Chapter I

Literature Review

I. INTRODUCTION

I.1 OROBANCHE LIFECYCLE

I.1.1 General

Orobanche (spp.) are root holoparasites that lack chlorophyll and well developed roots, and thus are entirely dependent on a host plant. *Orobanche* parasitizes many dicotyledonous crops in both temperate and semitropical regions. The seeds of *Orobanche* (approximately 0.3 mm long) germinate only in response to a chemical signal emitted from a nearby host root. The requirement for a germination stimulus ensures that a suitable host root will be available for attachment within a few millimeters of the germinated seed. Once this signal has been received, the parasite radicle, which can extend up to 3-4 mm, elongates via cell expansion towards the crop plant (Mussleman, 1980) and attaches to the host root.

From the parasite radical develops the haustorium, which penetrates the host root, and forms physical associations with host xylem and phloem tissue. This haustorial development is induced by phenolic compounds and cytokinins in the parasitic weed *Striga*, where this transition is well characterized (Estabrook and Yoder, 1998), but in *Orobanche* is manifested by the tip of the parasitic radicle enlarging and developing papillate cells at the point of host root contact (Joel and Losner-Goshen, 1994). The haustorium functions as a conduit transferring water and nutrients from the host plant to the parasite. Subsequently, the parasitic tissue outside the host root develops into a bulbous mass called a tubercle. The tubercle enlarges and produces a flowering shoot that is the only above ground structure of the parasite. This flowering shoot can produce up to 200,000 seeds (Parker and Riches, 1993).

I.1.2 Penetration

Penetration of the host root occurs by a combination of mechanical and enzymatic actions resulting in growth of the *Orobanche* haustorium between host cells. Studies indicate that the parasite penetration of the host tissue is aided by the secretion of lytic enzymes, which digest the middle lamella holding the cells together (Joel and Losner-Goshen, 1994; Losner-Goshen *et al*., 1998). These enzymes appear to include a combination of pectin methylesterase (PME) and polygalacturonase (PGA), which loosen

the adhesion between host root cells, allowing the intrusive parasitic cells to grow between them (Graham *et al*., 1993).

I.1.3 Pectin Methylesterase

PME is an enzyme that removes small branches from a pectin chain, thereby increasing its solubility and allowing attack by other enzymes. Losner-Goshen *et al.* (1998) have shown that sunflower (*Helianthus annuus*) root cell walls adjacent to young *Orobanche* haustorium cells show a decrease in the number of esterified pectins in the middle lamella adjacent to the parasite while host cell walls near mature parasite cells display a complete absence of pectins. Although it appears that sunflower root cell walls loose their pectins when in contact with the parasite, *Orobanche* cell walls are able to retain esterified pectins. An additional assay using gold-labeled PME indicated that PME was present in *Orobanche* cell walls attached to the host cell. Gold labeling was not found in distant host tissue, but was present in host cell walls that were attached to or located close to the parasite cells. These results suggest that the PME involved in parasitization is of parasitic origin (Losner-Goshen *et al*., 1998).

I.1.4 Polygalacturonase

PGA is a chain-splitting enzyme that attacks 1,4 linked D-galacturonide and hydrolyzes the linkage between two galacturonan molecules, thereby cleaving the chain. PGA results in the complete degradation of cell wall pectins and is thought to be the enzyme that allows *Orobanche* to penetrate the host root tissue (Losner-Goshen *et al*., 1998). During pathogenesis and infection, attacking fungi and bacteria secrete PGAs that degrade the pectin polymers in plant cell walls and facilitate penetration of the plant tissues (Stotz *et al*., 2000). PME and PGA are both necessary for the digestion of pectin. In tomato (*Lycopersicon esculentum* Mill), it has been shown that PME renders the polygalacturonic acid chain more accessible to attack by PGA (Keon *et al*., 1987).

In parasitism by *Orobanche*, PGA is also thought to work in concert with PME to facilitate the penetration event. Once PME has loosened cell wall adhesions, PGA completes pectin digestion allowing the intrusive parasitic cells to penetrate between host cells without rupturing them (Losner-Goshen *et al*., 1998). Another study supports PGA involvement in establishing a connection between *Orobanche* and the host root. Singh

and Singh (1993) demonstrated that PGA-specific activity was more than 2-fold higher in all parts of the parasite than in *Brassica campestris* host roots.

I.2 *OROBANCHE* CONTROL METHODS

Methods currently used to control *Orobanche* include mechanical methods (hand pulling), cultural methods (crop rotations, including the use of non-host crops), chemical methods (herbicides or fumigants), and biological control methods (mycoherbicides) (Musselman, 1980). Although many of these practices are used, few are effective at controlling *Orobanche*, and those that are effective are expensive.

I.2.1 Mechanical Methods

Hand pulling of emerged *Orobanche* flowers can be used to reduce the chance of seed spread, but it is only possible when sufficient labor is available. This method entails increased labor costs and can also damage roots of the crop plant due to the strong connections between *Orobanche* and the host plant (Musselman, 1980). This technique only limits further infestation, as most damage to yield has occurred before emergence of the floral shoot.

I.2.2 Cultural Methods

Crop rotation has also been used as a method to decrease the *Orobanche* seed bank in infected fields and is most efficient when used in combination with trap or catch-crops. These crops stimulate parasite germination and either do not allow further parasite development (trap-crops), or allow development of the parasite followed by removal of both parasite and host (catch-crops). These methods can be effective in decreasing the parasite seed bank, but the length of rotation needed to achieve significant reduction is not well established and trap and catch-crop varieties often vary in effectiveness. (Parker and Riches, 1993).

I.2.3 Solarization

An alternative method of control, solarization, uses polyethylene sheets and natural sunlight to heat moist infected soil. The increase in soil temperature leads to the degradation of *Orobanche* seeds and has been effective in decreasing the number of infections occurring in a field. In particular, Haidar and Sidahmed (2000) showed that solarization with chicken manure reduced *Orobanche* growth in infected cabbage fields

and increased cabbage yield. Although this method does provide some level of control, the relatively high cost of the polyethylene sheets is a limiting factor for most farmers.

I.2.4 Biological Control Methods

Bio-control agents such as *Fusarium oxysporum*, a fungal pathogen of *Orobanche*, have also been suggested as potential control agents. *F. oxysporum* attacks *Orobanche* seeds, tubercles, and shoots. Inoculation of infected soils with this fungus has provided a reduction in the number of *Orobanche* seeds occurring in the soil (Thomas *et al*., 1999). However, storable formulations of the fungi are not currently available and a better understanding of the *Orobanche-F. oxysporum* interaction in various environmental conditions is still needed (Muller-Scharer *et al*., 2000).

I.2.5 Chemical Methods

With respect to chemical control, fumigants such as methyl bromide are the most effective and have been used routinely in Israel and in the United States (Parker and Riches, 1993). These chemicals kill seeds in the soil prior to germination, but the toxicity of the gas to humans and damage to the ozone layer have led to an impending world-wide ban on methyl bromide. In addition, requirements for expensive plastic covers make this and other fumigants impractical for most farmers or where low value crops are grown.

Soil-applied herbicides and growth regulators have been tested as a means of inhibiting the parasite during its subterranean period. However, these chemicals tend to be more damaging to the host plant than to the parasite. Only one pre-emergence herbicide (dichloral urea) out of 13 tested was found to consistently control *Orobanche* (Foy *et al*., 1989). Also, two growth regulators, endothall and naphthaleneacetic acid suppressed the growth of the parasite, but were toxic to the host tomato plants.

Indirect control of *Orobanche* has been attempted by treating the parasite through the host plant just prior to or after attachment to the host roots (Foy *et al*., 1989). Translocatable herbicides such as 2,4-D and glyphosate have been used to control *Orobanche.* It has been demonstrated that 2,4-D accumulated to a level 14 times higher in the tubercle than in the faba bean (*Vicia faba*) host root (Whitney, 1973). Regardless of this increased concentration, no selective control was achieved, and the damage to the host plant was worse than that to the parasite. Glyphosate has also been used in crops such as common vetch (*Vicia sativa*), faba bean, carrot (*Daucus carota*), and celery

(*Apium graveolens*) which have some natural tolerance to the herbicide (Parker and Riches, 1993; Nandula *et al*., 1999). Although this herbicide has provided adequate control, the window of selectivity is so narrow that crop damage or inadequate control of the parasite are likely outcomes.

I.2.6 Herbicide Resistant Crops

Herbicide resistant crops offer a means of controlling *Orobanche* by allowing application of a translocatable herbicide at a rate that provides *Orobanche* control but does not damage the host plant. Joel *et al*. (1995) tested four herbicide resistant crops for their utility in controlling *Orobanche.* Of these plants, three were engineered with target-site resistances to chlorosulfuron, glyphosate, or asulum, while one had resistance to glufosinate based on degradation of the herbicide.

Foliar application of chlorosulfuron, glyphosate, and asulum to the corresponding resistant crops was effective in controlling attached *Orobanche*. On the other hand, glufosinate was not effective in deterring *Orobanche* parsitization of glufosinate resistant crops. Chlorosulfuron, glyphosate, and asulum resistant plants were effective because they allowed the movement of the herbicide through the host plant to the parasite without degradation of the herbicide. Glufosinate-resistant crops were ineffective in allowing control of *Orobanche* because they degraded the herbicide before it reached the parasite. The glyphosate-resistant tomato and ALS-resistant tobacco (*Nicotiana tabacum* L.) plants completely prevented *Orobanche* emergence and still maintained 95 % of their normal growth and flowering. The asulum-resistant tobacco plants were able to reduce the *Orobanche* population by only 70 % (Joel *et al*., 1995). However, in a more recent study using asulum on asulum-resistant potatoes, *Orobanche* parasitization was completely eliminated (Surov *et al*., 1998). Unfortunately, large chemical companies are not interested in investing time or money in engineering herbicide resistance into the low value or low acreage crops that are typically infected by *Orobanche*.

I.2.7 *Orobanche*-Resistant Cultivars

The best long-term control method involves the use of *Orobanche*-resistant crops. Such crops offer the advantages of requiring no toxic chemicals, no expensive materials, and no sophisticated technology on the part of the farmer. In addition, a crop that

triggered *Orobanche* germination but resisted parasite attachment or growth, would offer the benefits of a trap crop while producing a marketable yield.

Crop resistance to *Orobanche* may occur by several mechanisms, which may include avoidance of the parasite as well as resistance to parasitism. Mechanisms leading to crop avoidance may include low production of lateral roots and high compact root mass (Nassib *et al*., 1984); deeper growth of the root system (Cubero, 1991); or reduced production of germination stimulants (Aalders and Pieters, 1986; Wegmann, 1986). Resistance has also been attributed to the development of physiological barriers (Nassib *et al*., 1984; Aalders and Pieters, 1986; Wegmann, 1986). Additionally, Goldwasser *et al*. (1997) have shown that vetch resistance to *O. aegyptiaca* is characterized by the development of necrotic lesions in the area of parasite attachment. The *O. aegyptiaca* haustorium enters the vetch root epidermis but then is halted by a chemical or mechanical barrier at the endodermis cell layer. This barrier is accompanied by the accumulation of an unidentified material secreted at the host-parasite interface that somehow prevents further parasitism of the host plant (Goldwasser *et al*., 2000). These mechanisms of resistance are not completely understood and therefore, provide limited insight for engineering resistance. However, some crops have been successfully bred to produce cultivars resistant to *Orobanche*.

Useful levels of resistance have been found in several host plants such as sunflower (*Helianthus annuus*), faba bean, common vetch, eggplant (*Solanum melongena)*, and cucurbits (muskmelon, watermelon, squash, cucumber) (Alonso, 1998). Most research has focused on sunflower because of its particular interest to Russian breeders during its rapid expansion as a crop in Russia during the early 1900's and also its decline caused by *Orobanche*. In particular, this research has led to the development of sunflower lines with complete resistance to two particular races of *Orobanche cernua* and *O. cumana* (Pustovoit, 1967; Cubero, 1986; Cubero, 1991). Although these sunflowers were resistant to two races of *Orobanche*, they are susceptible to newly evolved races (Antonova, 1994). Unfortunately, the physiological basis for this interaction has not been elucidated.

I.3 PLANT DEFENSE MECHANISMS

I.3.1 General

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. For these inducible defenses, expression of defense genes may be confined to the region of pathogen attack, or may be expressed throughout the plant. It is believed that there are three major pathways that regulate the inducible response.

One of these inducible pathways, the wound response or jasmonic acid (JA) pathway, is triggered by wounding or insect feeding. This pathway is mediated by JA and ethylene, and is known to result in the induction of proteinase inhibitor (PI) genes (Ryan *et al*., 1990) as well as salicylic acid (SA)-independent pathogenesis-related (PR) genes (Fig. I.1). Studies have demonstrated that JA and ethylene are important in pathogen resistance. Plants that are mutated in their ability to detect ethylene such as the Arabidopsis *ein* and *etr* mutants show either an increased susceptibility (*ein*) or an increased tolerance (*etr*) to fungal pathogens (Vijayan *et al*., 1998). Additionally, plants that are mutated in their ability to synthesize jasmonate show an increased susceptibility to specific pathogens such as *Rhizoctonia* (Rance *et al*., 1998).

A second pathway, leading to systemic acquired resistance (SAR), is SA-dependent and is effective in conferring resistance in plant parts that have not been exposed to a pathogen. This pathway develops both locally and systemically in response to recognition of specific pathogens and may result in a

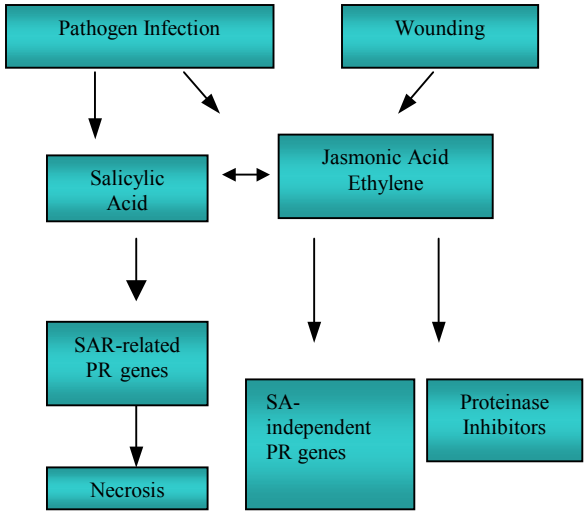

Figure I.1: Schematic illustrating the proposed interaction of plant responses related to the JA/wound response pathway and SAR. Pathogen infection may result in induction of the JA pathway and or SAR whereas wounding induces only the JA pathway. Diagram modified from Pieterse and Van Loon, 1995.

necrotic lesion at the site of infection. The resistance is associated with the production of PR proteins and is usually effective against a wide array of pathogens (Hammerschmidt, 1999). In this type of response, the plant cells around the point of infection may undergo cell death as a means to contain the area of infection. This process is called the hypersensitive response (HR).

It has been suggested that there is "cross-talk" between the SAR and JA pathways. Studies have shown that SA and JA signaling are mutually inhibitory (Creelman and Mullet, 1997; Harms *et al*., 1998). For instance, Doares *et al*. (1995) found that SA inhibited the synthesis of PIs that were produced in response to JA and ethylene. In another study using tomato, it was shown that the rate-limiting enzyme of JA synthesis was repressed by addition of SA (Pena-Cortes *et al*., 1993). However, researchers found that the inhibition of JA could be overcome by exogenous application of JA and ethylene. On the other hand, the synergistic effects of JA on SA-inducible responses has also been demonstrated in studies such as those performed by Xu, *et al*. (1994) who reported that a combined treatment of SA plus methyl jasmonate resulted in a stronger induction of the SA-inducible tobacco gene, *PR-1*, than treatment with SA alone. There is also evidence of a mutual antagonism between SAR and the induction of a basic PR gene in response to *Erwinia caratovora* in tobacco. Infection by this bacterial pathogen causes a delay in SAR induction while induction of SAR inhibits the response to *Erwinia* (Malek and Dietrich, 1999; Vidal *et al*., 1997). Wounding of a plant may turn on a JA-dependent pathway that inhibits SA-dependent gene induction, however additional studies need to be performed before this can be assumed (Reymond and Farmer, 1998).

A third and less studied pathway, induced systemic resistance (ISR), is induced by non-necrotizing mutualistic bacteria and does not induce any of the genes involved in the JA pathway or SAR (Malek and Dietrich, 1999; Pieterse *et al*., 1998). The resistance associated with ISR is independent of the production of SA and is not associated with the accumulation of PR proteins (Van Loon *et al*., 1998). Few defense-related compounds responsible for ISR have been identified and the level of protection provided by this pathway is usually less than that attainable through SAR (Pieterse, *et al*., 1998).

I.3.2 SAR Pathway

The SAR pathway is known as the SA-dependent pathway because its induction is often preceded by an increase in the level of SA. Induction of SAR by a predisposing infection with a necrotizing pathogen coincides with an early increase in endogenously synthesized SA, not only at the site of primary infection but also systemically in the uninfected tissues (Pieterse and Van Loon, 1995; Malamy *et al*., 1990; Durner *et al*., 1997). In tobacco and Arabidopsis, SA has been shown to be both necessary and sufficient for the induction of the SAR pathway (Glazebrook, 1999). Salicylic acid-deficient plants have been generated in several species by expressing a bacterial salicylic acid hydrolase gene (nahG) which inactivates salicylic acid by converting it to catechol. These plants are unable to accumulate SA and as a result are unable to develop SAR (Malek and Dietrich, 1999: Gaffney *et al*., 1993) indicating that SA accumulation is required for the expression of SAR (Gaffney *et al*., 1993). However, these plants also demonstrate that some defense responses are unaffected by the absence of SA and are therefore independent of SA (Pieterse and Van Loon, 1995; Vidal *et al*., 1997).

I.3.3 PR Genes

Pathogenesis-related proteins are host-encoded polypeptides that accumulate as a result of infection by a pathogen or in response to elicitors (Eyal and Fluhr, 1991). Many PR proteins are localized to the apoplast, suggesting that they are positioned to contact the pathogen during the initial parasitization (VanLoon, 1997). However, the great number of PR proteins identified makes generalizations difficult. PR proteins are divided into groups based on similar amino acid sequences, serological relationship, and enzymatic or biological activity (Van Loon *et al*., 1998). PR proteins are also divided into acidic or basic groups based on signaling and localization. Basic PR proteins are associated with JA signaling while acidic PR proteins are associated with SA signaling (Eyal and Fluhr, 1991; Reymond and Farmer, 1998). Some PR proteins such as PR-3, 4, 8, and 11 (chitinases), PR-2 (glucanases) and PR-5 (thaumatin-like protein) possess antimicrobial activity and can digest or permeabilize the cell walls of some fungi.

PR proteins can be expressed both locally and systemically. The systemic response has been demonstrated for TMV (tobacco mosaic virus)-mediated induction,

wherein tobacco mRNAs associated with TMV infection were shown to be induced within 2 days in the inoculated leaf tissue and within 8 days in the virus-free upper leaves (Hooft van Huijsduijnen *et al*., 1986). This initial localized response, followed by a systemic response has been explained by the existence of a mobile signal produced at the infection site that then spreads throughout the plant vascular system.

Although several of the PR proteins have been characterized, not all families of PR proteins have been identified and the specific subset of PR genes involved in SAR vary from species to species. In Arabidopsis, SAR correlated marker genes include PR-1, PR-2, and PR-5 (Uknes *et al*., 1992; Malek and Dietrich, 1999; Reymond and Farmer, 1998). In tobacco and tomato, there are approximately 11 families of PR proteins induced by pathogens (Van Loon *et al*., 1994) ranging from PR-1 to PR-11. In tobacco, *PR-1a* is known to have antifungal activity but is also induced in response to TMV. Joel and Portnoy (1998) recently provided evidence from transgenic tobacco plants containing promoter:GUS fusions that *Orobanche* induces *PRB-1b*, a basic PR1 protein known to be activated in correlation with pathogenic micro-organisms, ethylene, TMV, darkness or elicitors, as part of the SAR response (Eyal and Fluhr, 1991).

I.4 ISOPRENOID/MEVALONATE PATHWAY- RELATED GENES

I.4.1 General

The mevalonic acid pathway gives rise to over 10,000 plant-specific compounds such as cytokinin, phytotoxins and other specialized compounds (Weissenborn *et al*., 1995). Compounds derived from the isoprenoid pathway are involved in a range of physiological processes including the synthesis of membrane sterols, regulation of plant growth hormones, electron transport, and pathogen resistance.

Plant isoprenoids can be sub-classified as primary and secondary metabolites. Primary metabolites are essential for normal growth and development, and include substances such as sterols, carotenoids, and quinone (Chappell, 1995). Secondary metabolites such as monoterpenes, sesquiterpenes, and diterpenes are not essential, but play a role in plant defense. For example, the production of sesquiterpenes has been shown to be critical in localized disease resistance in several plant-pathogen interactions (Darvill and Albersheim, 1984; Dixon and Harrison, 1990; Kuć and Rush, 1985; Moesta and Grisebach, 1982; VanEtten *et al*., 1989).

I.4.2 3-Hydroxy-3-Methylglutaryl-CoA Reductase

The first committed step in the pathway leading to isoprenoid synthesis is mediated by 3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR). HMGR catalyzes the conversion of 3-Hydroxy-3-methylglutaryl coenzyme A to mevalonic acid, which is the precursor of all isoprenoid compounds (Fig. I.2). In situations requiring increased levels of pathway end products, elevated HMGR activity is necessary to commit more carbon to the isoprenoid pathway (Vögeli *et al*., 1988).

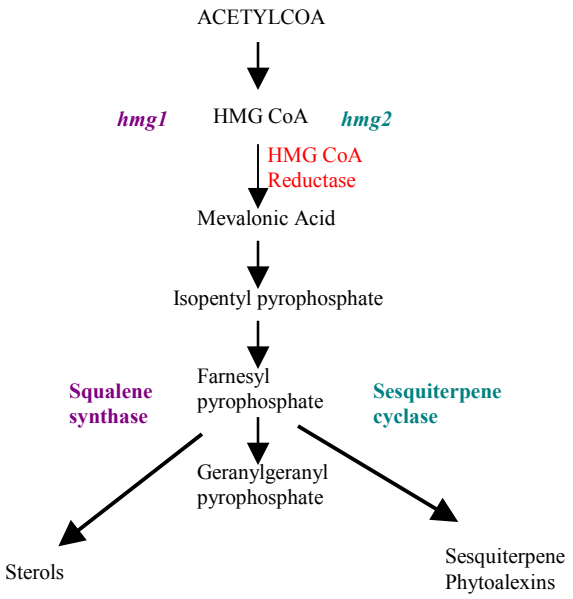

Figure I.2. Simplified diagram of the isoprenoid pathway. Genes discussed in this thesis are *hmg1* and *hmg2*, both of which encode HMGR, but are differentially regulated with squalene synthase and sesquiterpene cyclase respectively (Diagram adapted from Denbow *et al*., 1995).

In tomato, HMGR activity is regulated at the transcriptional level by the differential expression of a small family of genes encoding the enzyme (Cramer, *et al*., 1993) (Fig. I.2). These isogenes differ in expression pattern in response to developmental changes, pathogen attack, elicitor treatment, or wounding (Chappell, 1995; Weissenborn *et al*., 1995). Studies performed by Jelesko *et al*. (1999) on tomato plants containing *hmg1*:GUS fusions indicated that tomato *hmg1* is primarily expressed in processes related to cell division and growth. Additionally, Narita and Gruissem (1989) found that *hmg1* from tomato is also expressed in early stages of tomato fruit ripening.

A second HMGR isogene, *hmg2*, is expressed in response to wounding and a variety of pathogenic agents, suggesting a role in sesquiterpene phytoalexin biosynthesis (Denbow *et al*., 1995). Choi *et al*. (1992) reported that potato tubers elicited with the fungal pathogen *Phytophthora infestans* expressed *hmg2* at a much higher level than control tissue. Wound and pathogen induction of tomato *hmg2* was also analyzed using *hmg2* promoter:GUS reporter gene fusions introduced into transgenic tobacco plants.

Transgenic tobacco leaves treated with *Erwinia carotovora* showed high levels of GUS expression in the area surrounding the lesions (Weissenborn *et al*., 1995). Another study using transgenic tobacco expressing *hmg2*:GUS promoter fusions showed temporal and spatial regulation of *hmg2* in response to parasitism by *Orobanche* (Westwood *et al*., 1998).

I.4.3 Branch Pathways

Farnesyl diphosphate (FPP) is another important branch intermediate that is found at a later step in the mevalonic acid pathway (Fig. I.2). Under normal growing conditions, a portion of FPP is devoted to the production of sterols, and the remaining is used to synthesize ubiquinone and other lipid moieties (Brown and Goldstein, 1980). However, when a plant is stressed or challenged by microbial or viral pathogens, antimicrobial phytoalexins are produced (Stoessl *et al*., 1976). Sesquiterpene cyclase is the branch point enzyme responsible for diverting FPP toward the production of sesquiterpenoids (Fig. I.2). These phytoalexins are not found in healthy or control tissues, but only accumulate in response to elicitation or pathogen challenge (Bailey *et al*., 1975; Watson and Brooks, 1984; Brooks *et al*., 1986; Chappell and Nable, 1987).

Another enzyme, squalene synthase, is the branch point enzyme that diverts FPP toward the production of sterols (Fig. I.2). Studies using potato tuber disks treated with elicitor provided evidence that steroidal glycoalkaloid accumulation was suppressed while sesquiterpenoid activity increased (Tjamos and Kuć, 1982; Brindle *et al*., 1988). The branch pathway leading to the production of compounds such as sterols is primarily regulated by controlling the activity level of HMGR via *hmg1* (Chang, 1983), but squalene synthase also provides a secondary level of control for this pathway (Faust *et al*., 1979). In untreated or control tissue, squalene synthase shows expression coordinate with *hmg1* (Denbow *et al*., 1995), but is suppressed in tissue treated with elicitor.

I.5 SIGNIFICANCE AND OBJECTIVES

The long-term goal of this project is the development of *Orobanche*-resistant crops. Critical to this endeavor is an understanding of the molecular events that mediate the interactions between *Orobanche* and its host. The specific objective of this research was to characterize host plant defense responses in plants parasitized by *Orobanche aegyptiaca* focusing on genes involved in isoprenoid metabolism as well as genes

involved in SAR. Gene expression was studied using transgenic plants containing promoter-GUS inserts as well as northern analysis on *O. aegyptiaca*-infected tissue as compared to control tissues.

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