils, and stamens of developing flowers, with darkest staining in the ovary wall, ovules, stigmas, and pollen. The developmental and spatial expression pattern of tomato *hmg2* is similar to some extent, but in most part is different from the *Camptotheca hmg1*, suggesting that tomato *hmg2* serves a different role in isoprenoid metabolism.

*Arabidopsis thaliana hmg2* expression is restricted to meristematic (root tip and shoot apex) and floral tissues (secretory zone of the stigma, mature pollen grains, gynoecium vascular tissue, and fertilized ovules; Enjuto *et al.* 1995). It is not induced by

wounding. The floral localization of *Arabidopsis thaliana hmg2* activity is very similar to tomato *hmg2* suggesting a common controlling element(s) and possible analogous biological functions during floral development. However, strong activation of tomato *hmg2* to wounding and to multiple pathogen challenges also suggests a unique role in defense responses. The major phytoalexins in *Arabidopsis* are thought to be of the polyacetylene class and not terpenoids (Darvill and Albersheim 1984). These results suggest that *Arabidopsis hmg2* and tomato *hmg2* may be functional homologues with respect to HMGR isozyme function in floral tissues and development. However, tomato *hmg2* has evolved (or *Arabidopsis* lost) an additional function associated with host defense. This predicts that the pathogen response and tissue-specificity elements within the promoter are distinct. Initial promoter dissection of the tomato *hmg2* promoter indicates that the promoter is quite complex with multiple enhancers and at least one silencer element (Weissenborn and Cramer, unpublished results). Consistent with our prediction of distinct developmental and defense *cis* elements, one truncated *hmg2* promoter construct retains pollen expression but has lost both wound and pathogen (*Ecc*) inducibility (Weissenborn and Cramer, unpublished results).

#### *hmg2 is a defense-specific isogene*

Phytoalexins are an important line of defense during plant-microbe interactions. The *Solanaceae* plants use sesquiterpenoid phytoalexins (phytuberol, phytuberin, rishitin, and capsidiol in potato (Stolle *et al.* 1988)) in their defense. HMGR catalyzes the first rate limiting step in the pathway. Detailed analyses in this study revealed the defense specific regulation of the *hmg2* promoter. The *hmg2* gene of tomato is expressed in response to wounding, bacterial, fungal, and viral infection, and establishment of feeding sites by root-

knot nematodes (Cramer *et al.* 1993; Weissenborn *et al.* 1995). Therefore, our analyses of *hmg2*:GUS reporter gene expression in transgenic tobacco and tomatoes indicate that tomato *hmg2* is a defense specific isogene that responds to wide variety of pathogenic agents.

The patterns of developmental expression of tomato *hmg2* may also suggest its association with defense. For example, it was found that *hmg2* was expressed in the pollen, stigma of pistils, trichomes, cotyledons and at the soil/air interface of seedlings (the hypocotyl), site of lateral root initiation in the root. All of these sites are places of potential pathogen attack or predation. Trichomes are organs clearly associated with defense in tomato and tobacco. Defense related PAL (phenylalanine ammonia-lyase) and pathogenesis protein promoter analysis showed similar expression pattern (Liang *et al.* 1989; Uknes *et al.* 1993).

Our hypothesis is that the predominant role of isogene *hmg2* may be associated with production of defense compounds and its tissue specificity may reflect a "developmental defense system". One test of this hypothesis would be to specifically block *hmg2* expression and analyze effects on disease resistance. Inhibitors (mevinolin) are non-specific with isozymes, therefore isogene-specific antisense strategy may be the most effective approach.

## **Experimental procedures**

### *Plant material and plant transformation*

Tomato cvs. Gardner and Vendor and tobacco cvs. Xanthi nc and NC-95 were used in these experiment. The tobacco cultivar Xanthi nc is tobacco mosaic virus (TMV) resistant while NC-95 is TMV susceptible. The responses of *hmg2* to TMV in these two cultivars will facilitate the understanding of defense related gene expression during compatible and incompatible host-pathogen interaction. Tobacco and tomato plants used for transformation were grown on full strength Murashige-Skoog media (Murashige and Skoog 1962) in sterile Magenta boxes. Transformation was by the method of leaf disc co-cultivation (Burow *et al.* 1990; McCormick *et al.* 1986).

*Plant DNA isolation, Southern hybridizations and determination of transgene copy numbers*

Genomic DNA was isolated from transgenic tobacco line 002 R0 and R1 generation using a modified CTAB procedure (Dellaporta *et al.* 1984; Rogers and Bendich 1988). Approximately 3 grams of leaf tissue was freeze-dried and ground to a fine powder with a mortar and pestle in liquid nitrogen. The powder was transferred to a 50 ml centrifuge tube which contained 15 ml of 65°C prewarmed extraction buffer (1% CTAB, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 0.7M NaCl, 0.1% β-mercaptoethanol). The mixture was incubated at 65°C for two hours and was extracted with equal volumes of chloroform/isoamyl alcohol (24:1). After treating the aqueous phase with twenty µl of ten mg/ml RNase A for 30 minutes at room temperature, an equal volume of isopropyl alcohol was added to the aqueous phase and the precipitated DNA was hooked out and washed overnight with 70% ethanol. The pellet was resuspended in 0.7 ml of TE (50 mM Tris/HCl, 10 mM EDTA, pH 8.0) and transferred to a microfuge tube. After precipitating the DNA with 0.3M sodium acetate, two volumes of 100% ethanol and washing with 70%

ethanol, the DNA was resuspended in TE (10 mM Tris-HCl, 1 mM EDTA pH 8.0). DNA concentration was measured by using a DU640 spectrophotometer (Beckman, CA).

For Southern hybridization analyses, Analysis: 12 to 20 µg of genomic DNA were digested with *EcoRI* or *HindIII* overnight at 37°C. The DNA samples were electrophoresed in a 0.8% agarose gel using TAE buffer and capillary transferred to Nytran membrane (Schleicher & Schuell, NH) following the manufacturer's instructions. GUS fragment was gel purified from *SmaI* and *SacI* digested pBI121 (Clontech, CA) for use as hybridization probe. Probe GUS DNA was radioactively labeled with <sup>32</sup>P-dCTP (NEN, Du Pont) by random-primed labeling following the manufacturer's instructions (GIBCOBRL, MD). The blot was UV crosslinked (Bioslink, Bios Corporation, CT). Hybridization and washing was at 65°C (Two 30-minute washes in 2X SSPE/1%SDS at room temperature and final wash with 0.1X SSPE/1%SDS at 65°C) with a hybridization incubator (Model 310 Robbins Scientific, CA). X-ray film was exposed to the blot for 72 hours at -70°C using intensifying screens.

Transgene copy numbers were determined either by counting Southern bands digested with different restriction enzymes probed with GUS or by R1 generation seedling segregation analysis stained with X-Gluc or plated on 150 µg/ml kanamycin containing MS plates. Chi square analysis was used to determine the significance of segregation data.

#### *GUS assay*

GUS expression was histochemically localized following three times one minute vacuum infiltration by incubating tissues in the chromogenic substrate X-Gluc for 12 hours

at 37°C as described (Jefferson 1987; Jefferson *et al.* 1987). Stained tissues were bleached with 95% ethanol for several hours and pictures were taken with a Leitz photomicroscope which has Leica cameras for both 35 mm and 4x5 photomicroscopy.

*Tobacco mosaic virus and Erwinia carotovora subsp. carotovora inoculation*

TMV (strain U1) was purified by the method previously described (Asselin and Zaitlin 1978) [provided by Ms. Elizabeth Schwartz and Dr. Sue A. Tolin (VPI&SU)]. Leaves of about the same age and position on the selected transgenic tobacco plants (the top three fully expanded leaves) were inoculated with 50 µg/ml purified TMV [The inoculation solution was 1% K<sub>2</sub>HPO<sub>4</sub> with 1% w/v Celite 545 (Fisher Sci. Com. Fair Lawn, NJ) as an abrasive] with a cotton swab dipped in inoculum (Trevathan *et al.* 1982). After four days, the region where TMV lesions were developed was cut out and GUS activity was assayed. The soft rot pathogen, *Erwinia carotovora* subsp. *carotovora*. (*Ecc*) strain EC14 (provided by Dr. G. H. Lacy, VPI&SU) was cultured in LB medium as described (Yang *et al.* 1989). For maceration assays, tobacco stem sections and leaf petioles were excised, weighed, and placed in a test tube with 300 µl ( $A_{600\text{nm}} = 1.0$ ; ca.  $2 \times 10^8$  cfu) of *Ecc* suspension. Following incubation at 28°C for 16 hours, the sections were rinsed prior to exposure to X-Gluc. An *Ecc* suspension was introduced to the detached tobacco leaves by gently wounding the leaf with a pipet tip while introducing 5 µl of *Ecc* strain EC14 ( $A_{600\text{nm}} = 1.0$ ; ca.  $4 \times 10^6$  cfu). The leaves were then incubated in a moist chamber at 28°C to let the lesion fully developed (36hr to 48 hr). Infected tissues were then assayed for GUS activity with X-Gluc.

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**Chapter 3. Tomato *hmg2* overexpression in tobacco affects resistance to bacterial soft rot and tobacco mosaic virus diseases**

## ABSTRACT

3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR) is a key regulatory enzyme in the biosynthesis of isoprenoids including sterols, growth regulators, and defense-related phytoalexins. The tomato genome contains at least four *hmg* isogenes encoding HMGR isozymes. Among them, *hmg2* appears to be specifically associated with plant defense responses and is activated in response to wounding, fungal elicitors, and pathogen challenge. In this study we report the effect on disease resistance of constitutive expression of tomato *hmg2* in transgenic tobacco. Tomato *hmg2* cDNA was cloned by PCR, expressed in *E. coli* to confirm its HMGR activity, inserted behind the double enhanced CaMV 35S promoter, and engineered into tobacco (*Nicotiana tabacum* cv. Xanthi nc). Southern and northern analyses confirmed transformation and *hmg2* mRNA expression. Enzyme activity was elevated compared to nontransformed plants. Analysis of selected transgenic plants revealed significantly reduced tissue maceration by *Erwinia carotovora* subsp. *carotovora* (*Ecc*) assessed by *in vitro* leaf petiole and stem section maceration assays. The mean size of necrotic lesions induced by TMV was also significantly reduced compared to the nontransformed or vector controls. Thus, genetic manipulation of the rate-limiting step in a major defense pathway provides an effective strategy for enhancing disease resistance.

*Additional keywords:* HMGR, overexpression, disease resistance, tomato *Lycopersicon esculentum*, tobacco *Nicotiana tabacum* cv. Xanthi nc, *Erwinia carotovora* subsp. *carotovora*, tobacco mosaic virus (TMV).

## INTRODUCTION

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC 1.1.1.34) is a key regulatory enzyme involved in isoprenoid pathways leading to compounds critical for membrane structure, electron transport, photosynthesis, protein modification, protection against biological and physical stresses, and growth and differentiation. Specific isoprenoid compounds are synthesized depending on developmental and environmental cues. Differential regulation of multiple forms of HMGR encoded by a *hmg* multigene family and associated with several subcellular compartments has been described (Bach 1987; Bach *et al.* 1991). Therefore, study of plant HMGR regulation will increase our understanding of many critical cellular processes as well as differential regulation of multigene families.

In the *Solanaceae*, the predominant defense-related phytoalexins (sesquiterpenoid phytoalexins) are synthesized via the isoprenoid pathway. The tomato (*Lycopersicon esculentum* Mill.) genome contains at least four genes encoding HMGR isozymes which appear to be differentially regulated during development and defense responses (Gillaspy *et al.* 1993; Weissenborn *et al.* 1995). In order to study the role of HMGR in isoprenoid phytoalexin biosynthesis and disease resistance, we cloned and characterized the tomato *hmg2* gene which is specifically expressed at a high level, primarily during host defense response (see Chapter 2; Park *et al.* 1992; Yang *et al.* 1991). Based on expression patterns of *hmg2* promoter-GUS fusions in transgenic tobacco, we found that *hmg2* was activated by wounding and a broad array of microbial pathogens including bacteria, fungi, viruses, and root-knot nematodes (Cramer *et al.* 1993)(Cramer *et al.* 1993; Weissenborn *et al.* 1995; Chapter 2). The expression pattern of *hmg2* is consistent with a role in sesquiterpenoid phytoalexin biosynthesis and disease resistance (Chapter 2; Weissenborn *et al.* 1995).

Genetic engineering approaches involving overexpression of a specific gene have been used to study the function of a particular isogene from a multigene family. In order to evaluate the role of HMGR in plant defense response and to determine whether manipulation of *hmg* expression can be used to generate plants with enhanced disease resistance, we attempted to modify the pattern of tomato *hmg2* expression in transgenic plants by overexpression of *hmg2*. Since the timing of the defense response is important in determining the final outcome of the interaction between host and pathogen (Bell *et al.* 1986) we eliminated this factor in *hmg2* gene expression by expressing *hmg2* constitutively. Chappell *et al.* (1991) engineered tobacco (*Nicotiana tabacum* L.) plants for high level, constitutive expression of the hamster *hmg* (35S:*hmg*). Transgenic plants had 3- to 10-fold higher total HMGR activities than control plants and consequently increased sterol accumulation 3- to 5-fold. Although normal sterol end products such as sitosterol, campesterol, and stigmasterol were increased only 2-fold, cycloartenol, a sterol biosynthetic intermediate, was increased 10- to 100-fold. In contrast, the level of other isoprenoids (sesquiterpene phytoalexins and carotenoids) remained relatively unaltered in the transgenic plants. These results suggest that either HMGR is a controlling step for a subset of sterols but is not a rate determining step for overall isoprenoid metabolism or there may be organizational channels for isoprenoid biosynthetic enzymes dedicated to distinct branch pathways or particular isoprenoid end-products (Chappell *et al.* 1991a). Stringent control of branch pathway enzymes, subcellular compartmentalization, or protein-protein interactions may be important in controlling the flux of isoprenoid pathway intermediates. Overexpression of hamster *hmg* increased tobacco resistance to insects and, possibly, to environmental stress (Chappell 1991). The next obvious application would be crop improvement through *hmg* isogene manipulation.

Taking a similar approach, we introduced a chimeric 35S:*hmg2* gene into tobacco plants to produce high constitutive levels of tomato HMG2 . By overexpressing tomato *hmg2*, which is specifically associated with disease resistance (*i.e.*, *hmg2* may be the isogene that is rate-limiting specifically in phytoalexin biosynthesis, Chapter 2), we hypothesized that constitutive expression of *hmg2* will facilitate more rapid accumulation of isoprenoid defense compounds upon pathogen induced activation of the pathway. This hypothesis suggests that transgenic plants showing elevated and/or constitutive expression of *hmg2* will be more disease resistant. An analogous strategy, *i.e.*, manipulation of rate-limiting step in complex defense pathway, was attempted for the isoflavonoid phytoalexin pathway. However, overexpression of genes for phenylalanine ammonia-lyase (PAL) or chalcone synthase (CHS) led instead to transgene co-suppression and reduction in pathway end products (Elkind *et al.* 1990; Napoli *et al.* 1990). We have successfully overproduced the tomato HMG2 isozyme in tobacco and tested these 35S:*hmg2* tobacco plants for disease resistance to the phytopathogens, tobacco mosaic virus (TMV) and the soft-rotting bacterium, *Erwinia carotovora* subsp. *carotovora* (Jones) Bergey *et al.* (*Ecc*) .

*Erwinia carotovora* subsp. *carotovora* (*Ecc*), one of the most important bacterial phytopathogens, causes hand rot and hollow stalk diseases of tobacco (*Nicotiana tabacum* L.), soft rot of potato (*Solanum tuberosum* L.) tubers and tomato fruit, and black leg of potato stems in the field. In fact, *Ecc* is capable of rotting succulent tissues of almost any dicotyledonous and monocotyledonous plant. It probably has the widest host range of any pathogen (Yang 1990). Affected tissues show reduced turgidity, increased electrolyte leakage, and, eventually, become soft. No chemical nor genetic methods are known to provide efficient protection against soft rot bacterial infection. Wild *Solanum* spp. which

cannot be sexually crossed with potato cultivars have been reported as sources of genetic resistance against *Erwinia* soft rot (Austin *et al.* 1988). Previous studies showed that in potato, HMGR mRNA levels were greatly elevated in tuber slices inoculated with *Ecc* (Yang *et al.* 1991). By overexpressing tomato *hmg2* in transgenic tobacco plants we attempted to develop a model to improve resistance against this phytopathogenic bacterium in solanaceous crops.

Our results indicate that plant HMGR plays a major role in the general resistance of plants and support the hypothesis that specific *hmg* isozymes control synthesis of particular subsets of isoprenoid products. These results further demonstrate that manipulation of a major rate-limiting step in an important defense pathway significantly impacts disease resistance.

## RESULTS

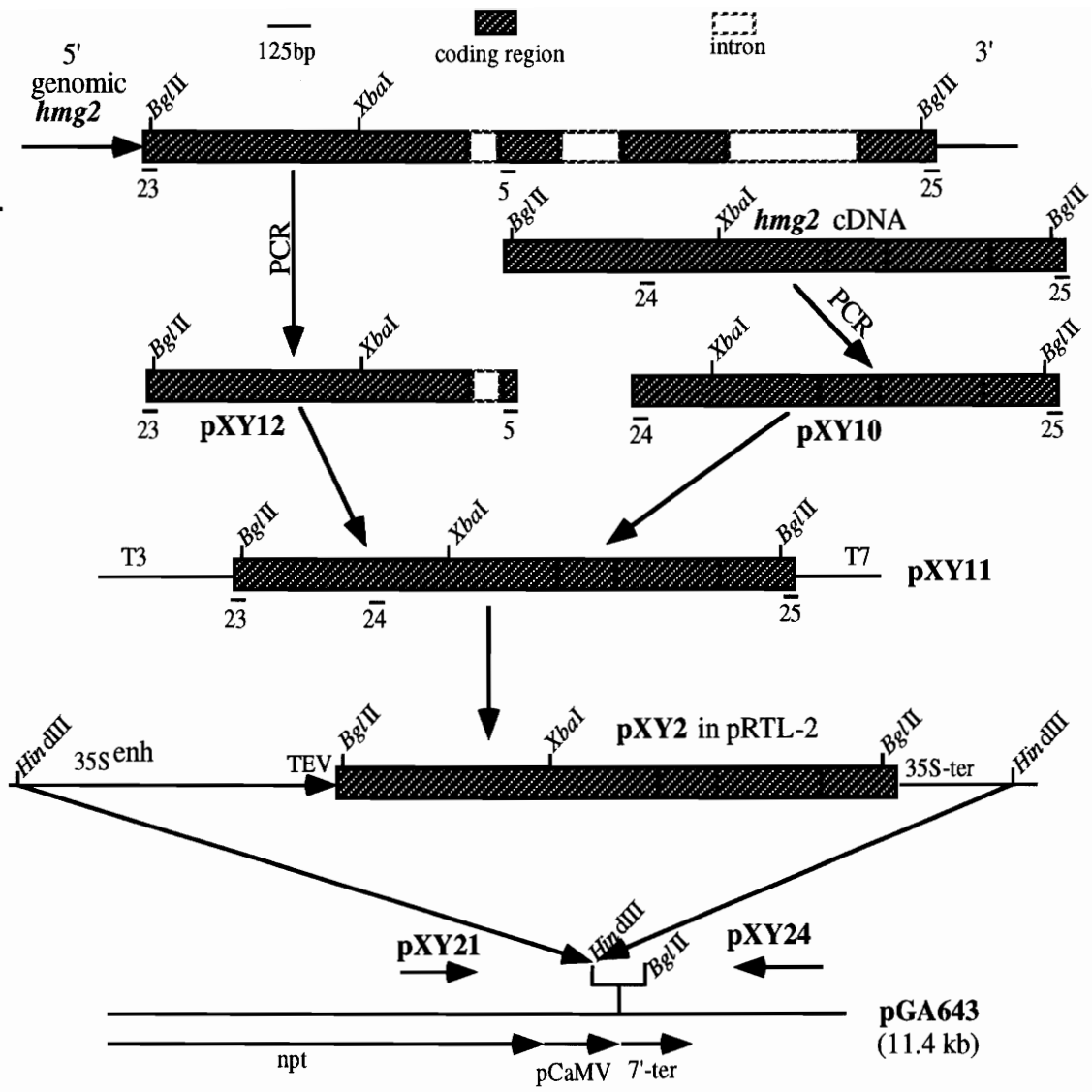
### **Tomato *hmg2* is a functional *hmg* isogene as verified by expression in *E. coli***

To confirm whether a cloned plant *hmg* gene is a functional isogene encoding HMGR rather than a pseudogene or a gene coding for another protein which shares sequence similarity, it is necessary either to perform genetic complementation (for example using a yeast *hmg*-mutant strain; Learned and Fink 1989; Vollack *et al.* 1994) or to express the gene in *Escherichia coli* (Migula) Castellani & Chalmers and demonstrate HMGR activity (Enjuto *et al.* 1994; Ferrer *et al.* 1990). We used an *E. coli* expression system to confirm that the cloned tomato *hmg2* gene was a functional isogene. We generated the

tomato *hmg2* full length cDNA as shown in Figure 3.1 and cloned it into expression vector pET-8c (Studier *et al.* 1990) and pT7-7 (Tabor 1992) (see Materials and Methods for details). Two fragments of tomato *hmg2* cDNA (the *Bgl*III fragment which corresponds to almost full length *hmg2* coding sequence and the *Xba*I/*Eco*RV fragment which encodes the carboxyl-terminal catalytic domain of *hmg2*; Figure 3.1) were also cloned into expression vector pT7-7 (pEXY10 and pEXY1, Table 3.1). The full length cDNA starting from the original start codon (ATG) was cloned into expression vector pET-8c (pEXY11, Table 3.1). These constructs were expressed in *E. coli* strain BL21(DE3). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of crude *E. coli* extracts revealed new, plasmid-dependent protein bands of the expected sizes for the expressed HMGR proteins (65 kD for full length and 40 kD for the catalytic domain only, data shown in Appendix Figure A.2). It was shown that the expressed catalytic domain crossreacted with maize (*Zea mays* L.) HMGR antibody (Moore 1995). All of these expression constructs had HMGR activity when *E. coli* was grown at 37°C or 25°C, whereas the host [*E. coli* BL21(DE3)] or host plus vector pT7-7 had no HMGR activity (Figure 3.2). Based on these results and previous promoter analysis (Chapter 2), we concluded that tomato *hmg2* is indeed a functional *hmg* isogene. Additionally, the results confirmed that the PCR-generated tomato *hmg2* cDNA was intact and functional.

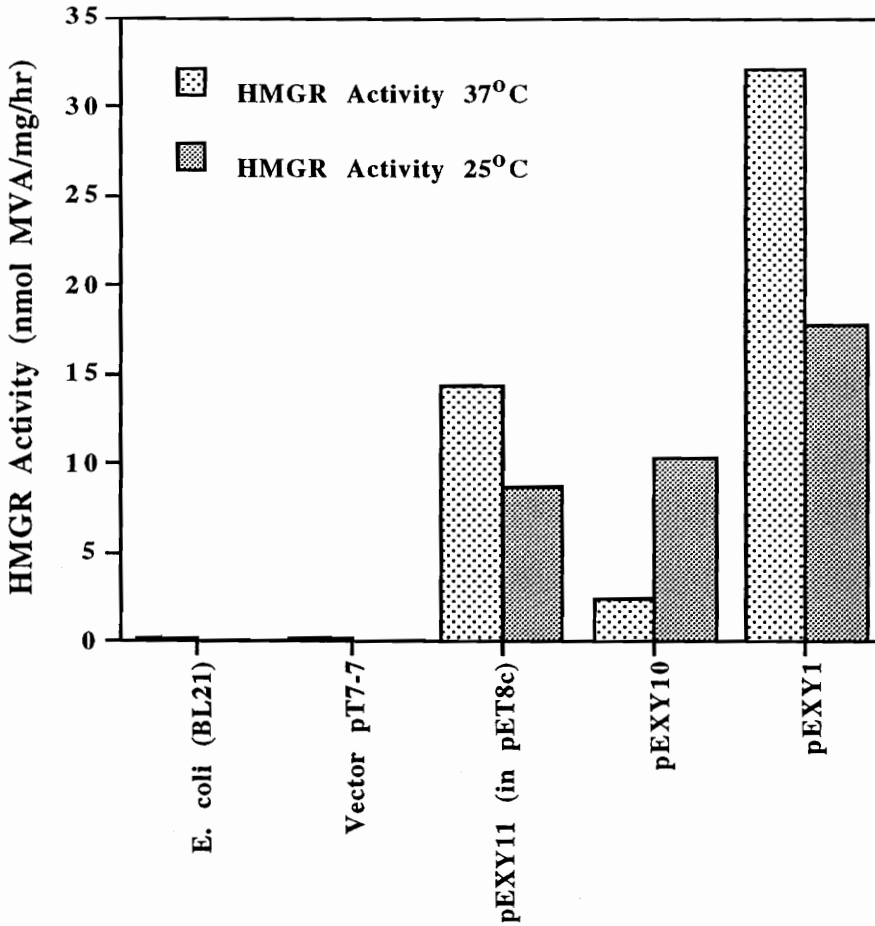






**Table 3.1.** Vector plasmids, *E. coli* expression constructs, and plant overexpression constructs used in this study.

| <b>Plasmid</b> | <b>Description</b>  | <b>Reference</b>              |
|----------------|---|-------------------------------|
| pT7-7          | <i>E. coli</i> expression vector  | Tabor, 1992                   |
| pET-8c         | <i>E. coli</i> expression vector  | Studier <i>et al.</i> 1990    |
| pXY10          | 3' end of tomato <i>hmg2</i> cDNA coding for catalytic domain   | This work                     |
| pXY12          | 5'end of tomato <i>hmg2</i> from start codon to the beginning of exon II                                | This work                     |
| pXY11          | Full-length <i>hmg2</i> cDNA constructed from pXY10 and pXY12   | This work                     |
| pEXY11         | Full-length <i>hmg2</i> cDNA inserted into <i>E. coli</i> expression vector pET-8c                      | This work                     |
| pEXY10         | cDNA fragment from pXY10 which encodes the catalytic domain of <i>hmg2</i> , cloned in frame into pT7-7 | This work                     |
| pEXY1          | Near full-length <i>Bgl</i> III fragment of cDNA cloned into pT7-7                                      | This work                     |
| pGA643         | Plant binary expression vector  | An <i>et al.</i> 1988         |
| pRTL-2         | Plasmid contains 35S double enhancer and TEV leading sequence (35S <sup>enh</sup> )                     | Carrington <i>et al.</i> 1990 |
| pXY21          | 35S <sup>enh</sup> : <i>hmg2</i> cDNA cloned with promoters in tandem to pGA643 promoter                | This work                     |
| pXY24          | 35S <sup>enh</sup> : <i>hmg2</i> cDNA cloned in reverse orientation to pGA643 promoter                  | This work                     |



**Figure 3.2.** HMGR activity of tomato *hmg2* cDNA in *E.coli*. Results from one representative experiment are shown. HMGR activity was expressed as nmol mevalonate /mg protein/hr. *E.coli* strain (BL21) expressing three different *hmg2* constructs or control plasmids were grown at 37°C or 25°C. HMGR activity was measured with crude cell-free extracts from host *E.coli* (BL21), vector control pT7-7, expression constructs pEXY11 (containing full-length *hmg2*), pEXY10 (catalytic domain of *hmg2*) and pEXY1 (*Bgl*II fragment, near full length *hmg2*).

## Gene construction and plant transformation for *hmg2* overexpression

To evaluate the effect of overexpression of the defense-specific tomato *hmg2* on disease resistance, an expression cassette for *hmg2* overexpression was constructed. The *hmg2* cDNA was joined in frame to an enhanced 35S promoter [35S<sup>enh</sup>, a modified CaMV 35S promoter containing a double enhancer and leader sequences from tobacco etch virus (Carrington and Freed 1990)] (Figure 3.1). The 35S<sup>enh</sup>:*hmg2* construct was inserted as a *Hind*III fragment into the binary vector pGA643 yielding plasmids with the *hmg2* cassette in both orientations with respect to the endogenous CaMV 35S promoter in the vector (pXY21 and pXY24). Both plasmids were used for *Agrobacterium tumefaciens*-mediated transformation of tobacco leaf disks. The resulting transgenic tobacco lines were designated as 21# or 24# to indicate which orientation was inserted into the plant genome (Figure 3.1 and Table 3.1). Thirty-six plants were generated (Table 3.2). Two pXY24 plants died before producing seeds and one pXY21 plant died after producing seeds. Southern analysis of fifteen plants of the 24# series and two of 21# series were performed with a neomycin phosphotransferase (NPT II) probe to detect pGA643 T-DNA inserted into the plant genome. A series of Southern hybridization analyses were conducted to analyze overexpression lines 2101, 2421 and 2426 using the NPT II probe, 5' end 0.7 kb *Bgl*III-*Xba*I probe and 3' end 1.1 kb, *Xba*I-*Bgl*III probe from *hmg2* cDNA, digesting the genomic DNA with *Hind*III, *Xba*I, and *Sst*I respectively. A representative Southern blot is shown in Figure 3.3A. Based on these analyses, I concluded that one copy of the transgene was inserted in plant 2421, 2426 and 2101. However plant 2101 contained an aberrant insertion involving integration of the kanamycin selectable marker but loss of part of the *hmg2* gene (Figure 3.3A lane 7). Consistent with this interpretation, no *hmg2* mRNA was detected from plant 2101 (Figure 3.3B lane 4). Northern analysis using the 5' end 0.7 kb *Xba*I to

*Bgl*III fragment from the *hmg2* cDNA, as probe, confirmed expression of the transgene in plants 2421 and 2426 (Figure 3.3B).

### ***hmg* transgene expression results in elevated HMGR activity**

Significant difficulty was experienced in developing a reliable assay system for HMGR enzyme activity from tobacco tissues, although several assay strategies were tested (Chappell *et al.* 1991b; Ji *et al.* 1992; Moore and Oishi 1993). Early assays, although highly variable, indicated elevated HMGR activities (two to five-fold) for transgenic plants 2401, 2421, and 2426 compared to vector control plants or nontransformed tobacco at the same developmental stages (Ji *et al.* 1992). However, subsequent experiments suggested that the assay system of Moore and Oishi (1993) gave more reproducible activity values (for detail, see appendix). Results using this system consistently demonstrated that plant 2421 had greater HMGR activity than either nontransformed or vector-transformed control plants (Figure 3.4). To establish specific values for the *hmg2* overexpression plants 2401, 2414, 2421, 2424, 2426, and 2427, additional assays are required (experiments in progress).

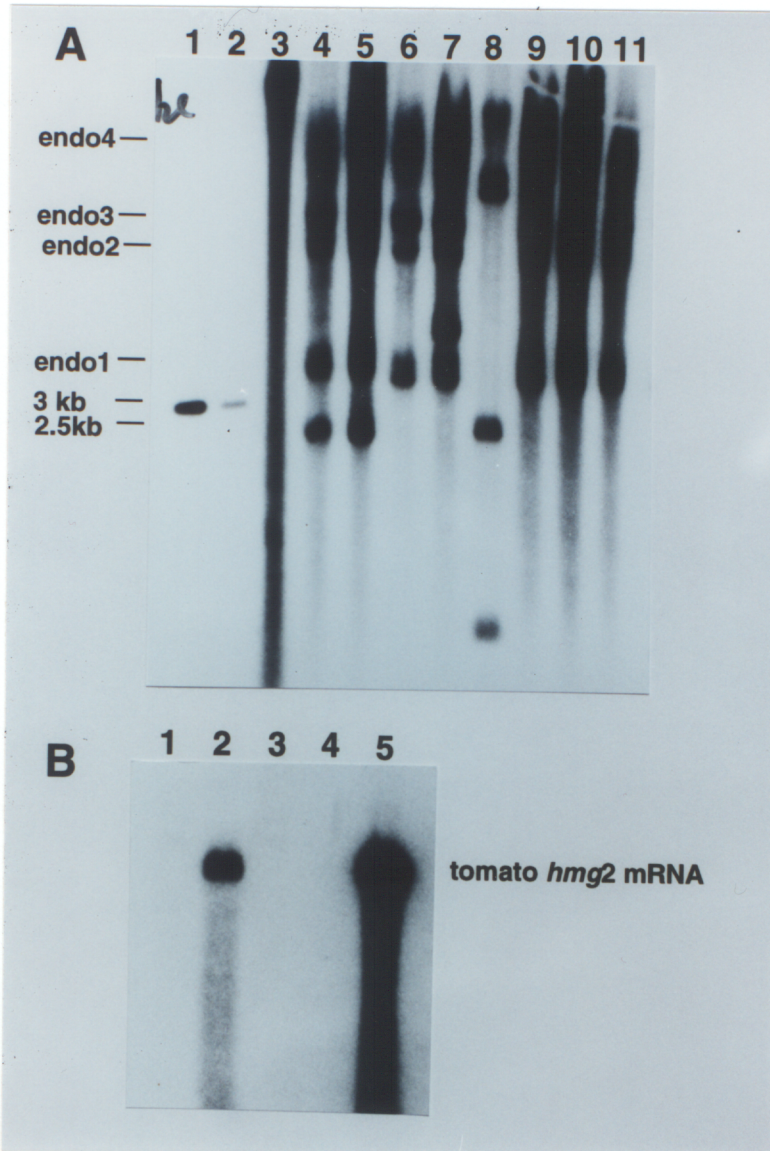
**Table 3.2.** Summary of the transgenic tobacco plants generated using pXY21 and pXY24

| Plasmids | Number of plants | Phenotype           | Gene Copy Number <sup>a</sup> |
|----------|------------------|---------------------|-------------------------------|
| pXY21    | 3                | normal              | 1                             |
| pXY24    | 33               | normal <sup>b</sup> | 1-8                           |

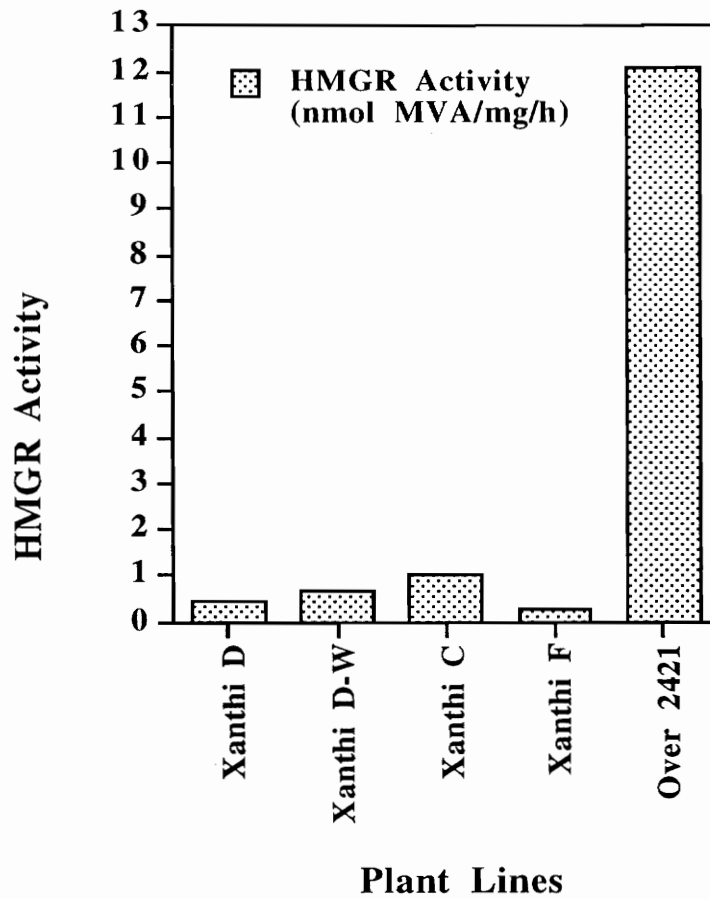
<sup>a</sup> Copy number determined by genomic Southern hybridization using NPTII and/or *hmg2* (5' end 0.7 kb, *Bgl*III-*Xba*I fragment or 3' end 1.1 kb, *Xba*I-*Bgl*III fragment) as probes.

<sup>b</sup> plant 2414 was chlorotic mosaic, plant 2421 had delayed floral tissue development and excessive height and plant 2426 was incomplete sterile.

**Figure 3.3.** Southern and northern analyses of tobacco lines 2421 and 2426. (A) For Southern hybridizations, 30  $\mu$ g of genomic DNA from nontransformed tomato cv. Vendor, tobacco cv. Xanthi nc, vector control and lines 2101, 2421 and 2426 were digested with *Sst*I (except lane 3). Probe used was the 3' end 1.1 kb, *Xba*I-*Bgl*II fragment of pXY11 (Figure 3.1) which encodes the conserved catalytic domain of tomato HMG2; Lane 1, 2 are plasmid pXY21 digested with *Hind*III which should give a 3 kb *Hind*III fragment containing 35S<sup>enh</sup>:*hmg2* (Figure 3.1). DNA in lane 1 is five times more than that in lane 2. Lane 3 is 2421 genomic DNA digested with *Kpn*I (Incomplete digestion is indicated by the smear). Lane 4 is 2421 and lane 5 is 2426 genomic DNA digested with *Sst*I which should give a 2.5 kb *Sst*I fragment from the *hmg2* internal *Sst*I (300 bp from the 3' *Bgl*II) and the *Sst*I site in pGA643 multiple cloning site located between *Hind*III and *Bgl*II (Figure 3.1, *Sst*I site was not shown). Lane 6 is vector control plant DNA. Lane 7 is an aberrant overexpression line 2101, which instead of showing a 500 bp *Sst*I fragment and another unknown size band (5' of *hmg2* from the *Sst*I site + 35S<sup>enh</sup> + npt from pGA643 + genomic DNA of tobacco until the nearest *Sst*I site), it gives a 6kb band which might be the expected unknown band. Lane 8 is tomato genomic DNA. Lane 9, 10, and 11 are Xanthi nc DNA controls. The probe hybridized to at least four bands to tobacco *hmg* isogenes as shown by the vector and Xanthi nc controls (lane 6, 9, 10, 11). (B) Northern analysis of total RNA (20  $\mu$ g/lane) from young tobacco leaves (the top three mature leaves). Lane 1, Xanthi nc control; lane 2, plant 2421; lane 3, pGA643 vector control; lane 4, the aberrant overexpression line 2101 (see legend on lane 7 panel A this Figure); Lane 5, plant 2426. Probe for the northern was the 5' end 0.7 kb *Bgl*II-*Xba*I fragment from pXY11 (Figure 3.1) which encodes the membrane domain of tomato HMG2.







**Figure 3.4.** HMGR activity in overexpression line 2421 and control plants. A representative experiment comparing HMGR activities from tobacco leaves overexpressing *hmg2* (plant 2421), and nontransformed Xanthi nc controls is shown. Three Xanthi nc plants (plant C, D, and F) were used as controls. A branch of Xanthi nc plant D was gently wounded (D-W) with carborundum. HMGR was assayed by the method (Moore and Oishi, 1993) using  $^3\text{H}$  mevalonate as controls.

## **Overexpression of tomato *hmg2* in tobacco plants reduces sizes of necrotic lesions induced by TMV**

In order to test whether *hmg2* overexpression affects disease interactions, two model pathogens, tobacco mosaic virus (TMV) and *Erwinia carotovora* subsp. *carotovora* (*Ecc*) were used. The parent cultivar, Xanthi nc, is resistant to TMV showing necrotic lesions typical of the hypersensitive response. In Chapter 2, I demonstrated that *hmg2*:GUS constructs are expressed in the tissues directly surrounding the TMV lesions. In order to test the effect of constitutive *hmg2* expression on TMV lesion development, detached leaves at about the same physiological and developmental stages as control plants (nontransformed and vector transformed plants) and transgenic tobacco plants overexpressing tomato *hmg2* were inoculated with the TMV (strain U1). The parent cultivar, Xanthi nc, produces visible localized necrotic lesions three to four days after inoculation with this strain (Lucas 1975). TMV lesion sizes were determined (under a binocular dissecting microscope equipped with an eye piece containing a micrometer) by measuring the diameter of the lesions (average of the length and width) and calculating the area by assuming circular lesions. Statistical analysis indicates that the TMV lesion diameter and lesion area of *hmg2* overexpression lines were significantly smaller than in the untransformed Xanthi nc wild type or the vector control plants (Table 3.3). Four different overexpression lines showed reductions (25 to 29%) in TMV lesion diameter. Calculated lesion areas were reduced 42 to 47%. Variability among the overexpression lines was not significant based on Duncan grouping at  $\alpha = 0.05$ . Leaves from whole plants growing in the greenhouse showed similar responses to TMV inoculation as the detached leaves (Figure 3.5).

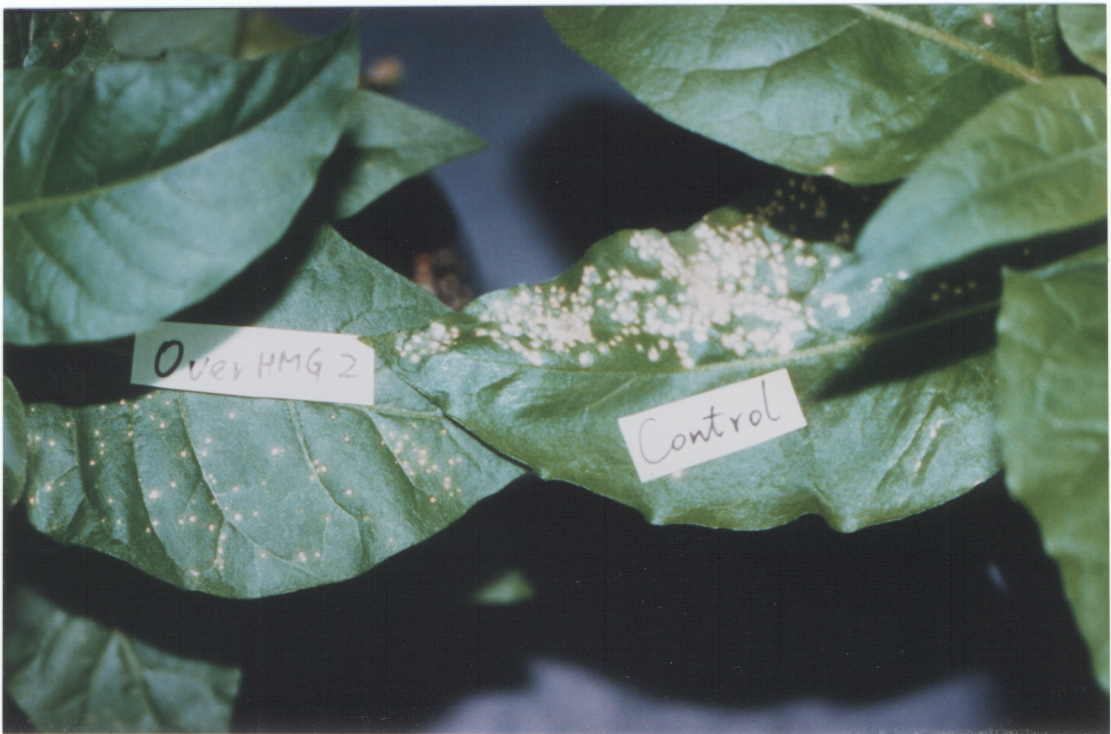
**Table 3.3.** TMV lesion sizes on detached tobacco leaves. Mean sizes of necrotic lesions were measured (under a binocular dissecting microscope equipped with an eye piece containing a micrometer) from detached tobacco leaves induced by tobacco mosaic virus (TMV strain U1) four days after inoculation. Values are expressed as the means of TMV necrotic lesion sizes (expressed in lesion diameter and lesion area)  $\pm$  standard error. A Xanthi nc plant was the untransformed control. Plant 64302 is the vector control. Plants 2426, 2421, 2427, and 2414 represent four different overexpression lines.

| Plant line | Lesion diameter (mm) |                                      |
|------------|----------------------|--------------------------------------|
| Xanthi nc  | 1.14 $\pm$ 0.02      | (A <sup>a</sup> , 100 <sup>b</sup> ) |
| 64302      | 1.07 $\pm$ 0.02      | (B, 161)                             |
| 2426       | 0.86 $\pm$ 0.02      | (C, 160)                             |
| 2421       | 0.83 $\pm$ 0.02      | (C, 160)                             |
| 2427       | 0.83 $\pm$ 0.02      | (C, 80)                              |
| 2414       | 0.82 $\pm$ 0.03      | (C, 74)                              |

<sup>a</sup> Figures in columns followed by the same letters are not significantly different at  $p \leq 0.05$  level by Duncan grouping.

<sup>b</sup> number of observations.

**Figure 3.5.** Lesion development of transgenic plants inoculated with TMV. Vector control and overexpression line 2421 at the same developmental stage were inoculated with 50 µg/ml TMV strain U1 on one side of the leaf and incubated in the greenhouse for one week.



## **Overexpression of *hmg2* in tobacco reduces tissue maceration induced by *Ecc***

Two types of disease resistance assays were conducted with *Ecc*: 1) *Ecc* lesion sizes were determined on detached leaves and 2) tissue maceration assays based on percent of tissue lost were determined using leaf petioles or stem sections. An *Ecc* suspension was introduced into the detached tobacco leaves by gently wounding the leaf with a pipet tip while introducing 5  $\mu$ l of *Ecc* strain EC14 ( $A_{600\text{nm}} = 1.0$ ; ca.  $4 \times 10^6$  cfu). The leaves were then incubated in a moist chamber at 28°C until the lesion fully developed (36 hours to 48 hours). *Ecc* lesion sizes were measured under a binocular dissecting microscope equipped with an eye piece containing a micrometer. The *Ecc* lesion sizes were significantly different between the vector controls and the two overexpression lines (Table 3.4). Sizes (diameter) of *Ecc* lesions on overexpression lines were reduced by 9% (plant 2426) or 12% (plant 2421) in comparison with the vector control (plant 64302). Areas of the *Ecc* lesion were reduced by 16% (plant 2426) and 21% (plant 2421), respectively. The differences among various overexpression lines were not significant for *Ecc* lesion sizes.

**Table 3.4** Lesion sizes on detached transgenic tobacco leaves inoculated with *Erwinia carotovora subsp. carotovora* (*Ecc*). Values are expressed as *Ecc* lesion diameter (mm)  $\pm$  standard error. Detached transgenic tobacco plant leaves were inoculated with 5  $\mu$ l of *Ecc* strain EC14 ( $A_{600\text{nm}} = 1.0$ ; ca.  $4 \times 10^6$  cfu) and incubated at 28°C for 48 hours, then blot dried and measured for their *Ecc* lesion sizes under a binocular dissecting microscope equipped with an eye piece containing a micrometer.

| Plant line | Lesion diameter (mm)                                 |
|------------|--|
| 64302      | 2.08 $\pm$ 0.03 (A <sup>a</sup> , 148 <sup>b</sup> ) |
| 2426       | 1.89 $\pm$ 0.04 (B, 138)                             |
| 2421       | 1.84 $\pm$ 0.03 (B, 141)                             |

<sup>a</sup> Figures in columns followed by the same letters are not significantly different at  $p \leq 0.05$  level by Duncan grouping.

<sup>b</sup> number of observations.

## **TMV-induced systemic acquired resistance (SAR) leads to greater resistance to *Ecc* in transgenic plants overexpressing *hmg2* than in control plants**

We developed an *Ecc* maceration assay for tobacco petiole and stem sections (see Materials and Methods) by measuring the loss of fresh weight. In order to establish the degree of variability in this assay, we tested the *Ecc* maceration experimental system with twelve wild type Xanthi nc tobacco plants of about the same size and physiological stage. Leaf petioles and stem sections were cut from these plants and subject to the *Ecc* inoculation and incubation. After 16 hours the maceration percentage was not statistically significantly different among the plants even at the  $p \leq 0.15$  level (data not shown). Therefore the *Ecc* maceration assay was used for subsequent analyses with the transgenic lines.

Overexpression of tomato *hmg2* in transgenic tobacco plants enhanced the resistance toward *Ecc* tissue maceration (expressed as percentage rotting). Leaf petioles from plants not inoculated with TMV or plants inoculated one week earlier (preinoculation) with TMV to induce systemic acquired resistance (SAR) were inoculated with 250  $\mu$ l of *Ecc* strain EC14 ( $A_{600nm} = 1.0$ ; ca.  $1.75 \times 10^8$  cfu) and incubated at 28°C for 16 hours. The *Ecc* maceration assay revealed that *hmg2* overexpression did not provide protection from a direct *Ecc* challenge using this assay procedure (Table 3.5). However, if the plants were inoculated one week earlier with TMV on (five to six) mature leaves, the *hmg2* overexpressing plants rotted significantly less than control plant similarly manipulated (Table 3.5 and Figure 3.6). Figure 3.7 shows that six *hmg2* overexpression lines (preinoculated with TMV for a week) gave different ranges of protection against *Ecc*



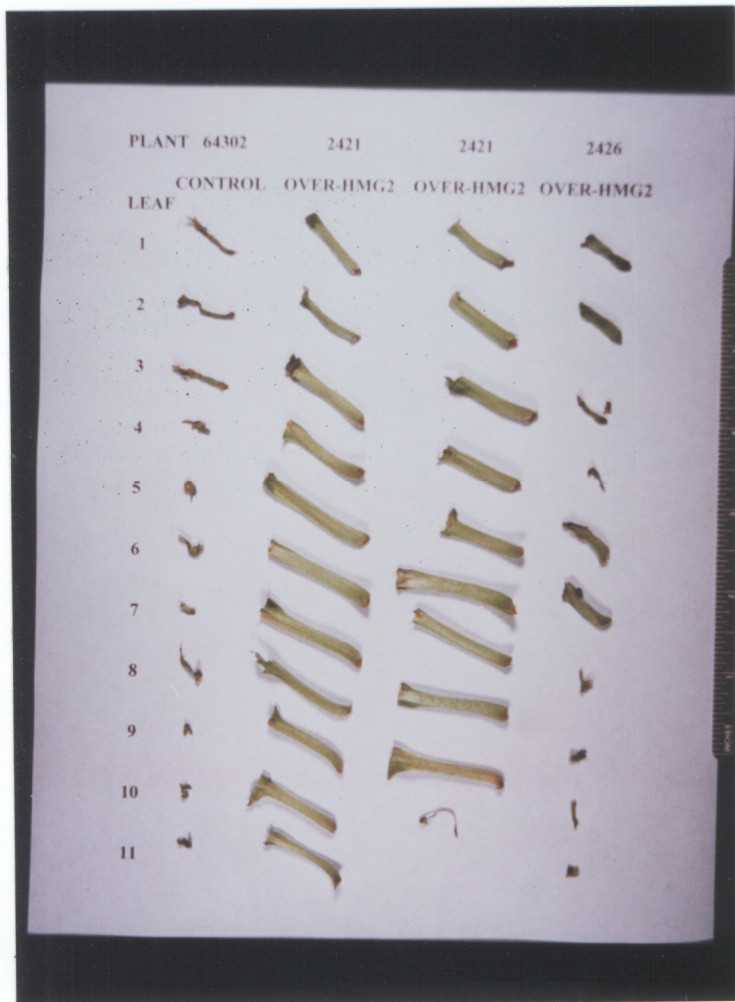
**Table 3.5.** *Erwinia carotovora* subsp. *carotovora* (*Ecc*) maceration analysis of leaf petiole tissues. Leaf petioles from plants with no-TMV preinoculation (the left column) or from plants inoculated with 50 µg/ml TMV strain U1 for seven days (the right column) were excised and placed in 250 µl of bacterial suspension of *Ecc* strain EC14 ( $A_{600nm} = 1.0$ ; ca.  $1.75 \times 10^8$  cfu). Maceration or percent of tissue loss was determined 16 hours after *Ecc* inoculation. Values are mean  $\pm$  standard error. Data are expressed as the mean of percentage of maceration  $\pm$  standard error.

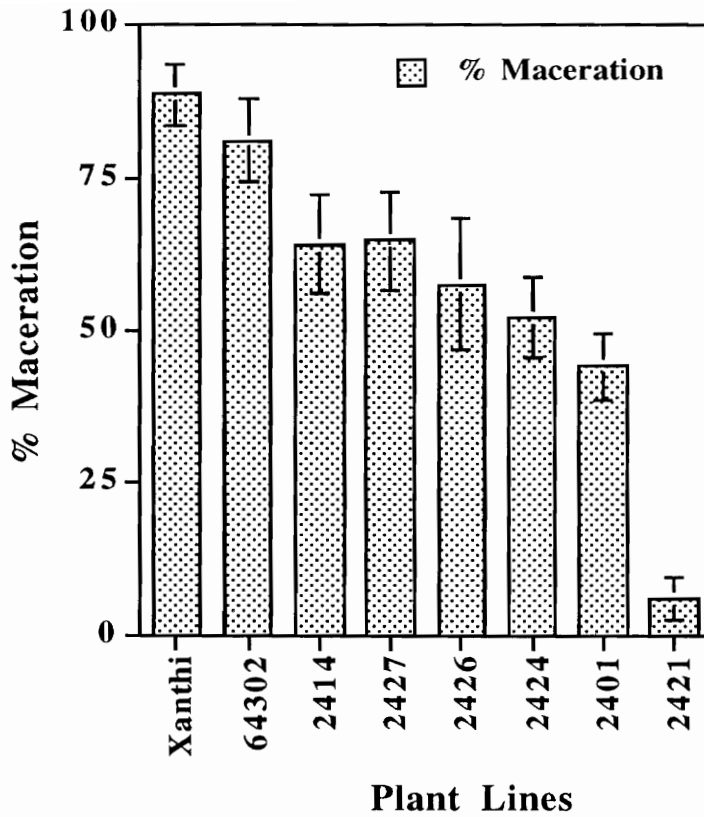
| Plant line           | % of maceration      |                                    | % maceration        |         |
|----------------------|----------------------|------------------------------------|---------------------|---------|
|                      | (no TMV inoculation) |                                    | (TMV-preinoculated) |         |
| Vector Control       | 82.8 $\pm$ 3.7       | (B <sup>a</sup> , 7 <sup>b</sup> ) | 81.1 $\pm$ 6.7      | (A, 11) |
| Overexpressor (2426) | 96.0 $\pm$ 1.5       | (A, 12)                            | 57.6 $\pm$ 10.7     | (B, 11) |
| Overexpressor (2421) | 56.0 $\pm$ 1.9       | (C, 10)                            | 5.8 $\pm$ 3.6       | (C, 21) |

<sup>a</sup> Figures in columns followed by the same letters are not significantly different at  $p \leq 0.05$  level by Duncan grouping.

<sup>b</sup> number of observations.

**Figure 3.6.** *Ecc* maceration of tissues from control and *hmg2* overexpression plants. Leaf petioles from vector control (plant 64302) and overexpressing lines 2421 and 2426 were incubated with 250  $\mu$ l of *Ecc* strain EC14 ( $A_{600\text{nm}} = 1.0$ ; ca.  $1.75 \times 10^8$  cfu) for 16 hours at 28°C. After maceration, the tissues were washed to remove macerated tissues and blotted with paper towels and weighed prior to photography. Numbers represent the leaf position number on the plants from top to the bottom.





**Figure 3.7.** Percentage of *Ecc* maceration of leaf petioles from TMV preinoculated tobacco plants. Tobacco plants tested were transgenic tobacco lines overexpressing tomato *hmg2* (plant 2426, plant 2421, plant 2401, plant 2424, plant 2427, plant 2414), vector control (plant 64302) and nontransformed Xanthi nc control. Leaf petioles (sample size 7 to 21) were incubated with 250  $\mu$ l of *Ecc* strain EC14 ( $A_{600nm} = 1.0$ ; ca.  $1.75 \times 10^8$  cfu) for 16 hours at 28°C.

rotting. This supports the conclusion that *hmg2* gene product is a crucial enzyme in defense against pathogen attack. With the defense signal transduction pathway activated via SAR, the overexpression line has an advantage over the controls because it is not limited by this first rate-limiting step in production of isoprenoid defense compounds.

## DISCUSSION

In this paper, the role of tomato *hmg2* in disease resistance and the feasibility of enhancing plant disease resistance by overexpressing a defense specific *hmg* isogene was studied. Overexpression of tomato *hmg2* in tobacco enhances disease resistance to two classes of pathogen, viruses (as represented by TMV) and phyto bacteria (represented by *Ecc*).

### **Tomato *hmg2* is an active defense related isogene**

Although many plant *hmg* genes have been cloned, only four have been confirmed by yeast complementational *hmg*-mutants (Learned and Fink 1989) or by expression in *E. coli* (Enjuto *et al.* 1994; Ferrer *et al.* 1990). We successfully expressed the PCR cloned tomato *hmg2* cDNA in *E. coli*, detected the predicted peptides in the *E. coli* extracts (see Appendix Figure A.2), and subsequently detected the HMGR activity in the expressed clones. This indicates that the cloned *hmg2* cDNA encodes an active HMGR. Our promoter analysis using the *hmg2*:GUS constructs demonstrated that tomato *hmg2* expression is developmentally regulated and defense related (Chapter 2 and Weissenborn *et al.* 1995). The combination of *hmg2* cDNA *E. coli* expression analysis and promoter analysis has lead us to conclude that *hmg2* is a fully functional *hmg* isogene.

## Tomato *hmg2* overexpression enhanced disease resistance

Three sets of experiments (Table 3.3, Table 3.4 Table 3.5, Figure 3.5, Figure 3.6, and Figure 3.7) with two pathogens (TMV and *Ecc*) indicated that transgenic tobacco lines that overexpress tomato *hmg2* are more disease resistant than the vector control. TMV induces typical hypersensitive necrotic lesions on the leaves of Xanthi nc, an incompatible host. Overexpression of *hmg2* leads to smaller necrotic lesions suggesting that these plants respond to pathogen challenge more quickly or effectively compared to controls. Based on the knowledge of isoprenoid defense compounds and tobacco-TMV interactions, the molecular basis of this enhanced resistance response is not evident. However, a more rapid elevation of isoprenoid phytoalexins (yet to be determined experimentally) may lead to accelerated localized cell death in infected cells leading to smaller lesions. Consistent with the results observed in transgenic tobacco with TMV, the *hmg2* overexpressing plant also exhibited increased resistance to bacterial soft rot. *Ecc* lesion assays also demonstrated that *hmg2* overexpressing plants responded in a more resistant manner to the pathogen challenge than the controls.

We conducted the *Ecc* maceration assay after the systemic acquired resistance (SAR) was induced by TMV preinoculation thus generating two sets of data from the same plants. SAR or induced resistance is the phenomenon that an initial inoculation by a necrotizing pathogen can result in broad, systemic immunity to subsequent (bacterial, fungal and viral) infections in many plant species (Lawton *et al.* 1993). SAR was triggered by TMV inoculation of fifth to 10th mature leaves from the top. Transgenic plants that were not inoculated with TMV were resistant to *Ecc* as demonstrated by the *Ecc* lesion assay and the tissue maceration assay. Although the protection was not as great as the SAR on plants,

plant 2421 still showed 32% reduction in tissue maceration compared to the vector control (Table 3.5 left column). Plant 2426 was surprisingly more susceptible to *Ecc* maceration which might indicate a cosuppression because of the introduction of *hmg2*, or positional effect because plant 2426 produced less seeds compared to other overexpression lines. However after the SAR was activated, the *Ecc* resistance of 2426 was much greater than the controls which suggests that the *hmg2* is operating on a different platform from the SAR superimposed on the SAR from the native defense system. Test of this hypothesis would be using the *nahG* transgenic tobacco plants (Chapter 1 and Lawton *et al.* 1993), crossed with our overexpression lines to see the effect of overexpression *hmg2* on disease resistance of SAR inhibited plants.

Stolle *et al.* (1988) studied the relationship between sesquiterpenoid phytoalexin accumulation and restricted colonization by *Peronospora tabacina* in SAR-activated tobacco (immunized tobacco). They concluded that immunized and non-immunized tobacco leaves accumulated the sesquiterpenoid phytoalexins phytuberol, phytuberin, rishitin, and capsidiol, but not solavetivone or lubimin, after challenge inoculation. Because the accumulation of these phytoalexins was low compared with reports of accumulations for other solanaceous plants and because they did not accumulate earlier in the immunized leaves than in the controls, it is unlikely that they are the primary cause of restricted colonization by *P. tabacina* in SAR-induced tobacco (Stolle *et al.* 1988).

We have not yet determined the levels of isoprenoid phytoalexins produced in plants overexpressing *hmg2* nor developed a direct correlation of HMGR activity and enhanced disease resistance for all of our transgenic plants. However, the experiments described here support a key role for the HMG2 isozyme in defense. Our results also

suggest an involvement of the isoprenoid pathway in the systemic acquired resistance response in tobacco. Most importantly, our results provide a novel strategy for engineering enhanced disease resistance by manipulating a single rate-limiting step in a highly complex pathway for multiple defense compounds. Unlike strategies involving expression of a single defense-related enzyme or viral protein that targets a specific pathogen or pathogen group, *hmg2*-overexpression appears to simultaneously enhance resistance to diverse and unrelated pathogens. Although enhanced disease resistance based on *hmg2*-overexpression must be confirmed in the field and tested in additional crop species, these initial results suggest that this strategy may be broadly applicable to complex disease problems in crop species that utilize isoprenoid defense compounds.

## **MATERIALS AND METHODS**

### **Chemicals, enzymes and reagents.**

Restriction enzymes, bacteriophage T4 DNA ligase, and Klenow fragment polymerase were obtained from GIBCO-BRL (Gaithersburg, MD). The bacterial expression vector pET-8c (Studier *et al.* 1990) and pT7-7 (Tabor 1992) and *E. coli* strain BL21(DE 3) were kindly provided by Dr. Dennis Dean (Biochemistry and Anaerobic Microbiology, VPI & SU). Chemicals were obtained from Sigma (St. Louis, USA).

### **Generation of *hmg2* cDNA clones and plant overexpression constructs.**

A full length cDNA for tomato *hmg2* was generated by ligation of two PCR-derived fragments generated from *hmg2* genomic clone pTH295 (5' end) and *hmg2* cDNA (3' end).



Figure 3.1). Cloning of *hmg2* partial cDNA for the conserved catalytic carboxyl-terminal domain (the 3' end) was achieved by RT-PCR. Total RNA was extracted from tomato suspension culture (cultivar EP-7) treated with *Fusarium oxysporum* f.sp. *lycopersici* (Sacc.) Snyder & Hans. cell wall elicitor for 9 hours (Park 1990). 15 µg of total RNA was subjected to reverse transcription to make first strand cDNA primed with random oligo dTs. The cDNA was subjected to PCR primed with primers 24 and 25 (Figure 3.1). PCR was performed with the Ericomp TwinBlock system for 40 cycles (melting at 95°C 1 min, annealing at 58°C 2 min, extending at 72°C 3 min). Primer 24, a 24-mer (ACATAAGCTTCGTCTGGGGAAGAC) complementary to coding sequence for the linker region (the region between the amino-terminal membrane spanning domain and the carboxyl-terminal catalytic domain) of *hmg2*, started at 540 bp downstream from the start codon. Primer 25 was a 25-mer (ACTAAGCTTTAGGAGGACGCCTTGG) complementary to 3' end covering the stop codon. The generated PCR fragment contains all the coding sequence for HMGR catalytic domain and eliminates the three introns of the genomic version. This PCR fragment was cloned into the pBS SK- (Stratagene) vector digested with *Sma*I. The derived plasmid was called pXY10. The genomic version of *hmg2* in plasmid pTH295 (Park *et al.* 1992), was subjected to PCR primed with primer 23 and primer 5. Primer 23 was a 30-mer (CTTTGTCGACCATGGACGTTCCGGAGAT) which included from amino terminal ATG start codon (underlined). Primer 5 was a 33-mer antisense within Exon II of pTH295 (Park *et al.* 1992). The derived PCR fragment was subcloned into pBS SK- digested with *Sma*I. The derived plasmid was called pXY12. The full length cDNA of *hmg2* was constructed by inserting the *Eco*RV/*Xba*I fragment from pXY12 into pXY10 as the vector (cut with *Not*I, ends filled in by Klenow fragment polymerase, further digested with *Xba*I and purified by gel electrophoresis). The derived plasmid which contain the full length

cDNA of *hmg2* was called pXY11. Full length *hmg2* cDNA was subcloned into a high-level plant expression cassette containing the 35S<sup>enh</sup> promoter in pRTL-2 (Carrington and Freed 1990). The expression cassette containing the cDNA in a sense direction was subcloned into the *Hind*III site of the *A. tumefaciens* binary vector pGA643 (An *et al.* 1988). The *Hind*III overexpression cassette was inserted at the *Hind*III site of binary vector pGA643 in two directions resulting two constructs (pXY21 and pXY24 Figure 3.1). These chimeric 35S<sup>enh</sup>:*hmg2* genes were subsequently introduced into *Agrobacterium tumefaciens* (Smith & Townsend) Conn (strain LBA4404) by direct transformation (An *et al.* 1988) and transformed *A. tumefaciens* cells were used to inoculate leaf discs of tobacco (*N. tabacum* cv. Xanthi nc) (Burow *et al.* 1990). The transgenic tobacco lines derived from pXY21 or pXY24 are *hmg2* overexpression lines, which were designated as 21# or 24# (Table 3.2)

### **Construction of expression constructs and expression of *hmg2* in *E. coli*.**

In order to confirm of tomato *hmg2* isogene encodes a functional HMGR, we expressed the *hmg2* cDNA in *E. coli* prior to introduction into tobacco plants. Table 3.1 listed the plasmids used and the gene constructs generated in this study. pEXY11 was constructed from ligating the *Nco*I/*Xba*I fragment from pXY12, *Xba*I/*Eco*RV fragment from pXY10 and vector pET-8c (Studier *et al.* 1990) digested with *Bam*HI, blunted and further digested with *Nco*I. This is a full length cDNA expression construct spanning the original tomato *hmg2* from start codon and to stop codon. pEXY1 was constructed by cloning the *Bgl*III fragment of *hmg2* into pT7-7 (Tabor 1992) digested with *Bam*HI. The first *Bgl*III site starts at 16bp downstream of start codon. The second *Bgl*III site is located 27 bp before the stop codon. The fused in frame construct would encode eight amino acids at

the amino terminal from the vector pT7-7 in place of the first five amino acids from *hmg2*. The carboxyl terminal would have 13 amino acids encoded by the vector in place of the nine original amino acids. pEXY10 was constructed by cloning the *XbaI/EcoRV* fragment from pXY10 into pT7-7 *HindIII* site by partial fill-in. The derived construct would have 18 amino acids encoded by the vector at the amino terminal. The carboxyl terminal would be the original *hmg2* sequence. This construct would give rise to a partial *hmg2* eliminating the membrane-spanning domain (700 bp downstream from the start codon).

All the clones of interest were initially selected in *E. coli* strain DH5a and further introduced into *E. coli* BL21 (DE3) for expression studies. Expression in *E. coli* was done as described by Ferrer *et al.* (1990) with modification. *E. coli* strain BL21 (DE3) itself and those transformants with pEXY11, pEXY1, pEXY10, or vector pT7-7 were grown at 37°C or room temperature on LB medium with 100 µg/ml ampicillin to  $A_{600\text{nm}} = 0.6$  and induced by the addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG). The sampling and sonication of the samples were done as described (Ferrer *et al.* 1990).

### **Protein concentration and HMGR enzyme activity assays.**

Protein content of the samples was measured by using the Coomassie plus protein assay reagent (Pierce, Rockford, IL), which employs the Bradford method for protein determination, with BSA as a standard (Bradford 1976). HMGR activity was determined by the radiometric assay described by Ji *et al.* (1992) and Moore and Oishi (1993).

## **Nucleic acid isolations and hybridization analyses.**

Genomic DNA was isolated from tobacco plant leaves using three different protocols: CTAB (Rogers and Bendich 1988), SDS (Dellaporta *et al.* 1984) and 7M urea (personal communication, Claudia Kay, University of Florida) procedures. Tomato genomic DNA (30 µg) was digested and size separated on 0.8% agarose gel with 1xTAE (Sambrook *et al.* 1989) for use in genomic Southern blotting. Total RNA from young leaves was isolated using the procedure provided by the manufacturer of RNazol (Tel-test, Inc. Friendswood, Texas, U.S.A.) with modifications. 600 to 1,000 mg of young tobacco leaves (the first three leaves) were homogenized in 3 ml of RNazol with a polytron tissue homogenizer three times for one minute on ice/water slurry (0°C). Chloroform:isoamyl alcohol (24:1, 350 µl) was added to the homogenate and sample was homogenized for another 30 seconds. The homogenate was centrifuged at 11,950g (Sorvall RC-5B, SS-34 rotor) for 10 minutes at 4°C. The top 2.5 ml aqueous phase was transferred to a fresh tube and 2.5 ml of 7M urea buffer [7M urea, 0.05M Tris HCl (pH7.0), 0.01M EDTA (pH7.0), 0.35 M NaCl, and 1% (w/v) of sarkosyl] was added, mixed together and equal volume of phenol/chloroform was added. After spinning for 10 minutes at 11,950g, the aqueous phase was transferred to a fresh tube and two volumes of 100% ethanol was added to precipitate the nucleic acids. The pellet was washed with 70% ethanol, dried and resuspended in 350 µl of TE buffer. 20 µg of RNA from each sample was used for northern analysis. The Southern and northern gel blotting and hybridization were carried out following the procedures previously described (Sambrook *et al.* 1989) using the Nytran plus membrane (Schleicher & Schuell). Radioactive probes were labeled using a random primer kit from GIBCO-BRL.

## **TMV inoculation and lesion measurement with detached leaves**

Four to eight leaves of about the same age and position in the R0 transgenic plants (the primary transformant regenerated directly from leaf disks) and the nontransformed control or vector transformed control plants were cut at the base of the leaf petioles and were inoculated with 50 $\mu$ g/ml TMV (strain U1). TMV was purified by the method previously described (Asselin and Zaitlin 1978) [provided by Ms Elizabeth Schwartz and Dr. Sue. A. Tolin (VPI&SU)]. Leaves were inoculated either on one side of the leaf or sometimes both sides of the leaf (Trevathan *et al.* 1982). The inoculations were done in 1% K<sub>2</sub>HPO<sub>4</sub> + 1% Celite 545 (Fisher Sci. Com. Fair Lawn, NJ). Leaves were put into a 150x25 mm style petri dish (Corning, NY) with a wet filter paper to maintain the moisture. After four days incubation in the growth chamber, the leaves were heated in microwave oven for 15 seconds at high power and then dried with paper towels. TMV lesion sizes were measured under a binocular with an eye piece micrometer to accurately measure the diameters of at least twenty randomly selected lesions per leaf. Lesions were usually irregularly shaped, therefore, the length and the rectangular width were measured for each lesion and the diameter = (length + width)/2 was calculated (mm). The calculated lesion area (mm<sup>2</sup>) was the area of a circle with the measured diameter.

## ***Ecc* lesion assay**

*Ecc* lesions were introduced individually on the detached leaves by inoculating the leaf with 5  $\mu$ l of *Ecc* strain EC14 ( $A_{600\text{nm}} = 1.0$ ; ca.  $4 \times 10^6$  cfu) using gentle application of the pipet tip to create the wound site, leaves were sandwiched in wet paper towels and incubated at 28 $^{\circ}$ C for 36 to 48 hours to let the lesions fully develop (transparent in lesion

area). Generally 10 to 20 lesions separated by the veins were introduced on to one detached leaf. The leaves were heated at high power in microwave oven for 15 seconds to stop the metabolism of pathogens and host and dried in paper towels for a week. Lesion sizes were measured the same way as the TMV lesions. Calculations were the same as for TMV lesions.

### ***Ecc* tissue maceration assay.**

We established a leaf petiole and stem section maceration assay with *Ecc* for assessing the levels of maceration (weight loss due to rot caused by *Ecc*) in transgenic and control plants. The assay was designed to mimic natural *Ecc* infection (hand rot) which started rot at the base of leaf petioles of tobacco plants at the time of harvesting. Leaf petioles or stem segments (each about 2.5 cm long) from greenhouse grown young suckers of primary transformants were inoculated with 250  $\mu$ l of *Ecc* strain EC14 ( $A_{600nm} = 1.0$ ; ca.  $1.75 \times 10^8$  cfu). Incubation was performed for 16 hours in glass test tubes at 28°C. Several independent transgenic plants overexpressing tomato *hmg2* and wild type as well as vector transgenic control plants were assayed. The fresh weights of the leaf petioles and/or stem sections were measured with an analytical balance. After 16 hours of *Ecc* incubation at 28°C (in glass test tubes with 250  $\mu$ l of *Ecc* strain EC14 ( $A_{600nm} = 1.0$ ; ca.  $1.75 \times 10^8$  cfu), the leaf petioles or stem section were rinsed with deionized water and wrapped in a piece of paper towel to gently blot and then weighed. The percent maceration was calculated as (Fresh weight before rotting - Fresh weigh after the maceration)/Fresh weight before rotting times 100.

### ***Ecc* tissue maceration assay following TMV preinoculation:**

When plants had 10 to 20 leaves and about three months old grown in the plastic greenhouse with natural sunlight, the mature leaves (4th leaf from the top down) were inoculated with 50µg/ml TMV (strain U1) on both side of the leaf. Inoculated plants were grown in the greenhouse for one week to let necrotic lesions fully develop. All the leaf petioles of the uninoculated top leaves and the inoculated bottom ones were individually cut from the stem. The stem section of the corresponding uninoculated and inoculated portion were cut into segments about 2.5 cm long and subjected to the same maceration assay.

### **Data calculation and statistics analyses.**

The collected data were calculated using Lotus 123. The SAS system was used for statistical analyses. General linear models (GLM) procedures was used to perform the analysis of variance. The hypothesis tested was that all groups had the same mean and the probability of it having occurred by chance. Duncan's multiple range test for variables was used to group the different treatment at a = 5%. Means of the *Ecc* maceration percentage, TMV or *Ecc* lesion diameters (mm) and lesion area (mm<sup>2</sup>) were compared and the mean ± standard error were presented.

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**Chapter 4. Effects of antisense tomato *hmg1* and *hmg2* on disease resistance of transgenic tobacco and tomato**

## ABSTRACT

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) mediates a major rate-limiting step in the isoprenoid pathway which in the *Solanaceae*, leads to the synthesis of sesquiterpenoid phytoalexins associated with disease resistance. The tomato genome contains at least four differentially regulated *hmg* genes. We generated transgenic tobacco (*Nicotiana tabacum* L. cv. Xanthi nc) and tomato (*Lycopersicon esculentum* Mill. cv. Vendor) containing tomato *hmg1* and *hmg2* antisense constructs to study their effect on disease resistance. Full-length *hmg2* (a defense-specific isoform) and presumptive gene-specific 5' regions of *hmg1* or *hmg2* were inserted in the antisense orientation behind a CaMV 35S promoter. Southern and northern analyses confirmed successful transformation and antisense message expression. Transgenic tomato plants expressing the full-length *hmg2* antisense showed lower HMGR enzyme activity and were more susceptible to soft rot by *Erwinia carotovora* subsp. *carotovora* (*Ecc*) than were control plants. This confirms previous results suggesting that tomato *hmg2* plays a critical role in disease resistance. In contrast, expression of either anti-*hmg1* or anti-*hmg2* in the heterologous tobacco system resulted in plants with enhanced resistance to *Ecc* and significantly reduced TMV lesion sizes. These results, while surprising, may indicate that antisense inhibition is non-specifically exerted on isogenes other than the defense-specific HMGR gene which is quite divergent from tomato *hmg2*.

*Additional keywords:* *Lycopersicon esculentum*, *Nicotiana tabacum*, *Erwinia carotovora* subsp. *carotovora*, TMV

## INTRODUCTION

Plant 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC 1.1.1.34) mediates the first regulatory step in the synthesis of a diverse array of isoprenoid compounds including sterols, carotenoids, electron transport components, rubber, gibberellins, abscisic acid and isoprenoid phytoalexins (Gray 1987). Specific isoprenoid compounds are synthesized depending on both developmental and environmental cues. Multiple forms of HMGR associated with several subcellular compartments and differential regulation have been described (Bach 1987).

In the *Solanaceae*, the predominant phytoalexins, sesquiterpenoid phytoalexins, are synthesized via the isoprenoid pathway (Weissenborn *et al.* 1995). We seek to determine how regulation of plant HMGR, as the first rate-limiting enzyme in the production of isoprenoid defense compounds, affects disease resistance. The detailed genetic background on tomato and relative ease for transformation make it a good choice as a model system to study effects of HMGR on disease resistance of Solanaceous plants. The tomato genome contains at least four *hmg* genes encoding HMGR isozymes critical for metabolism, growth, development, and disease resistance (Cramer *et al.* 1993; Narita *et al.* 1991; Weissenborn *et al.* 1995). Among them, *hmg2* appears to be associated with plant defense responses and is activated in response to wounding, fungal elicitors, and pathogen challenge (Cramer *et al.* 1993; Weissenborn *et al.* 1995a; Chapters 2, 3). The inducibility of *hmg2* by diverse stresses might reflect its specialized role in the synthesis of specific defense isoprenoids. *hmg2* is not induced in association with sterol biosynthesis or carotenoid pigment formation. In contrast, tomato *hmg1* seems to be closely related to sterol biosynthesis (Gillaspy *et al.* 1993; Narita and Gruissem 1989). Other tomato *hmg*



isogenes might be involved in more distinct developmental and environmental responses (Gillaspy *et al.* 1993; Weissenborn *et al.* 1995). Directly analyzing the specific functions of different *hmg* isogenes will help us to understand the regulatory mechanisms which mediate isoprenoid synthesis in plants.

One common approach to assess the function of members of a multigene family is antisense inhibition of specific isogenes. The strategy of the antisense approach is to generate "mutants" which silence or weaken gene functions by inducing the plant to produce antisense RNA capable of annealing to the normal (sense) mRNA to form a non-translatable RNA duplex while leaving other isogenes unperturbed. The precise mechanism of how antisense inhibition works still remains unresolved. Antisense genes expressed in transgenic plants have proved to be effective for inhibiting the translation of genes and inhibiting mRNA synthesis, maturation, or accumulation. Antisense inhibition has been used successfully to alter fruit ripening in tomato targeting genes encoding polygalacturonase (DellaPenna and Giovannoni 1991; Sheehy *et al.* 1988; Smith *et al.* 1990; Smith *et al.* 1988) and 1-aminocyclopropane-1-carboxylate synthase (Oeller *et al.* 1991)); flower pigmentation (Krol *et al.* 1990; Meer *et al.* 1992); ADP-glucose pyrophosphorylase (Muller-Rober *et al.* 1992); proteinase inhibitors I and II (McGurl *et al.* 1992); stearoyl-ACP desaturase (Knutzon *et al.* 1992); small subunit of Rubisco (Hudson *et al.* 1992); and  $\beta$ -glucuronidase (GUS) (Robert *et al.* 1989). Antisense techniques have also been used to identify genes encoding enzymes for carotenoid biosynthesis (phytoene synthase) and ethylene biosynthesis (the ethylene-forming enzyme) (Hamilton *et al.* 1991; Hamilton *et al.* 1990).

Our knowledge of plant HMGR structures led us to attempt the antisense approach

to study specific functions of different *hmg* isogenes. The domain structure of plant HMGR, like that of the animal and fungal enzymes, is divided into two domains connected by a divergent and flexible linker (Nelson *et al.* 1994; Rogers *et al.* 1986). The N-terminal membrane domain contains two transmembrane spans such that both carboxy- and amino-termini are located on the cytosolic face as recently demonstrated in tomato microsomal HMGRs (Denbow *et al.* 1995). Although there is significant sequence identity within the actual transmembrane span, the N-terminal 200 amino acid residues are relatively divergent both among plant species and among isozymes within a species (Table 4.1). Table 4.1 compares the amino acid identity between cloned HMGR isoforms of the *Solanaceae* and several other species. The catalytic domain (about 400 residues) shows much greater sequence conservation (83 - 98% identity). In general, hybridization probes generated from the catalytic domain recognize all isoforms in the genome while those derived from the amino-terminal membrane-domain region function as isoform-specific probes under high stringency (Yang *et al.* 1991). The sequence similarity at the nucleic acid level reflects the observation at protein level but with more divergence, especially within the 5' region encoding the amino terminal membrane-bound domain. This 5' region was therefore targeted as a potential gene specific sequence for antisense approaches.

In order to pinpoint the specific function of tomato *hmg* isogenes, especially in disease resistance, we generated antisense constructs for two isogenes, tomato *hmg1* and *hmg2*, for expression of antisense RNA in tobacco (cultivar Xanthi) and tomato (cultivar Vendor) plants. The 5' membrane-spanning domains of *hmg1* or *hmg2* were used as putative gene-specific regions. These regions and full length *hmg2* were cloned in the antisense orientation under the direction of a 35S promoter. These antisense constructs were transferred to tomato and tobacco via the *Agrobacterium*-mediated transformation

**Table 4.1.** Amino acid similarity/identity (%) of the membrane and catalytic domains of plant HMGRs. Lower lane: catalytic domain (201 to 600 amino acids); upper lane: membrane domains (1 to 200 amino acids). Tomato *hmg1* is marked as (*Tom1*; partial sequence), tomato *hmg2* (*Tom2*), potato *hmg1* (*Pot1*), potato *hmg3* (*Pot3*; incomplete sequence), *Nicotiana sylvestris* (*Nic*, isogene unknown), *Arabidopsis thaliana hmg1*(*Ara1*). Comparison is based on the sequences available from GenBank Databases under reported accession numbers. Parentheses indicate comparison of smaller regions due to partial sequence information. N.A. indicates that data is not available. Computer facilitated manipulation of DNA sequences were performed using Sequence Analysis Software Package by the University of Wisconsin Genetics Computer Group (Devereux *et al.* 1984). This Table is adapted from Marco Laine's thesis (Laine 1993).

|             | <i>Tom1</i> | <i>Tom2</i> | <i>Pot1</i> | <i>Pot3</i> | <i>Nic</i> | <i>Ara1</i> |
|-------------|-------------|-------------|-------------|-------------|------------|-------------|
| <i>Tom1</i> | 100         | 83/75       | 99/98       | (80/68)     | 85/77      | 72/57       |
|             | 100         | (92/86)     | (98/97)     | (94/85)     | N.A.       | N.A.        |
| <i>Tom2</i> |             | 100         | 82/74       | N.A.        | 90/85      | 71/50       |
|             |             | 100         | 95/90       | 95/90       | 98/95      | 91/81       |
| <i>Pot1</i> |             |             | 100         | N.A.        | 83/76      | 72/58       |
|             |             |             | 100         | 96/90       | 95/93      | 93/83       |
| <i>Pot3</i> |             |             |             | N.A.        | N.A.       | N.A.        |
|             |             |             |             | 100         | 96/92      | 93/82       |
| <i>Nic</i>  |             |             |             |             | 100        | 69/54       |
|             |             |             |             |             | 100        | 92/83       |

system and tested for disease phenotypes following inoculation with the soft-rotting bacterium, *Erwinia carotovora* subsp. *carotovora* (Ecc).

## RESULTS

### **Transformation of antisense *hmg1*, *hmg2* and full-length *hmg2***

In order to generate antisense constructs that may function in an isoform specific manner, restriction fragments from the less conserved 5' region encoding the membrane domain were selected (Figure 4.2). The resulting antisense constructs (pXY61, pXY31 and pXY39, Figure 4.2, see Material and Methods for detail) were transformed into tomato cv. Vendor and tobacco cv. Xanthi plants using *Agrobacterium*-mediated transformation (Burow *et al.* 1990; McCormick *et al.* 1986). Table 4.2 summarizes the resulting transgenic plants and gene copies of the antisense constructs. Southern and northern blot hybridization confirmed the successful transformation and expression. As shown in Figure 4.1, northern hybridization analyses confirmed expression of the tomato *hmg2* and tomato *hmg1* antisense RNA in the transgenic plants. The tomato plant with full length antisense *hmg2* had a smear of the message suggesting antisense inhibition of the endogenous tomato *hmg2* expression.

### **Transgenic plants expressing *hmg* antisense constructs are phenotypically normal**

Because HMGR is the first regulatory enzymes in the isoprenoid pathway and because there are so many isoprenoids synthesized in plants, some vital for growth of the

plant, we expected to see some dramatic changes in the appearance of transgenic plants containing antisense *hmg* constructs. Quite surprisingly, most of the regenerated transgenic plants were normal (Table 4.2). Only one tobacco plant (plant 603) out of the 150 regenerated plants (148 of these were tobacco plants, two of them were tomato plants) was chlorotic, which could be due to a positional effect of the transformation. The antisense tomato produced fewer seeds compared to the vector control and the seeds we collected were not viable. However, seeds from the vector control tomato collected at the same time and treated the same way were also not viable. On the other hand the transgenic tobacco seeds germinated normally and at the same rate as the nontransformed Xanthi seeds.

### **HMGR activity in antisense tomato plants was lower than in vector-transformed control plants**

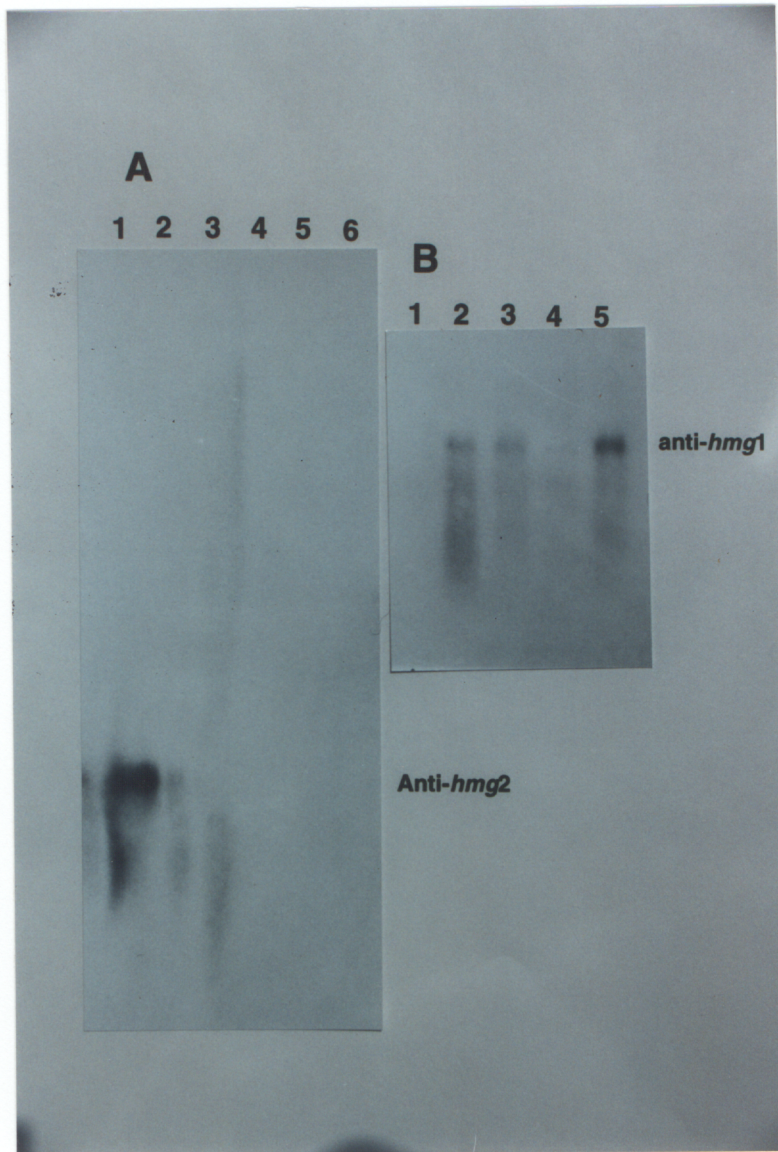
Significant difficulty was experienced in developing a reliable assay system for HMGR enzyme activity from tobacco and tomato tissues (Ji *et al.* 1992; Moore and Oishi 1993). Early assays using the method developed by Ji *et al.* (1992) for maize HMGR analyses, although highly variable, indicated that transgenic tomato plants had a partial block of HMGR activity as the result of antisense inhibition. It was observed that HMGR enzyme activity of microsomal preparations from transgenic tomato containing full length antisense *hmg2* was about 50% lower than the vector control tomato plant (Table 4.3). The loss of the sole transgenic tomato containing antisense *hmg2* due to fungal infection was very unfortunate (see Discussion). Recent assays with Moore and Oishi's method using previously purified microsomal preparation preserved at -70°C (8 months old) did not show any activity, most probably due to the loss of activity during handling and storing. HMGR activity following wounding or pathogen challenge (which should specifically

**Table 4.2.** Gene constructs and transgenic plants generated in this study.

| Construct | Region taken                      | Number of plants | Phenotype           | Gene Copy # |
|-----------|-----------------------------------|------------------|---------------------|-------------|
| pGA643    | vector                            | 15 <sup>a</sup>  | normal              | 1-2         |
| pXY61     | <i>hmg1</i> 5' antisense          | 41               | normal <sup>b</sup> | 1-7         |
| pXY31     | <i>hmg2</i> 5' antisense          | 44               | normal              | 1-10        |
| pXY39     | <i>hmg2</i> full length antisense | 50 <sup>c</sup>  | normal              | 1-3         |

a. including one tomato plant T64301; b. plant 603 was chlorotic; c. including one tomato plant T3901.

**Figure 4.1.** Northern analysis of transgenic tobacco and tomato plants expressing *hmg2* and *hmg1* antisense constructs. 20 µg total RNA/lane. Panel A: Lane 1, antisense *hmg2* plant line 3117; lane 2 plant 3115; lane 3 antisense *hmg2* in tomato; lane 4 and lane 5 tomato vector controls; lane 6 tobacco Xanthi control. Panel B: 4 independent transformants carrying the antisense *hmg1* (lane 2, 3, 4, and 5). Lane 1, plant 629 which is a false positive during regeneration, serve as a nontransformed Xanthi control. Lane 2, plant 627; lane 3, plant 644; lane 4, plant 642; lane 5, plant 640.





**Table 4.3.** HMGR activities in the microsomal fraction prepared from young leaf tissues of transgenic tomato plants; containing antisense full-length *hmg2*. Values represent results from a single experiment measured with the previously described method (Ji *et al.* 1992).

| Plant line   | HMGR activity (nmol MVA / mg protein / hr) |
|--|--|
| Tomato vector control (plant 64301)                    | 1.72                                       |
| Tomato antisense full-length <i>hmg2</i> (plant T3901) | 0.83                                       |

assess *hmg2* suppression) was not tested.

### **Expression of antisense full length *hmg2* in tomato resulted in more susceptible plants**

In order to study the specific function of tomato *hmg2* in plant defense, we tested tomato plants constitutively expressing antisense full-length *hmg2* RNA for disease responses. Transgenic tomato plants containing the full length antisense *hmg2* showed increased susceptibility to the phytopathogenic bacterium *Erwinia carotovora* subsp. *carotovora* (*Ecc*) strain EC14. *Ecc* is the bacterial causal agent of plant soft rot disease. Leaf petioles from transgenic tomatoes containing antisense full-length *hmg2* showed 48.6% more tissue loss due to maceration than the control plants which were transformed with the vector pGA643 to eliminate variability from the regeneration process (Table 4.4). Stem sections showed 40.8% greater maceration compared to the vector control sixteen hours after inoculation. However, statistical analyses indicated that this difference was not significant at the level of  $P \leq 0.05$  (the p value = 0.064; the difference was significant at  $p \leq 0.1$ ).

Analysis of *Ecc* lesion sizes also suggested higher susceptibility in antisense *hmg2* tomato than in controls. The *Ecc* infection was initiated by inoculating interveinal leaf surfaces on detached tomato leaflets (of similar size and development) with 5  $\mu$ l of *Ecc* strain EC14 ( $A_{600nm} = 1.0$ ; ca.  $4 \times 10^6$  cfu) and incubating in a humid chamber for 36 hours at 28°C. *Ecc* lesion sizes were measured under a binocular dissecting microscope equipped with an eye piece containing a micrometer. The mean sizes of *Ecc* lesions on antisense tomato leaflets were bigger than lesions on the vector controls (Table 4.5). The increased

**Table 4.4.** Percentage of tissue maceration by *Ecc* from transgenic tomato plants expressing full-length antisense *hmg2*. Leaf petioles and stem sections were inoculated with 250  $\mu$ l of *Ecc* strain EC14 ( $A_{600nm} = 1.0$ ; ca.  $1.75 \times 10^8$  cfu) and incubated at 28°C for 16 hours. Data are expressed as the mean of percentage of maceration + standard error.

| Plant line                        | % maceration            |                                     |                         |         |
|-----------------------------------|-------------------------|-------------------------------------|-------------------------|---------|
|                                   | % loss of leaf petioles |                                     | % loss of stem sections |         |
| Full-length antisense <i>hmg2</i> | 57.9 $\pm$ 2.6          | (A <sup>a</sup> , 38 <sup>b</sup> ) | 60.5 $\pm$ 4.7          | (A, 12) |
| Vector control                    | 39.0 $\pm$ 2.4          | (B, 29)                             | 43.0 $\pm$ 8.4          | (A, 8)  |

<sup>a</sup> Figures in columns followed by the same letters are not significantly different at  $p \leq 0.05$  level by Duncan grouping.

<sup>b</sup> number of observations.

**Table 4.5.** *Erwinia* lesion sizes on detached tomato leaflets;. Lesions were generated by inoculating the leaflets with 5  $\mu$ l of *Ecc* strain EC14 ( $A_{600nm} = 1.0$ ; ca.  $4 \times 10^6$  cfu) using gentle application of the pipet tip to create a wound site and incubating in a humid chamber for 36 hours at 28°C. Leaflets were then sandwiched in wet paper towels and incubated in a 28°C incubator till the lesions were well developed (36 to 48 hours). Lesion diameters were measured under a binocular dissecting microscope equipped with an eye piece containing a micrometer. Values are presented as *Ecc* lesion diameter (mm)  $\pm$  standard error.

| Plant line                        | Lesion diameter (mm)                                 |
|-----------------------------------|--|
| Full-length antisense <i>hmg2</i> | 0.92 $\pm$ 0.02 (A <sup>a</sup> , 128 <sup>b</sup> ) |
| Vector control                    | 0.88 $\pm$ 0.01 (A, 188)                             |

<sup>a</sup> Figures in columns followed by the same letters are not significantly different at  $p \leq 0.05$  level by Duncan grouping.

<sup>b</sup> number of observations.

susceptibility of the *hmg2* antisense plant is statistically significant at  $p \leq 0.1$  but not at the level of  $p \leq 0.05$ .

Based on these results, we conclude that antisense tomato *hmg2* rendered the plants more susceptible than controls. Perturbation of *hmg2* expression clearly impacts the defense reaction of tomato to phytopathogens such as erwinias. These data further support results presented in Chapters 2 and 3 that tomato *hmg2* plays a critical role in disease resistance.

#### **Transgenic tobacco plants carrying antisense tomato *hmg2* and antisense tomato *hmg1* showed enhanced disease resistance to *Ecc* and TMV**

Transgenic tobacco carrying the introduced antisense tomato *hmg1*, *hmg2* and *hmg2* full-length were also analyzed by *Ecc* maceration assays and TMV inoculation assays. Quite surprisingly, the antisense tobacco lines either did not differ or were more resistant than vector controls, in contrast to the increased susceptibility observed in homologous *hmg2* antisense tomato plant. Table 4.6 shows that TMV lesion diameters and lesion areas of antisense *hmg1* and *hmg2* were significantly smaller than the untransformed Xanthi wild type. Tobacco plant leaves with the 5' antisense *hmg2* (represented by plant 3117) were most resistant showing the smallest TMV lesions on detached leaves. While antisense *hmg1* transformed tobacco plants (plant 644) also showed smaller lesions compared to Xanthi controls, the lesion sizes were bigger than on plants expressing the 5' antisense *hmg2*. On the other hand, the antisense full-length *hmg2* containing plants (represented by plant 3925) were the same as the vector controls; The lesions on plant 3925 were significantly smaller than nontransformed plants but bigger than plants containing

antisense *hmg1* and 5' antisense *hmg2*. These results although quite unexpected, did suggest that manipulating *hmg* expression by antisense constructs could have a dramatic impact on the TMV disease resistance. The difference in disease responses in the homologous tomato system versus the heterologous tobacco system may reflect a lack of isogene specificity in tobacco. There is significant divergence in nucleic acid sequences (see Introduction and gene construction section) of the tomato and *N. sylvestris* HMGRs although no sequence data is available for *N. tabacum*.

*Ecc* lesions were also smaller on the leaves from tobacco antisense lines versus vector controls as shown in Table 4.7. Once again plant 3117, which carries the 5' antisense *hmg2* has the smallest *Ecc* lesions while plant 644 which has 5' antisense *hmg1* showed smaller lesions compared to vector control plant 64302. Sizes (diameter) of *Ecc* lesions on antisense lines were statistically significant with reductions of 21% (plant 644) to 27% (plant 3117) compared to the vector control (plant 64302).

In an alternate assay procedure which tested tissue loss due to *Ecc* infection, leaf petioles and stem sections from primary transformants of antisense *hmg1* and antisense *hmg2* responded to *Ecc* maceration differently. Leaves from antisense *hmg2* were most resistant showing about 16.8% less tissue loss compared to the vector control, while antisense *hmg1* was not significantly different from the vector control (Table 4.8).

Therefore, the results from two pathogen studies (TMV lesion sizes, *Ecc* lesion sizes and leaf petioles *Ecc* maceration) indicate that plant 3117 which is an antisense *hmg2* line is the most resistant plant, while plant 644, an antisense *hmg1* line, is also resistant but not as much as antisense *hmg2*.

**Table 4.6.** TMV necrotic lesion sizes were reduced on detached transgenic tobacco leaves;. Detached leaves from transgenic tobacco plants containing antisense 5'-*hmg1* (plant 644), 5'-*hmg2* (plant 3117) or *hmg2* full-length (plant 3925) inoculated with 50 µg/ml TMV (strain U1). Lesions were measured 4 days postinoculation. Xanthi plant is the untransformed control. Vector control (plant 64302) contains the T-DNA from binary vector pGA643. Lesion sizes are expressed in diameter (mm) ± standard error.

| Plant line                      | Lesion diameter (mm)                             |
|---------------------------------|--|
| Xanthi                          | 1.14 ± 0.02 (A <sup>a</sup> , 100 <sup>b</sup> ) |
| Vector control (64302)          | 1.07 ± 0.02 (B, 161)                             |
| Antisense full-length (3925)    | 1.05 ± 0.02 (B, 137)                             |
| 5'-antisense <i>hmg1</i> (644)  | 0.89 ± 0.02 (C, 160)                             |
| 5'-antisense <i>hmg2</i> (3117) | 0.76 ± 0.02 (D, 145)                             |

<sup>a</sup> Figures in columns followed by the same letters are not significantly different at  $p \leq 0.05$  level by Duncan grouping.

<sup>b</sup> number of observations.

**Table 4.7.** Soft rot interactions of transgenic tobacco leaves inoculated with *Ecc*;. Inoculation of detached leaves (the 5th to 10th leaf from the top) was identical to that described for tomato (Table 4.5). Values are mean of diameter or area + standard error (S.E.).

| Plant line                       | Lesion diameter (mm)                             |
|----------------------------------|--|
| VecCN (64302)                    | 2.08 ± 0.03 (A <sup>a</sup> , 148 <sup>b</sup> ) |
| 5'- <i>hmg1</i> antisense (644)  | 1.64 ± 0.04 (B, 120)                             |
| 5'- <i>hmg2</i> antisense (3117) | 1.52 ± 0.03 (C, 125)                             |

<sup>a</sup> Figures in columns followed by the same letters are not significantly different at  $p \leq 0.05$  level by Duncan grouping.

<sup>b</sup> number of observations.



**Table 4.8.** Tissue maceration by *Ecc* of transgenic tobacco plants expressing 5'-*hmg2* or 5'-*hmg1* antisense constructs;. Leaf petioles and stem sections were inoculated with 250  $\mu$ l of *Ecc* strain EC14 ( $A_{600nm} = 1.0$ ; ca.  $1.75 \times 10^8$  cfu) and incubated at 28°C for 16 hours. Data are expressed as the mean of percentage of maceration + standard error.

| Plant line                      | Leaf petioles  |                                    |
|---------------------------------|----------------|------------------------------------|
| Vector control (64302)          | 82.8 $\pm$ 3.7 | (A <sup>a</sup> , 7 <sup>b</sup> ) |
| 5'-antisense <i>hmg1</i> (644)  | 76.3 $\pm$ 4.1 | (AB, 10)                           |
| 5'-antisense <i>hmg2</i> (3117) | 68.8 $\pm$ 3.1 | (B, 11)                            |

<sup>a</sup> Figures in columns followed by the same letters are not significantly different at  $p \leq 0.05$  level by Duncan grouping.

<sup>b</sup> number of observations.

## DISCUSSION

Plants have evolved a broad array of defense mechanisms important for disease resistance. These include synthesis of phytoalexin antibiotics and proteinase inhibitors, deposition of cell wall materials, and accumulation of hydrolytic enzymes such as chitinases (Keen 1993). Resistance appears to depend on the ability of the host to rapidly recognize the pathogen and induce these defense responses in order to limit pathogen spread (Cramer *et al.* 1985). HMGR, encoded by four *hmg* isogenes in tomato, is the first regulatory enzyme mediating the production of isoprenoid defense compounds. In order to study the specific effects of *hmg* isogenes on plant disease resistance, antisense approaches were employed in this study. Narita *et al.* (1991) previously reported the cloning and sequencing of tomato *hmg1*, *hmg2*, and *hmg3*. However, only tomato *hmg2* sequence has been published (Park *et al.* 1992). The 5' end cDNA of tomato *hmg1* was also cloned in our lab and characterized (Denbow *et al.* 1995). Based on the available sequence of tomato *hmg2* and partial sequence of *hmg1* we designed the antisense constructs by isolating the *hmg1* or *hmg3*-containing fragments coding for the HMGR amino-terminal domain which is presumably specific for each isogene.

HMGR is encoded by a multigene family in all plant species studied (two to sixteen genes) and in fungal systems (two *hmg* genes for fungi) (Burmester and Czempinski 1994). It was shown that sequence similarity between homologous isogenes from different but related species is higher than the similarity between the member isogenes from the same species (Burmester and Czempinski 1994; Choi *et al.* 1992). We thought that the antisense constructs we designed might work as gene specific antisense genes in a heterologous tobacco system. In retrospect, this experiment indicated that the antisense constructs were

not specifically inhibiting the targeted tobacco defense specific *hmg* isogene. The experiment should have been done in the homologous tomato systems. Due to the relative ease of tobacco transformation, we simultaneously introduced our tomato antisense constructs into the related but heterologous host tobacco. We surprisingly found that our antisense constructs resulted in enhanced disease resistant plants in tobacco. We speculate that the effect might be due to interactions among divergent *hmg* sequences between tomato and tobacco and that the tomato antisense constructs do not function as gene-specific inhibitors in tobacco. The regulation of HMGR isoforms in plants is very complex with evidence for differential control at the gene and protein levels, including mounting evidence suggesting pathway partitioning and HMGR isozyme specialization (Weissenborn *et al.* 1995). Constitutive expression of tomato 5'-*hmg1* (non-defense isoform) and 5'-*hmg2* (defense isoform) antisense in the heterologous system may result in suppression of non-defense-related HMGRs leading to the potential of an increase in substrate availability for the defense-pathway upon pathogen challenge. Because neither tobacco *hmg* gene sequences, isoform-specific antibodies, or isoform-specific activity assays are available, direct testing of this hypothesis must await development of additional molecular tools.

Results in the homologous tomato system with full-length antisense *hmg2* support an important role for tomato *hmg2* in disease resistance consistent with results of Chapter 2 and 3. These results were not as dramatic as the over-expression studies, however, the direct effect of anti-*hmg2* on defense-induced *hmg2* message levels or phytoalexin accumulation was not tested; antisense approaches rarely generate "complete" mutants.

Perhaps most telling, although not statistically valid, the *hmg2* antisense tomato plant was lost to fungal infection. A combination of hot, humid conditions and

overcrowding in the transgenic plant greenhouse in August, 1994, led to significant leaf mold problems. Although fairly aggressive control measures were undertaken, and very few plants were lost, the sole *hmg2* antisense tomato succumbed rapidly to this pathogen. It was unfortunate that this plant could not be tested further either for its disease responses or for effects of the antisense construct on endogenous mRNA levels or phytoalexin production during defense responses. The results obtained, however, do support an important role for tomato *hmg2* in disease interactions.

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## **MATERIALS AND METHODS**

### **Generation of antisense constructs and transformation of tobacco and tomato**

The first 806 bp of tomato *hmg1* exon I was cloned into pBS by PCR designated pCD5 (provided by Cindy Denbow, VPI&SU; Denbow *et al.* 1995). At the nucleic acid

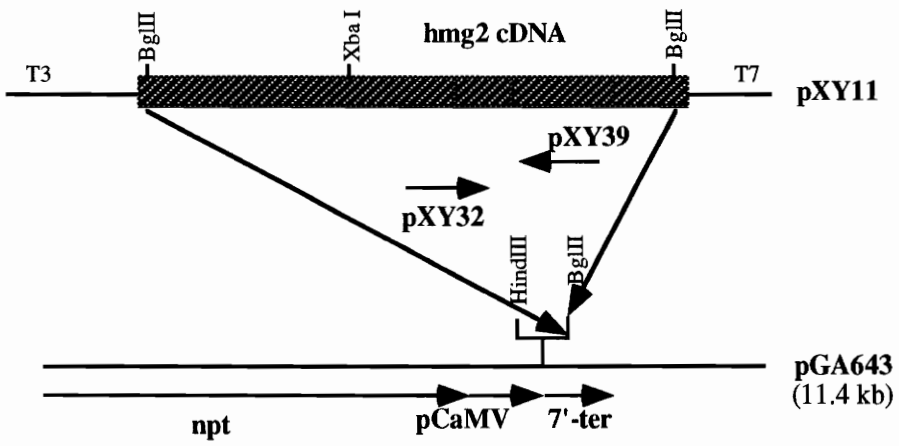
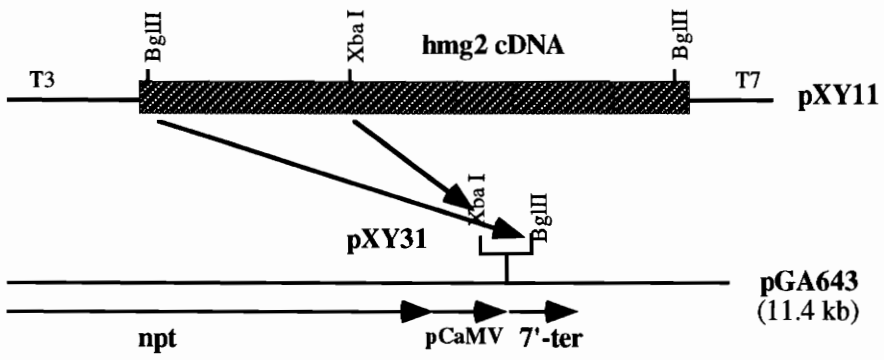
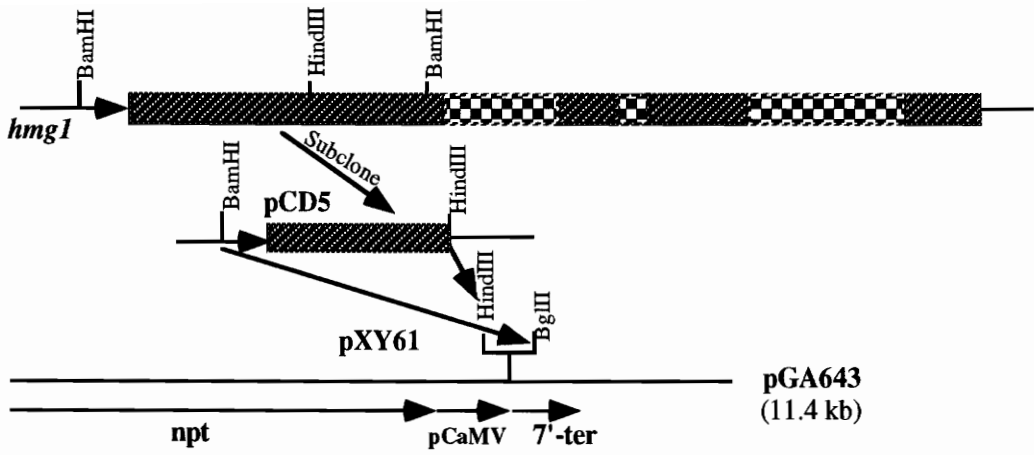
level this region of tomato *hmg1* has only 57% similarity with tomato *hmg2* (Park *et al.* 1992) and 62.7% similarity with *Nicotiana sylvestris hmg* (Genschik *et al.* 1992). Therefore the antisense *hmg1* construct utilized the first 800 bp of tomato *hmg1* contained within two convenient restriction sites *Bam*HI and *Hind*III on pCD5 (Figure 4.2). This fragment corresponds to the 5' end of *hmg1* coding sequence for HMG1 membrane bound domain. The *Bam*HI - *Hind*III fragment from pCD5 was inserted into pGA643 (An *et al.* 1988), a plant expression binary vector, at the sites of *Hind*III and *Bgl*II which resulted in the *hmg1* sequence in the antisense orientation (pXY61, Figure 4.2).

The 5' 803 bp of tomato *hmg2* cDNA located between *Bgl*II and *Xba*I sites were excised from tomato *hmg2* cDNA clone pXY11 (see Chapter 3). This fragment codes for the tomato HMG2 N-terminal membrane bound domain and was inserted into pGA643 at the *Xba*I and *Bgl*II sites (pXY31, Figure 4.2). This fragment of tomato *hmg2* has 73.5% sequence similarity to the *Nicotiana sylvestris hmg*. The resulting antisense *hmg2* construct served as 5' *hmg2* antisense construct (Figure 4.2).

Antisense sequences to the near full length tomato *hmg2* were constructed by inserting the *Bgl*II fragment (1761 bp while the full length is 1803 bp) from pXY11 into the *Bgl*II site of pGA643 (pXY39 and pXY32, Figure 4.2). The antisense orientation construct pXY39 was subsequently used in plant transformation. This portion of the *hmg2* has 84% of similarity to the *Nicotiana sylvestris hmg*. It served as antisense *hmg2* and possibly antisense to all of the *hmg* isogenes due to the high homology at the 3' end among all of the cloned plant *hmg* genes (Figure 4.2).

Tobacco transformation followed the protocol previously described (Burow *et al.*

**Figure 4.2.** Construction of antisense *hmg1*, *hmg2*, *hmg2* full-length in binary vector pGA643. Antisense *hmg1* (pXY61) was constructed by inserting the *Bam*HI & *Hind*III fragment from pCD5 which contains tomato *hmg1* 5' of exon I. Antisense *hmg2* to 5' region (pXY31) was constructed by inserting the 5' *Bgl*III and *Xba*I fragment in reverse orientation into pGA643. Antisense *hmg2* (pXY39) was constructed by inserting the near full-length *hmg2* *Bgl*III fragment into pGA643.



1990). Tomato transformation was followed the procedure of McCormick *et al.* (1986).

### **Nucleic acids isolation and hybridization**

DNA was isolated from tobacco plant leaves using the procedure of Dellaporta *et al.* (1984). Total RNA from young leaves was isolated using the procedure provided by the manufacturer of RNazol (Tel-test, Inc. Friendswood, Texas, U.S.A.) with modifications. A detailed protocol was described in Chapter 2. 20 µg of RNA from each sample was used for northern analysis. The southern and northern gel blotting and hybridization were carried out following the procedures in Sambrook *et al.* (1989) using the Nytran plus membrane. Radioactive probe was labeled using a random primer kit from BRL.

### **TMV inoculation and lesion measurement with detached leaves**

The TMV inoculation and lesion measurement for tobacco plants (transgenic plants or nontransformed controls) followed the methods previously described in Chapter 3.

### ***Ecc* lesion assay; *Ecc* tissue maceration assay**

*Ecc* lesion analysis, *Ecc* maceration assay for both tomato and tobacco plants were done by the methods previously described in Chapter 3.

### **Data calculation and statistical analyses**

Data analysis followed the protocol previously described in Chapter 3.



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## **Chapter 5. Conclusions and future directions**

3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, EC 1.1.1.34) catalyzes reduction of HMG-CoA to mevalonate. This is the key regulatory step in the isoprenoid biosynthesis pathway to cholesterol biosynthesis in mammalian systems (Nakanishi *et al.* 1988). Animal systems have only one *hmg* gene coding for HMGR (Chin *et al.* 1984; Goldstein and Brown 1990). Multiple *hmg* genes have been found in other systems; yeasts have two *hmg* genes (Basson *et al.* 1986; Basson *et al.* 1988). In plants, single *hmg* genes were analyzed (Caelles *et al.* 1989; Learned and Fink 1989; Narita and Gruissem 1989), but later multigene *hmg* gene families were found in several plants (Park 1990; Chye *et al.* 1992; Choi *et al.* 1992; For details, see Chapter 1). Differential expression of *hmg* isogenes under developmental and/or environmental conditions were investigated using mRNA analyses and enzyme activity assays (Choi *et al.* 1992; Chye *et al.* 1992; Yang *et al.* 1991). Promoters of *hmg* isogene using the GUS reporter gene were carried out for the *Catharanthus acuminata hmg1* promoter (Burnett *et al.* 1993), *Arabidopsis thaliana hmg2* (Enjuto *et al.* 1995), and our tomato (*Lycopersicon esculentum*) *hmg2* promoter (Weissenborn *et al.* 1995; Chapter 2). We found that tomato *hmg2* promoter is regulated developmentally showing expression in seedling cotyledons and hypocotyls, in trichomes, and in a variety of reproductive tissues including pollen, stigmas, ovules, petals, and mature seeds of unstressed plants (Chapter 2, Figure 2.2). *hmg2*:GUS activity was rapidly induced by wounding or in response to plant pathogenic viruses and bacteria (Chapter 2, Figure 2.3). *hmg2*:GUS expression was localized to tissue directly surrounding lesions generated through interactions with tobacco mosaic virus (TMV) or plant pathogenic bacterium, *Erwinia carotovora* subsp. *carotovora* (Ecc) (Chapter 2, Figure 2.3). The expression pattern of tomato *hmg2* is consistent with a role in sesquiterpenoid phytoalexin biosynthesis and disease resistance.



Engineering for plant disease resistance has been a major goal ever since beginning of plant genetic engineering. The first genetically manipulated plants with enhanced disease resistance were tobacco plants constitutively expressing TMV coat protein gene (Beachy *et al.* 1987). Since then, genes encoding fungal cell wall lytic enzymes such as chitinase genes were utilized to generate disease resistant plants (for review, see Lamb *et al.* 1992). Our study of overexpressing tomato *hmg2* to affect disease resistance represents the first demonstration that manipulation of the rate-limiting step in a major defense compound biosynthesis pathway provides a successful strategy for enhancing disease resistance (Chapter 3). We cloned tomato *hmg2* cDNA by PCR, expressed it in *E. coli* to confirm its HMGR activity, inserted the active cDNA behind the double enhanced CaMV 35S promoter, and engineered the overexpression construct into tobacco (*Nicotiana tabacum* cv. Xanthi nc). Southern and northern analyses confirmed transformation and *hmg2* message expression. Enzyme activity was enhanced compared to nontransformed plants. Analysis of selected transgenic plants revealed significantly reduced tissue maceration by *Ecc* assessed by *in vitro* leaf petiole and stem section maceration assays (Chapter 3). The mean size of necrotic lesions induced by TMV was also significantly reduced compared to the nontransformed or vector controls (Chapter 3). Prior to publication of this study, additional assays of 1) basal HMGR activities, 2) HMGR activities 4 days after TMV inoculation, and 3) phytoalexin concentrations 4 days after TMV inoculation will be performed for all transgenic plants used for the disease assays.

As a control of the overexpression studies, we also generated transgenic tobacco and tomato containing antisense constructs for tomato *hmg1* and *hmg2* to study their effect on disease resistance (Chapter 4). Tomato plants expressing the full-length *hmg2* antisense showed lower HMGR enzyme activity and were more susceptible to soft rot by *Ecc* than

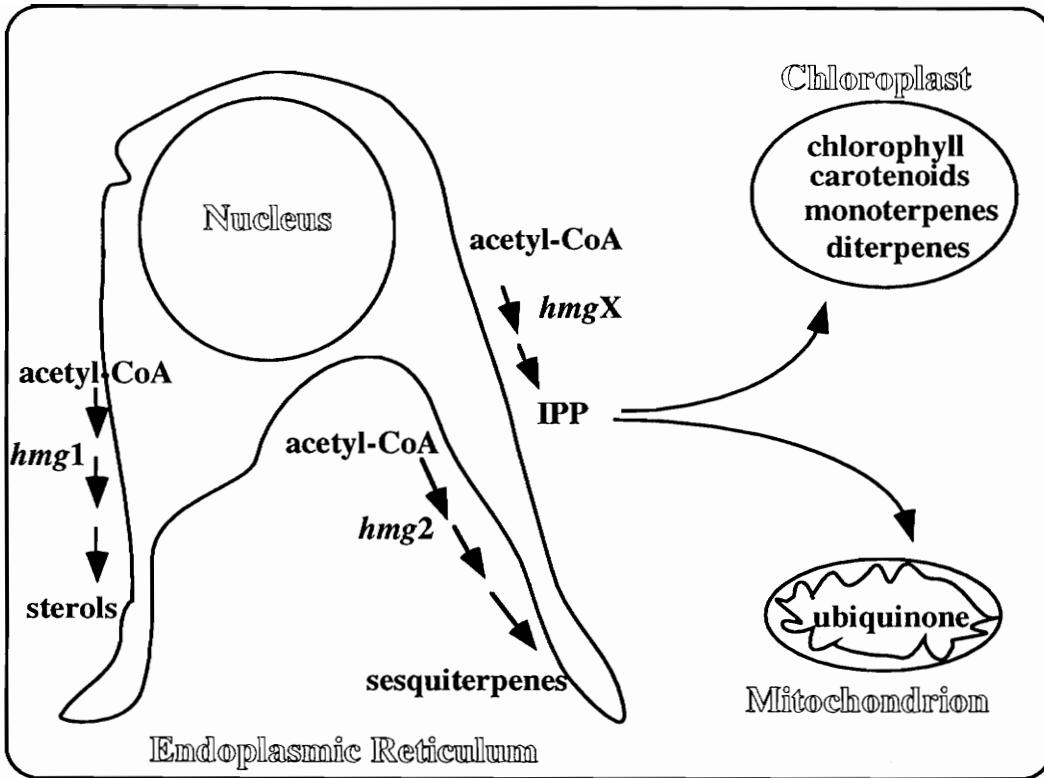
control plants. In contrast, expression of either antisense *hmg1* or antisense *hmg2* in the heterologous tobacco system resulted in plants with enhanced resistance to *Ecc* and reduced TMV lesion sizes. These results, while surprising, may indicate that antisense inhibition is non-specifically exerted on isogenes other than the tobacco defense-specific *hmg* which is quite divergent from tomato *hmg2* defense-specific gene.

Our detailed study of tomato *hmg2* isogene in plant defense responses suggest that specific *hmg* isogenes maybe involved in distinct group of isoprenoid biosynthesis (Figure 5.1; Chappell 1995). *hmg2* might involved in specific defense compound biosynthesis such as sesquiterpenoid phytoalexins (Figure 5.1; Chapter 1, Figure 1.1). Other important compound such as cytokinins, abscisic acid, and gibberellins might also be catalyzed by *hmg2*. Overexpression tobacco line 2421, which has the highest HMGR activity and has prolonged vegetative stages, imitates the giant tobacco photoperiod mutant "Maryland Mammoth". This suggests that may have some relationship with the phytochrome signal transduction pathway or has something to do with gibberellic acid biosynthesis. Overexpression line plant 2414 has a chlorotic phenotype. Only some of the branches are chlorotic. Within one primary plant, chlorotic and green branches exist at the same time suggest some mosaic pattern at the meristematic region. The chlorotic pattern mimics the natural "ghost" mutant in the virology textbook (Matthews 1991). Carotenoids and chlorophylls are two groups of isoprenoid synthesized in chloroplasts. Specific HMGR might involved in these photosynthetic pigment synthesis. The fact that plant 2414 is chlorotic and another 5' anti-*hmg1* line 603 is also chlorotic indicated the possible role of HMGR in pigment production.

Products of these *hmg* isogene might be in a loosely bounded membrane-bound

multienzyme complex which will be dedicated to specific branch pathway isoprenoid biosynthesis. Pathway aggregates in the living cells are well documented by the electron transport pathway in the mitochondria and chloroplasts. The electron transfer chains are buried in the membrane and transfer the electron along with the energy associated with the transfer step by step in an organized way. The mammalian fatty acid biosynthesis pathway is another example of enzyme complex in the biosynthesis of particular products by a group of enzymes. It is hard to imagine that a well organized living cell would depend on the random migration of crucial biosynthetic intermediates to specific enzyme location and the product of that catalysis would be randomly pass to the next enzymatic step in isoprenoid biosynthesis.

In the near future, useful projects related to this research would be the following: 1. To further test the disease resistance of overexpression lines with other pathogens such as root knot nematodes, fungi, and parasitic weeds. Extensive field trials will be needed for fully evaluate the potential of overexpression of tomato *hmg2* on disease resistance. 2. To transform potato plants with the overexpression constructs will test the effect of overexpression tomato *hmg2* on disease resistance and sesquiterpenoid phytoalexin biosynthesis in potato. There have been intensive research in potato on the fungal pathogen *Phytophthora infestans*. It would be worthwhile to test whether overexpression tomato *hmg2* will enhance disease resistance to this pathogen. 3. To further characterize plant lines 2421 and 2414 for information on relationships of *hmg2* with GA or other phytohormones or carotenoids biosynthesis. 4. To genetically cross overexpression and the antisense plants or cross overexpression lines or antisense lines with *hmg2*:GUS plants to understand the function of that particular isogene. 5. To use the *hmg2*:GUS transgenic plants to test the



**Figure 5.1.** A hypothetical model for the subcellular localization and metabolic channels of tomato isoprenoid biosynthesis pathways. Modified from Chappell (1995). Sesquiterpenes, such as capsidiol and debneyol, are involved in plant defense to pathogen challenge.

effect of bleaching herbicides such as 2-[(2-chlorophenyl)methyl]-4,4-dimethyl-3-isoxazolidone (clomazone) on the expression of *hmg2*. Bleaching herbicides which inhibit enzymes further down the pathway for carotenoids biosynthesis should have an effect on HMGR (Ji and Hatzios 1992). MUG assay will give the time course data while X-Gluc staining will give a rough idea of what kinds of effects the herbicides may have. Caution should be taken to figure out ways to eliminate the interference of wound response of *hmg2*. Herbicide safeners which are chemicals protecting crop plants from herbicide injury (Hatzios and Hoagland 1989) may serve also as a inducer of *hmg* gene expression. By applying safener to the tobacco seedlings and then monitor the activity of GUS, it should tell us whether safener has an effect.

Transgenic tobacco, tomato and potato which have the overexpression *hmg2* genes may be marketed as real crops for the market. There is certainly some potential for practical use for this gene construct.

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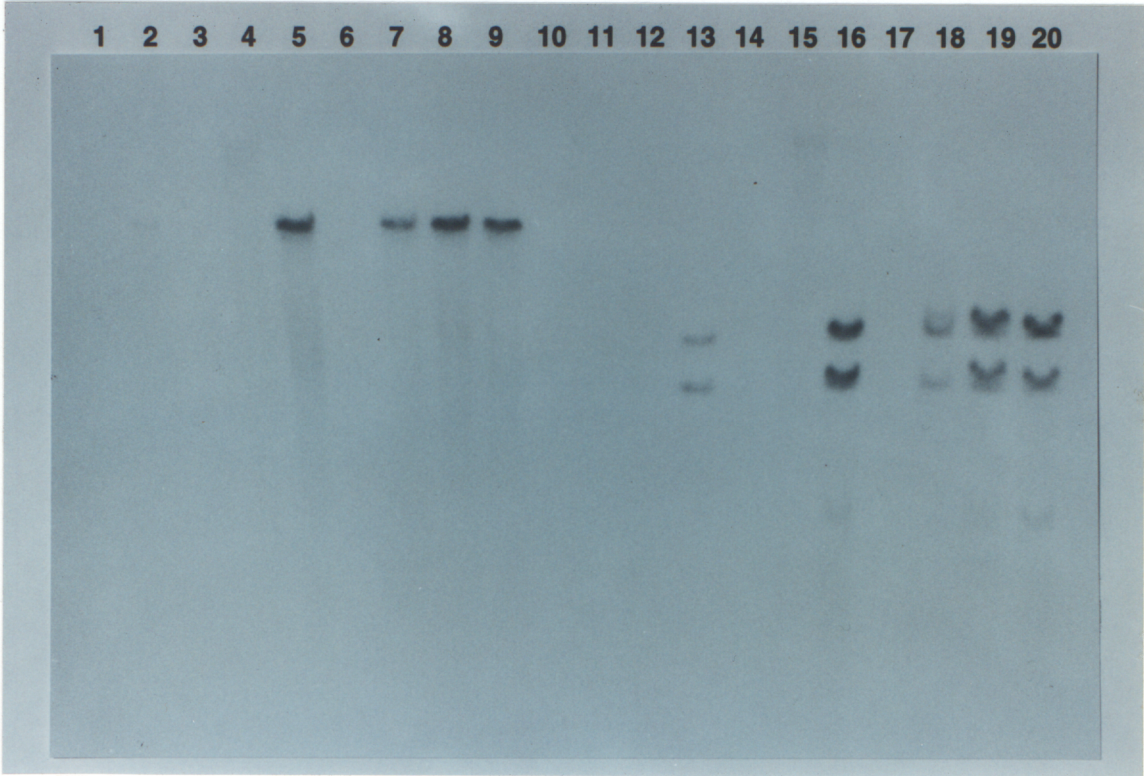


## **Appendix**

### **Plant 002 contains two copies of *hmg2*:GUS at one locus**

One transformed Xanthi tobacco line, 002, had two copies of the transgene linked head to head (Right border to left border : left border to right border) inserted at one locus (Figure A.1). DNA from eight 002 R1 generation plants (R1p1 to R1p8), 002 R0 plant, and control DNA from nontransformed tobacco (Xanthi) were analyzed using Southern blot analysis. The blot was probed with the <sup>32</sup>P labeled GUS gene fragment isolated by gel purification from *Sma*I and *Sac*I or *Xba*I and *Sac*I digested pBI221 from Clontech (CA). The genomic Southern blot analysis showed there was only one band when digested with *Eco*RI (about 20kb) and two bands when cut with *Hind*III. Based on the pattern of Southern (Figure A.1) and the structure of the construct (Chapter 2, Figure 2.1), it was concluded that there was a single insertion with two consecutively linked t-DNA into the same place (Right-left:left-Right). R1p1, R1p3 and R1p6 were GUS staining negative and the Southern analysis confirmed that there were no GUS insert in these R1 plants. R1p2, R1p4, R1p5, R1p7 and R1p8 and R0 were GUS staining positive and the Southern analysis confirmed the GUS existence. The GUS staining pattern was in the ratio of 3:1 (Table A.1). Therefore the one insertion conclusion is correct.

**Figure A.1.** Southern analysis of R0 and R1 generation of transgenic tobacco line 002. Primary transformant 002 (R0) and 8 of its R1 generation plants were analyzed. 12 µg of genomic DNA was digested with *EcoRI* or *HindIII*. Lane 1 to lane 8 are R1 plant genomic DNA digested with *EcoRI* (p1, p2, p3, p4, p5, p6, p7, p8); lane 9 is R0 plant DNA, lane 10 is control nontransformed Xanthi plant DNA digested with *EcoRI*; Lane 11 is 1 molecular marker; Lane 12 to lane 19 are R1 plant genomic DNA digested with *HindIII* (p1, p2, p3, p4, p5, p6, p7, p8); Lane 20 is R0 genomic DNA digested with *HindIII* and probed with GUS gene.



**Table A.1.** GUS staining pattern of R0 and R1 generation of transgenic tobacco line 002. "-" no blue staining; "+" positive staining; "++" strong positive staining. Segregation will occur in the R1 generation. "-" is recessive homozygous; "+" is hybrid; "++" is homozygous positive.

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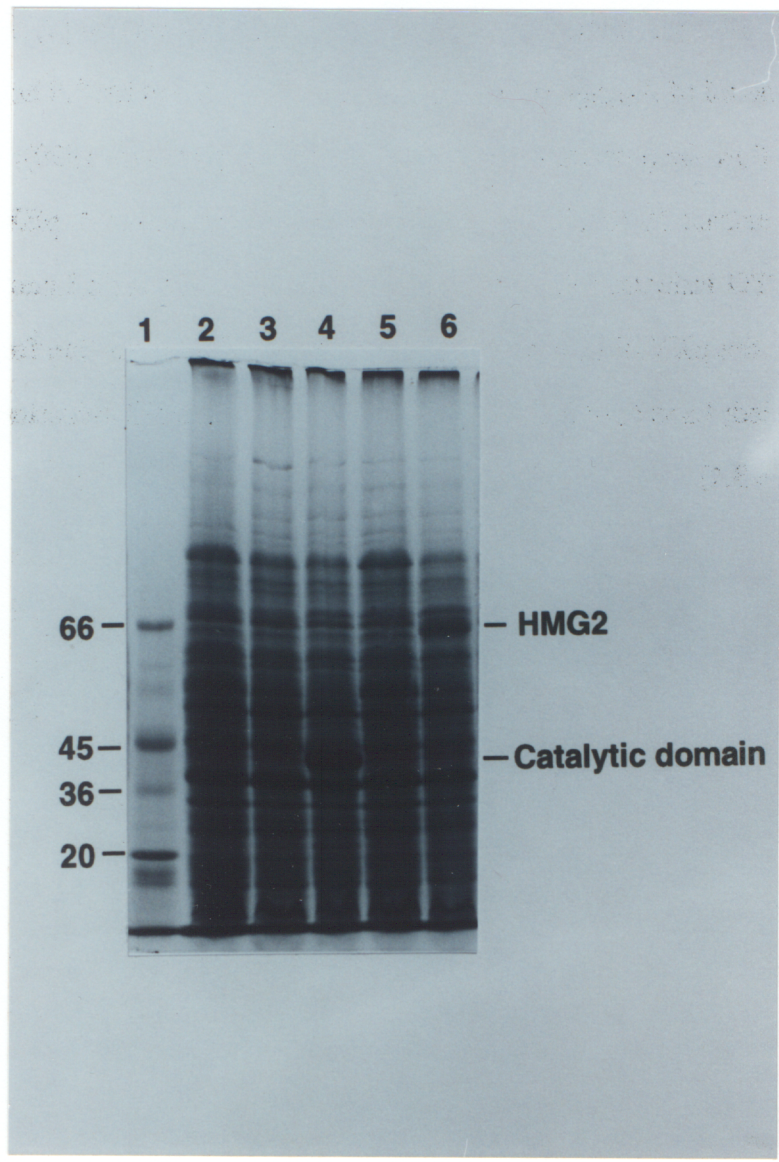
|              |   |    |   |   |    |   |   |   |
|--------------|---|----|---|---|----|---|---|---|
| GUS staining | 1 | 2  | 3 | 4 | 5  | 6 | 7 | 8 |
|              | - | ++ | - | + | ++ | - | + | + |

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## **Expression of tomato *hmg2* in *E.coli* revealed new plasmid dependent proteins**

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of crude *E. coli* extracts revealed new, plasmid-dependent protein bands of the expected sizes for the expressed HMGR proteins (65 kD for full length and 40 kD for the catalytic domain only Figure A.2).

**Figure A.2.** Expression of tomato *hmg2* in *E.coli* revealed new plasmid dependent proteins. Crude *E.coli* extracts were run with 4% stacking gel and 10% separation SDS-PAGE and stained with Coomassie blue R-250. Lane 1, protein standards 30  $\mu$ l; The molecular weight of the markers from top to bottom are 66 kD, 45 kD, 36 kD, 29 kD, 20 kD, 18.4 kD, and 14.3 kD; Lane 2, *E. coli* host strain BL21 extract 30  $\mu$ l, IPTG induction for 3.5 hours (One ml of  $A_{600nm} = 0.6$  culture in LB was induced for 3.5 hours with IPTG and processed follow the procedure previously described Ferrer *et al.* 1990); Lane 3, vector pT7-7 in BL21 extract 30  $\mu$ l IPTG induction for 3.5 hours ; Lane 4, pEXY10 in BL21 extract 30  $\mu$ l IPTG induction for 3.5 hours (Chapter 3, Table 3.1); Lane 5, expression construct with entire pXY10 fragment in pT7-7 in BL21 IPTG induction for 3.5 hours, 30  $\mu$ l of extract loaded; Lane 6, pEXY11 in BL21 extract 30  $\mu$ l IPTG induction for 3.5 hours (Chapter 3, Table 3.1)



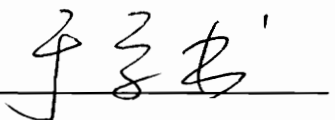


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