# Chapter III
# Localized Gene Expression in Response to Parasitization by *Orobanche*

## III.1 ABSTRACT

In order to control *Orobanche* spp. (broomrapes), it will be important to understand the molecular-level interaction between the host and the parasite. As *Orobanche* penetrates and forms connections to the host vascular tissue, the host reacts to this invasion with attempts at self-defense. Previous work has shown that genes involved in localized induction of isoprenoid phytoalexin synthesis (*hmg2*), are expressed at the site of *O. aegyptiaca* parasitization in transgenic tobacco. To expand on this finding, we have studied other key genes in the isoprenoid pathway including *hmg1*, which is an isogene of *hmg2*, but differs in that its expression is linked to plant development and is induced in response to wounding rather than by specific pathogen interactions. Squalene synthase is similar to *hmg1* in that it is also known to play a role in general cell growth and maintenance processes and is induced in response to wounding, but is repressed in response to pathogen elicitors. Host gene expression was studied using transgenic tomato plants containing *hmg1* promoter-GUS fusions, and northern hybridization analysis of tobacco roots using probes specific for *hmg1* or squalene synthase. Results indicated that expression of *hmg1* was induced, while squalene synthase was repressed in tissue parasitized by *O. aegyptiaca*. Together, these results indicate a complex response to the parasite. Whereas *hmg1* induction is consistent with *Orobanche* inflicting a simple wound-like injury, the repression of squalene synthase is consistent with plant recognition of a pathogen attack. By comparing the regulation of these defense genes in response to *Orobanche* attack, we are able to gain a greater understanding of host response to parasitization.

## III.2 INTRODUCTION

Parasitism of a plant by *Orobanche* spp. is a complex interaction that involves invasion by the parasite and subsequent responses (including defense-related) by the host. The parasite must physically enter the host root, a process that involves the *Orobanche* seedling adhering itself to the host root (Joel and Losner-Goshen, 1994) and penetrating with the aid of lytic enzymes (Ben-Hod *et al*., 1993). For *Orobanche,* these enzymes include a combination of pectin methylesterase and polygalacturonase to loosen the adhesion between host cells and allow the haustorium to grow between the host cells (Ben-Hod *et al*., 1993, Joel *et al*., 1994). At this time, the cells of the haustorium are

closely associated with the host cells, and *Orobanche* vascular tissue establishes connections with those of the host phloem (Dörr 1996). *Orobanche* then acts as a sink to withdraw carbon, water, and mineral nutrients from the host.

Plants have evolved multiple mechanisms to protect themselves against the wide array of pathogens with which they are confronted. These defenses may be pre-existing or may be specifically induced in response to pathogen attack. With respect to inducible defenses, a plant under attack must redirect metabolic resources from normal growth to a defense strategy. The strategy ultimately employed is determined by the plant's perception of the attacking agent, but generally two major signaling pathways are involved. One of these is the jasmonic acid (JA) pathway, which is triggered by wounding or insect feeding and is mediated by JA and ethylene. This pathway is known to result in the induction of proteinase inhibitor (PI) genes (Ryan, 1990), as well as salicylic acid (SA)-independent pathogenesis-related (PR) genes (Pieterse and Van Loon, 1995). A second defense-signaling pathway leads to systemic acquired resistance (SAR), which develops both locally and systemically such that resistance appears in plant parts distant from those originally exposed to the pathogen. This pathway develops in response to recognition of specific pathogens and is mediated by SA. Induction of this pathway is associated with SA-dependent PR proteins and the hypersensitive response (HR) which results in necrotic lesions (Hammerschmidt, 1999).

Phytoalexin production induced by wounding and pathogens can be attributed in part to altered regulation of the isoprenoid biosynthetic pathway (Fig. I.2). The first committed step of the isoprenoid pathway is mediated by the enzyme 3-hydroxy-3-methylglutaryl CoA reductase (HMGR), the activity of which is regulated at the transcriptional level by the differential expression of a family of genes (Fig. I.2). In tomato (*Lycopersicon esculentum* Mill.), HMGR consists of a family of at least four genes that differ in their expression pattern in response to developmental changes, pathogen attack, elicitor treatment, or wounding (Chappell, 1995; Weissenborn *et al*., 1995; Denbow *et al*., 1995). One of these isogenes, *hmg2*, is specifically activated during defense responses associated with the production of sesquiterpene phytoalexins (Cramer *et al*., 1993; Chappell, 1995). The expression of *hmg2* in response to parasitization by *O.*

*aegyptiaca* indicates that the host recognizes *O. aegyptiaca* as an attacker (Westwood *et al*., 1998).

In addition to phytoalexins, the isoprenoid pathway produces many important compounds needed for general metabolism and growth. A second isogene encoding HMGR, *hmg1*, is expressed in early stages of fruit ripening in tomato (Narita and Gruissem, 1989) and in processes related to cell division and growth (Denbow *et al*., 1995). Another enzyme, squalene synthase occurs further down the pathway and seems to be coordinately regulated with *hmg1* to divert carbon toward the production of sterols (Fig. I.2) (Zook and Kuć, 1991). Farnesyl pyrophosphate (FPP) is the intermediate positioned at this regulatory point and can be partitioned toward different end products (Vögeli and Chappell, 1988).

Our objective is to understand the molecular-level interactions that occur during *Orobanche* parasitization with the long-term goal of developing genetically engineered crops resistant to *Orobanche*. This chapter focuses on the expression patterns of key genes in the isoprenoid pathway in order to characterize host perception of parasitism.

## III.3 MATERIALS AND METHODS

### III.3.1 Tissue Preparation for Northern Analysis

Tobacco (*Nicotiana tabacum* L. var. Coker) plants were grown from seed in soil until approximately 6 cm in height, at which time they were transplanted into polyethylene (PE) bags [as described in Westwood *et al*., (1996)] containing glass fiber filter paper and watered with 0.5X Hoagland solution (Hoagland and Arnon, 1950). Plants were grown under 100 μmol/$m^2$/sec light, 12 hr. days at 25 ± 3°C. *O. aegyptiaca* seeds were surface sterilized using 70 % EtOH and 1 % sodium hypochlorite. Seven days after transplanting, plants were divided into treatment groups (Fig. III.1). One set was inoculated using a paint-brush to apply sterilized *O. aegyptiaca* seeds directly to the root. Seeds were brushed around and against the roots to form a nearly continuous band. Seeds were placed only on roots sections located 1 cm down from the shoots and 1 cm up from the bottom of the bag. Numerous seeds were applied in order to obtain a maximum number of attachments. The *O. aegyptiaca* seeds, which require a period of pre-conditioning prior to germination, were then allowed to precondition on the tobacco roots for seven days. Plants were subsequently stimulated to germinate by injecting 10 mL of

a 1 mg/L solution of GR-24, a known germination stimulant for *Orobanche* (Mangnus *et al*., 1992), into the back of each PE bag. Thirteen days after the addition of GR-24, unattached *O. aegyptiaca* seedlings were removed by rinsing the roots with a gentle stream of water. Entire root sections present in the region of inoculation were then harvested. Control tissues consisted of non-inoculated roots that had received either no treatment, one of two treatments applied directly to the roots prior to harvest: 10 mL cellulase (1µg/ml) simulated production of digestive enzymes by a pathogen, and 10 mL SA (50mM) simulated induction of SAR-related responses, or treatment by removing shoots from roots and then cutting roots with a scalpel into 1-cm sections to simulate a physical (wound) injury. Plants receiving SA, cellulase, or wounding treatments were placed at room temperature for 6 hr to allow for gene induction. Then SA and cellulase treated roots were excised from shoots, and all roots were blotted to remove excess moisture, weighed, frozen in liquid $N_2$, and stored at -80°C until extraction. Control and *O. aegyptiaca*-infected tissues were harvested at the same time.

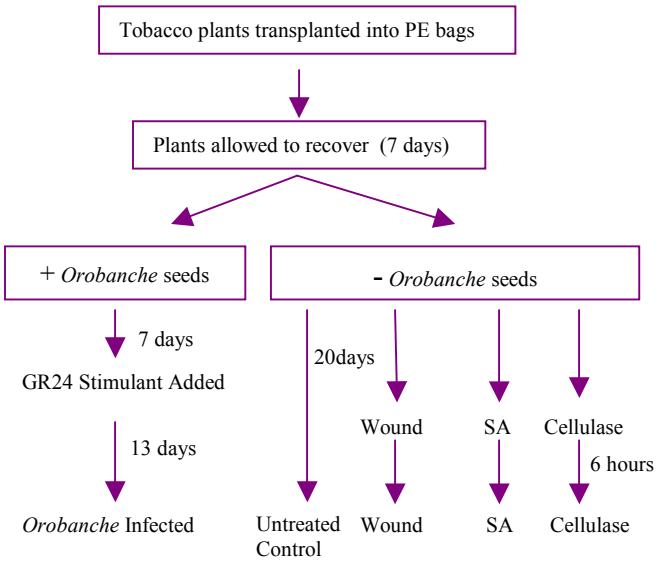


Figure III.1. Schematic diagram of plant treatments for mRNA analysis. All treatments were transplanted on the same day and harvested on the same day. Wound, salicylic acid, and cellulase-treated roots were included to represent different types of stresses that are known to elicit specific defense responses.

**III.3.2 Extraction and Northern Analysis of Total RNA**

Total RNA was isolated (Qiagen, Valencia, CA) and 20µg of each sample separated on a 0.8% agarose formaldehyde gel. Samples were concentrated using a speed-vac and mixed with 10X MOPS/EDTA, 3.5 µl formaldehyde, 10.0 µl de-ionized formamide, and 2 µl loading buffer to obtain a final RNA concentration of approximately 1 µg/ul. Samples were loaded onto the gel and run at 60V for one hour and then at 100V for an additional hour. RNA was then transferred to an uncharged nylon membrane (ICN, Costa Mesa, CA). Membranes were pre-hybridized in Church buffer (1 % BSA, 1 mM EDTA pH 8, 0.5 M

$NaHPO_4$ pH 7.2, 7 % SDS) for one hour and then hybridized overnight (60°C for squalene synthase and 50°C for *hmg1*) in the presence of 50 ng probe labeled using digoxygenin (dig)-labeled dUTPs (Roche Molecular, Indianapolis, IN). Wash steps were performed initially at the hybridization temperature (1 mM EDTA, pH 8, 40 mM $NaHPO_4$, 1 %SDS), and then at room temperature (1mM EDTA, 40 mM $NaHPO_4$). A blocking reagent (Roche Molecular, Indianapolis, IN) was then applied to the membrane. A 1:15,000 dilution of anti-dig antibody was prepared in blocking buffer and added to the membrane after the initial blocking step. At the end of this time, the membrane was washed with a buffer including 3 % Tween and equilibrated in a detection buffer (100 mM Tris, pH 9.5, 150 mM NaCl). The membrane was then agitated in a solution containing the detection buffer along with CDP-Star (Roche Molecular, Indianapolis IN) which allowed for chemiluminescent detection. The blot was exposed to x-ray film at varying time intervals and developed.

### III.3.3 Transformation and Probe Labeling

pBluescript plasmids containing a 0.9 Kb *Xba*1-*Hind*III fragment for tomato *hmg1* kindly provided by Dr. Cynthia Denbow (Virginia Tech), and a tobacco 1.2 Kb *Eco*RI-*Bam*HI fragment for squalene synthase, a gift from Dr. Chappell (University of Kentucky) were transformed into DH10B *E. coli* cells using a Beckman electroporator. Successful transformants were grown overnight in LB medium with 100 µg/mL ampicilin. Plasmids were purified for use in subsequent PCR reactions. Probes were labeled by incorporating 1 mM dig dUTP's (Roche Molecular, Indianapolis, IN) in the reaction mix along with 20 µM T3 and T7 primers, 2 mM dNTPs, and 2.5 units Taq. The PCR conditions were as follows: (1) 94°C, 1 minute; (2) 49°C, 1 minute; (3) 72°C, 2 minutes, 49 cycles. Dig labeled products were purified (Quiagen, Valencia, CA) and then quantitated against control DNA.

### III.3.4 Treatment of *hmg1*-GUS Tomato Plants

Transgenic tomato plants containing the 1.8Kb *hmg1* promoter fused to the GUS reporter gene were grown, transplanted to PE bags, and inoculated with *O. aegyptiaca* seeds as described above. The seeds were preconditioned in the bags for 1 week and then GR-24 was added. At various times after parasite attachment, plant root tissue was harvested, vacuum infiltrated in X-gluc (5-bromo-4-chloro-3 indolyl- β-D glucuronide)

solution, and incubated overnight at 37ºC (Jefferson, 1987). The stained tissue was observed using a stereomicroscope to determine GUS expression.

## III.4 RESULTS AND DISCUSSION

Studying the induction patterns of host plant genes in response to parasitization can provide a greater understanding of host-parasite interactions. With respect to local defense responses, it is known that *hmg2* is expressed in wounded and elicited tissue as well as in tissue that has been parasitized by *O. aegyptiaca* (Westwood *et al*., 1998). These findings provided evidence of host plant up-regulation of the isoprenoid pathway leading to the production of phytoalexin defense compounds in an attempt to combat parasitization of the host root. In order to expand on this research, a more complete analysis was made of genes involved in isoprenoid metabolism.

Transgenic plants containing the GUS reporter gene provide a means of studying temporal and spatial aspects of gene expression. Analysis of tomato plants containing an *hmg1* promoter fused to the GUS gene indicated that *hmg1* gene expression associated with tubercles was evident at 8, 11, and 14 days after *O. aegyptiaca* germination (Fig. III.2). This represents parasitic attachments ranging from young to well-established (Fig. III.2 A-C). The GUS expression was greatest at the point of parasite attachment and indicated a localized response. Furthermore, the staining appeared to be associated primarily with tissues of the stele. Expression patterns of *hmg1* in non-infected tissues (Fig. III.2 D) or those away from the point of parasite attachment were consistent with the known roles of *hmg1* in tissues associated with active cell division and growth (Jelesko *et al*., 1999).

To further study *hmg1* gene expression, mRNA levels expressed in response to *O. aegyptiaca* parasitization were analyzed using northern hybridization. Treatments included non-inoculated control, *O. aegyptiaca* parasitized, and cellulase-treated root tissue samples. *hmg1* was expressed in non-inoculated control tissues and at approximately the same level in the cellulase and *O. aegyptiaca* infected tissue samples (Fig. III.3 A). This similar level of expression in control and infected tissues is not surprising given the general metabolic role of *hmg1* discussed above, such that the shifts in *hmg1* expression to points of parasite attachment as observed in the promoter-GUS plants may not be easily detected as a change in total *hmg1* mRNA levels.

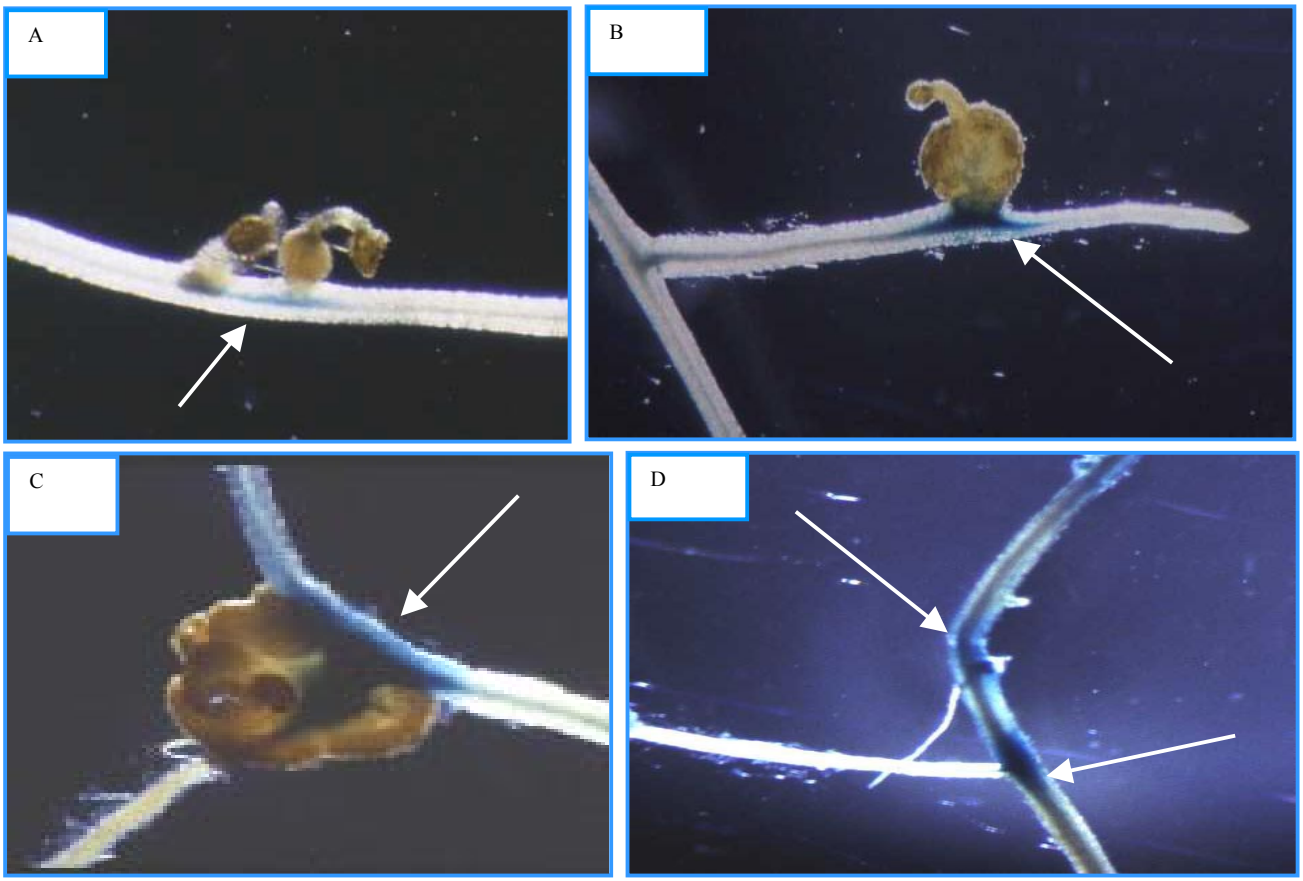


Figure III.2: GUS expression in transgenic *hmg1*:GUS tomato plants. **A**) 8 days (16X) **B**) 11 days (13X) **C**) 14 days after parasitic seedling germination (31X). **D**) *hmg1* gene expression in control (non-infected) tissue (10X). GUS expression is indicated by dark areas in the light roots, with major areas of expression indicated by arrows.

The method of harvesting infected tissue, which involved collecting lightly infected root sections along with heavily infected tissue also resulted in a dilution of final *O. aegyptiaca*-induced *hmg1*:GUS expression in infected samples. Since *hmg1* is a low expresser even in non-parasitized tissue, differentiating changes in mRNA levels in *O. aegyptiaca* infected tissue as compared to untreated control tissue is difficult.

Plants are able to discriminate wound and pathogen signals and possess the ability to alter gene expression in response to the specific type of attack (Stermer and Bostock, 1987, Vögeli and Chappell, 1988; Chappell *et al*., 1991; Zook and Kuć, 1991;Choi *et al*., 1994;). Results obtained from *hmg1* gene expression in response to parasitization suggest host plant recognition of a wound-like event. On the contrary, analysis of squalene synthase expression in response to parasitism is more consistent with a pathogen

interaction. Squalene synthase is known to be induced by wounding, yet suppressed in response to fungal pathogen elicitors (Chappell, 1995; Weissenborn *et al*., 1995). Our results demonstrate that squalene synthase was suppressed in parasitized tissue as compared to all other treatments (Fig. III.4 A). Control and wounded roots showed greatest squalene synthase expression, while cellulase and SA treatments reduced expression. These results are consistent with data obtained by Choi *et al*. (1994) in which a decrease in the level of steroid derivatives and an increase in the production of sesquiterpenoid phytoalexins was reported in wounded potato tubers exposed to elicitors or isolates of pathogens that induce HR.

The expression patterns of *hmg2*, *hmg1*, and squalene synthase in response to *O. aegyptiaca* indicate a coordination of metabolic events in the isoprenoid pathway. Upon parasitization, the host plant appears to recognize the mechanical wound that occurs during parasite attachment and penetration of the host vascular tissue. At the same time, it also appears that the host plant perceives *O. aegyptiaca* as an invasive pathogen,

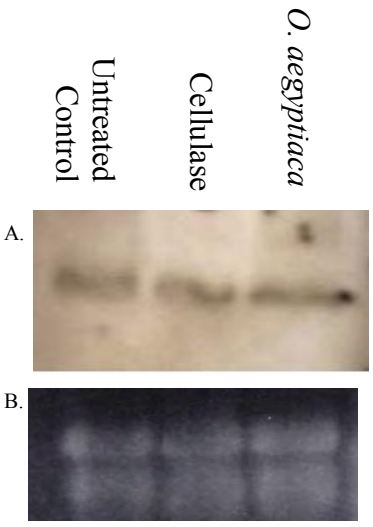


Figure III.3:A) Northern hybridization of tobacco root RNA with a *hmg1* probe. Treatments were untreated control, cellulase, and parasitized by *O. aegyptiaca*. B) Ethidium bromide stained gel demonstrating equal sample loading.

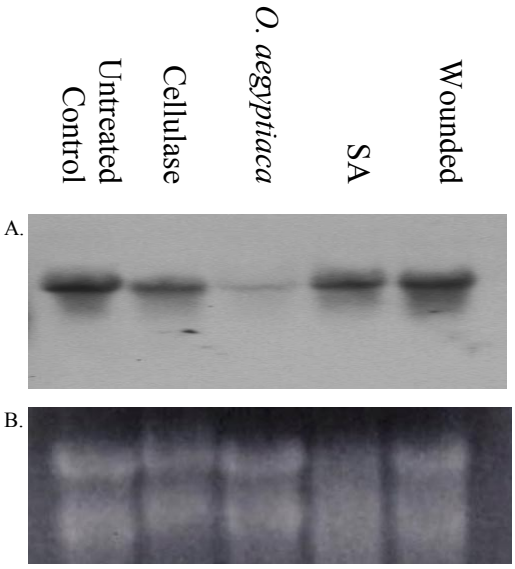


Figure III.4:A) Northern hybridization of tobacco RNA with a squalene synthase probe. Treatments were untreated control, cellulase, *O. aegyptiaca* parasitized, salicylic acid (SA), and mechanically wounded. B) Ethidium bromide stained gel demonstrating equal sample loading.

perhaps due to the production of lytic enzymes by the parasite. This recognition prompts the host plant to alter gene expression by repressing squalene synthase, and redirecting metabolic energy towards the production of plant defense compounds (Fig. I.2). It is not clear whether *O. aegyptiaca* parasitization causes the host to up-regulate the expression of other genes, such as sesquiterpene cyclase, involved in the branch pathway leading to the production of phytoalexins. Our attempts at studying sesquiterpene cyclase gene expression in response to *Orobanche* have yielded ambiguous results and further work is needed to characterize this gene.

These results have raised several questions as to why plants are unable to defend themselves against *Orobanche* parasitization despite the induction of defense-related genes. Perhaps the mRNA from these genes is not translated into enzymes, or the defense compounds produced may be degraded by the parasite. In addition, phytoalexins produced by the host could be specifically toxic to other classes of pathogens such as microbes or fungi and have little or no effect on an angiosperm such as *O. aegyptiaca*. Finally, in contrast to many fungi or bacteria, *Orobanche* avoids penetration of plant cells and may rely on avoiding the release of compartmentalized defense compounds such as phytoalexins. In order to more fully understand these host defense responses, experiments analyzing levels of defense enzymes and phytoalexins within the host plant are needed.

## III.5 ACKNOWLEDGEMENTS

This project was supported by USDA NRI award # 97-35315-4206. The authors are grateful to Dr. C. Denbow, and Dr. J. Chappell for probes and Dr. J. Jelesko for *hmg1*-GUS tomato plants.

## III.6 REFERNCES

Ben-Hod, G., Losner, D., Joel, D.M., and Mayer, M. 1993. Pectin methylesterase in calli and germinating seeds of *Orobanche aegyptiaca*. *Phytochemistry* 32:1399-1402.

Chappell, J. 1995. The biochemistry and molecular biology of isoprenoid metabolism. *PlantPhysiology* 107:1-6.

Chappell J., Vonlanken, C., and Vögeli, U. 1991. Elicitor-inducible 3-hydroxy-3-methylglutaryl coenzyme A reductase activity is required for sesquiterpene accumulation in tobacco cell suspension cultures. *Plant Physiology* 97:693-698.

Choi, D., Bostock, R.M., Avdiushko, S., and Hilderbrand, D.F. 1994. Lipid-derived signals that discriminate wound- and pathogen- responsive isoprenoid pathways in plants: Methyl jasmonate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3-methylglutaryl-coenzymeA reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. *Proceedings of the National Academy of Science USA* 91:2329-2333.

Cramer, C.L., Weissenborn, D.L., Cottingham, C.K., Denbow, C.J., Eisenback, J.D., Radin, D.N., and Yu, X. 1993. Regulation of defense-related gene expression during plant-pathogen interactions. *Journal of Nematology* 25:507-518.

Denbow, C.J., Lång S., and Cramer, C. 1995. The N-terminal domain of tomato 3-hydroxy-3-methylglutaryl-CoA reductase. *Journal of Biological Chemistry* 271: 9710-9715.

Dörr, I. 1996. New results on interspecific bridges between parasites and their hosts. In: *Advances in Parasitic Plant Research*, (MT Moreno, JI Cubero, D Berner, D Joel, LJ Musselman and C Parker, eds.), 195-201. Junta de Andalucia, Cordoba, Spain.

Hammerschmidt, R. 1999. Induced disease resistance: how do induced plants stop pathogens? *Physiological and Molecular Plant Pathology* 55:77-84.

Hoagland, D.R. and Arnon, D.I. 1950. The water-culture method for growing plants without soil. *California Agricultural Experiment Station Circular* 347:1-32.

Jefferson, R.A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Molecular Biology Reporter* 5:387-405.

Jelesko, J.G., Jenkins, S., Rodriguez-Concepción, M., and Gruissem, W. 1999. Regulation of tomato HMG1 during cell proliferation and growth. *Planta* 208: 310-318.

Joel, D.M. and Losner-Goshen, D. 1994. The attachment organ of the parasitic angiosperms *Orobanche cumana* and *O. aegyptiaca* and its development. *Canadian Journal of Botany* 72:564-74.

Mangnus, E.M., Stommen, P.L.A., and Zwaneburg, B.Z. 1992. A standardized bioassay for evaluation of potential germination stimulants for seeds of parasitic weeds. *Journal of Plant Growth Regulation* 11:91-98.

Narita, J.O. and Gruissem, W. 1989. Tomato hydroxymethylglutaryl CoA reductase is required early in fruit development but not during ripening. *The Plant Cell* 1:181-190.

Ryan, C.A. 1990. Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. *Annual Review of Phytopathology* 28:425-449.

Stermer, B.A. and Bostock, R.M. 1987. Involvement of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the regulation of sesquiterpenoid phytoalexin synthesis in potato. *Plant Physiology* 84:404-408.

Vögeli, U. and Chappell, J. 1988. Induction of sesquiterpene cyclase and suppression of squalene synthetase activities in plant cell cultures treated with fungal elicitor. *Plant Physiology* 88:1291-1296.

Weissenborn, D.L., Denbow, C.J., Laine, M., Läng, S., Yang, Z., Yu, X., and Cramer, C.L. 1995 HMG-CoA reductase and terpenoid phytoalexins: Molecular specialization within a complex pathway. *Physiologia Plantarum* 93:393-400.

Westwood, J.H., Yu, X., Foy, C.L., and Cramer, C.L. 1996. Parasitization by *Orobanche* Induces expression of a defense-related gene in tobacco. In: *Advances in Parasitic Plant Research*, (MT Moreno, JI Cubero, D Berner, D Joel, LJ Musselman and C Parker, eds.), 543-550. Junta de Andalucia, Cordoba, Spain.

Westwood, J.H., Yu, X., Foy, C.L., and Cramer, C.L. 1998. Expression of a defense-related 3-hydroxy-3-methylglutaryl CoA reductase gene in response to parasitization by *Orobanche* spp. *Molecular Plant-Microbe Interactions* 11:530-536.

Zook, M.N. and Kuć, J.A. 1991. Induction of sesquiterpene cyclase and suppression of squalene synthetase activity in elicitor-treated or fungal-infected potato tuber tissue. *Physiological and Molecular Plant Pathology* 39:377-390.