Chapter IV

Response of PR genes in Plants Parasitized By *Orobanche*

IV.1 ABSTRACT

Orobanche aegyptiaca is a parasitic plant that attacks the roots of many important crops. *Orobanche* penetrates the host root and forms connections to the host vascular tissue, from which it will draw all of its water and nutrient requirements. In order to investigate how the host plant responds to this attack, we are studying the patterns of expression of defense related host genes. These responses are complex and involve the coordinate regulation of several distinct defense pathways. One such pathway that leads to systemic acquired resistance (SAR), is activated by necrotizing pathogens and can provide resistance to a broad range of pathogens. SAR is also associated with the hypersensitive response (HR) and the production of pathogenesis related (PR) proteins. These PR proteins can be expressed both at the site of parasitization and systemically throughout the plant. In recent years, PR genes have been intensely studied due to their potential use in engineering long-term broad-spectrum resistance in crop plants. Tobacco and Arabidopsis serve as model systems for understanding PR gene expression and SAR. In this study, plant gene expression in response to parasitism was investigated using northern hybridization analysis of tobacco and Arabidopsis roots using probes specific for *PR-1a* (tobacco) and *PR-1*, *PR-2*, and *PR-5* (Arabidopsis). Additionally, leaves of infected tobacco plants were sprayed with known inducers of SAR, salicylic acid (SA) and benzothiadiazole (BTH), and the effects of SAR induction on parasite attachments were studied. Results from northern analysis indicated that *O. aegyptiaca* parasitization did not induce any of these genes. The failure of *Orobanche* to induce these PR genes suggests that *Orobanche* somehow avoids inducing the SAR response. Results from SA and BTH treated plants indicated that neither treatment affected *O. aegyptiaca* attachments. This ability to avoid activating the SAR pathway may hold the key to the success of *Orobanche* as a parasite, and hence aid in the development of *Orobanche* resistant crops.

IV.2 INTRODUCTION

IV.2.1 Parasitism by *Orobanche*

Orobanche spp. are root holoparasites that are totally dependent on a host plant for all water and mineral nutrients. These parasites attack many dicotyledenous crops in both temperate and semitropical regions (Parker and Riches, 1993; Musselman, 1980).

Orobanche reduces crop value and yield by diverting resources from the host plant to the parasite. The underground life cycle of *Orobanche* and its close association with the host crop roots minimizes the effectiveness of conventional control techniques. The best long-term strategy for limiting damage by *Orobanche* is the development of *Orobanche*-resistant crops.

The *Orobanche*-host interaction is a complex process which includes signaling and physiological responses. The initial communication between the parasite and the host involves the *Orobanche* seed perception of a germination signal exuded from the roots of a compatible host plant. Upon germination, a radical emerges from the seedling, elongates via cell expansion and adheres to a nearby host root. Once contact is achieved, *Orobanche* uses lytic enzymes to digest the middle lamella holding the host cells together (Mussleman, 1980; Joel, and Losner-Goshen, 1994; Losner-Goshen *et al*., 1998). *Orobanche* appears to use a combination of pectin methylesterase (PME) and polygalacturonase (PGA) to loosen the adhesion between host root cells, allowing the haustorium to grow between these host cells (Graham *et al*., 1993; Joel and Losner-Goshen, 1994). While *Orobanche* penetrates and forms connections to the host vascular tissue, the host reacts to this invasion with attempts at self-defense (Westwood *et al*., 1998).

IV.2.2 Plant Defense Responses

Plants are versatile and possess the ability to defend themselves against various types of pathogens. These defense responses may be pre-existing or may be induced in response to pathogen attack. In terms of inducible defenses, at least three types of microbial-induced resistance pathways appear to exist in plants (Van Loon and Van Strien, 1999). One of these pathways, the wound response or jasmonic acid (JA) pathway, is triggered by wounding or insect feeding and induces the expression of defense genes only in the region of wounding or pathogen invasion. This pathway is mediated by JA and ethylene, involves increases in JA, and ethylene, and is known to result in the induction of proteinase inhibitor (PI) genes as well as salicylic acid (SA)-independent pathogen-related (PR) proteins (Fig. I.1) (Ryan, 1990; Van Loon and Van Strien, 1999).

A second pathway, systemic acquired resistance (SAR), develops both locally and systemically in response to recognition of specific pathogens. This pathway is SA mediated, and may be effective in conferring resistance in plant parts distant from those originally exposed to a pathogen. The resistance expressed is associated with the production of SA-dependent PR proteins and the hypersensitive response (HR) which results in necrotic lesions (Hammerschmidt, 1999). Resistance induced by SAR is characterized by metabolic alterations within the host plant that impede the spread of the pathogen as well as an enhanced resistance to subsequent infection by other types of pathogens (Van Loon, 1997; Hammerschmidt, 1999).

A third and less studied pathway, induced systemic resistance (ISR), is independent of the production of SA and is not associated with the accumulation of PR proteins (Van Loon *et al*., 1998). Additionally, few defense-related compounds responsible for ISR have been identified. Please see Chapter I for details.

IV.2.3 Systemic Acquired Resistance

SAR has been best characterized in tobacco, cucumber, and Arabidopsis (Cameron *et al*., 1999). Studies with these species suggest that SAR occurs in three stages: 1) infection of a host plant by a necrotizing pathogen 2) localized necrosis in the area of attack 3) accumulation of SA and subsequent expression of a set of proteins (PR proteins) that act to limit further infection of the host (Cameron *et al*., 1999; Kuć, 1982; Malamy, *et al*., 1990; Uknes, *et al*., 1992; Ward *et al*., 1991). In tobacco and Arabidopsis, SA has been shown to be both necessary and sufficient for the induction of the SAR pathway as shown by salicylic acid-deficient plants (Glazebrook, 1999). These plants express a bacterial salicylic acid hydrolase gene (nahG) and thus are unable to accumulate SA. NahG plants are also unable to activate the SAR pathway, providing direct evidence that SA accumulation is required for the expression of SAR (Malek and Dietrich, 1999: Gaffney *et al*., 1993). Additionally, SAR can be induced by exogenous application of SA or synthetic compounds with structural similarities to SA such as 2,6-dichloroisonicotinic acid (INA) or benzothiadiazole (BTH). Application of SA, INA or BTH induces the same group of genes as in the SAR response and resistance to the same

range of pathogens is also achieved (Van Kan *et al*., 1989; Uknes *et al*., 1992; Dempsey and Klessig, 1994; Klessig and Malamy, 1994; Ryals *et al*., 1994; Hammerschmidt, 1999).

The PR proteins produced during SAR are host-encoded polypeptides that are grouped into families based on shared amino acid sequences, serological relationship, or enzymatic or biological activity (Van Loon and Van Strien, 1999). 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). Localization studies involving PR proteins in tobacco indicate that acidic PR proteins tend to be located extracellularly and are coordinately expressed upon pathogen infection whereas basic PR proteins are targeted to vacuoles and are expressed in a controlled cell-type and organ-dependent manner (Van Loon and Van Strien, 1999).

The biological function of most PR proteins is not known, but several groups have been shown to have antimicrobial/antifungal activity allowing them to act directly on the invading pathogen. Some examples of these PR proteins include PR-3, 4, 8, and 11 (chitinases), and the PR-2 family (β-1,3-glucanases) which can digest the cell walls of some fungi (Epple *et al*., 1995; Van Loon and Van Strien, 1999); the PR-5 family (thaumatin-like proteins) which are thought to possess the ability to permeabilize fungal membranes (Abad *et al*., 1996; Hajgaard *et al*., 1991; Vigers *et al*., 1992; Woloshuk *et al*., 1991); and members of the PR-6 family (proteinase inhibitors) which are thought to act by inhibiting proteolytic enzymes used by insects and microbes (Hammerschmidt, 1999; Koiwa *et al*., 1997; Ryan, 1990). Additionally, the PR-1 family is notable because related sequences have been identified in yeast (genes involved in hyphae formation), insects (allergens from the white-face hornet wasp, *Dolichovespula maculata*), and vertebrates (cysteine-rich secretory proteins), indicating that the function of PR-1 proteins may be essential to all living organisms (Fang *et al*., 1988; Schuren *et al*., 1993; Eberspaecher *et al*., 1995). Although the mechanism of action of most PR-1 proteins is not known, they are induced by pathogens or SA and are therefore commonly used as markers for SAR in most species (Van Loon and Van Strien, 1999).

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 varies 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, approximately 11 families of PR proteins (PR-1 to PR-11) are induced by pathogens (Van Loon *et al*., 1994).

Constitutive expression of other PR genes has provided enhanced resistance to a variety of pathogens (Broglie *et al*., 1991; Lin *et al*., 1995; Datta *et al*., 1999). Transgenic potato plants over-expressing osmotin, a member of the PR-5 family, showed enhanced resistance to the fungal pathogen *Phytophthora infestans* (Liu *et al*., 1994; Zhu *et al*., 1996). In another study, rice plants containing an over-expressed rice *PR-5* provided enhanced resistance to *Rhizoctonia solani*, the fungus causing sheath blight disease (Datta *et al*., 1999). Another method used to study PR genes focuses on plants that are mutated in their ability to resist pathogen invasion. Reuber *et al*. (1998) tested the ability of a set of non-expresser of PR (npr) and enhanced disease susceptibility (eds) mutant Arabidopsis plants to express PR-1, PR-2, and PR-5 upon infection by *Erysiphe orontii*, a known inducer of SAR. Several of these plants showed a reduced level of PR gene expression and an increased susceptibility to the pathogen, indicating that the expression of one or more of these genes is required for SAR.

The first PR gene purified and characterized was *PR-1a* from tobacco (Antoniw and Pierpoint, 1978). *PR-1a* encodes an acidic PR protein that is expressed in response to SA or specific pathogens, but not by wounding or other elicitors (Eyal *et al*., 1992). Transgenic tobacco plants containing a constitutively expressed *PR-1a* gene demonstrated an increased tolerance to the fungal pathogens *Phytophthora parasitica* var. *nicotianae* and *Peronospora tabacina* (Alexander *et al*., 1993; Van Loon and Van Strien, 1999). The gene encoding the basic PR protein, *PRB-1b* is JA-mediated and expression is correlated with pathogenic micro-organisms, ethylene, TMV, darkness or elicitors (Eyal and Fluhr, 1991). Joel and Portnoy (1998) recently provided evidence from transgenic tobacco plants containing *PRB-1b* promoter:GUS fusions that *PRB-1b* is also induced in host root tissue parasitized by *O. aegyptiaca*.

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*. Previous results from tobacco indicate that *Orobanche* induces host defense genes associated with response to wounding and pathogen elicitors (Westwood *et al*., 1998; Chapter III). This chapter discusses the role of PR genes in host plants parasitized by *O. aegyptiaca* focusing on *PR-1a* from tobacco, and *PR-1*, *PR-2*, and *PR-5* from Arabidopsis.

IV.3 MATERIALS AND METHODS

IV.3.1 Tissue Preparation for Northern Analysis

Tobacco (*Nicotiana tabacum* L. var. Coker) and *Arabidopsis thaliana* (var. Columbia) were grown from seed in soil for approximately 12 days at which time they were transplanted into polyethylene (PE) bags containing glass fiber filter paper [as described in Westwood *et al*. (1996)] 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. Seven days after transplanting, plants were divided into treatment groups (Fig. III.1) and plants were treated and harvested as described in Chapter III.

IV.3.2 Transformation and Probe labeling

pGEM plasmids containing a 0.8 Kb *EcoR*1 fragment for tobacco *PR-1a* (provided by Dr. Daniel Klessig, Rutgers University) 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 (Qiagen, Valencia, CA) and diluted for use in subsequent PCR reactions. Probes were labeled by incorporating 1 mM digoxygenin (dig) dUTP's (Roche Molecular, Indianapolis, IN) in the reaction mix along with 20 μM T7 and SP6 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 (Qiagen, Valencia, CA) and then quantitated against control DNA.

BAC clones T6B13, F28O9, F9E10 containing *PR-1*, *PR-2*, and *PR-5* Arabidopsis genomic DNA were acquired from the Arabidopsis Biological Resource Center (ABRC). BACs F2809 and F9E10 were grown overnight at 37˚C on LB agar plus 50 μg/ml kanamycin while BAC T6B13 was grown on LB plus 30 μg/ml chloramphenicol. Five

ml liquid LB plus antibiotic was then inoculated from these overnight cultures and grown for 20 h at 37˚C. The BACs were then purified using the recommended protocol provided by Ohio State University (http://aims.cps.msu.edu/aims). BAC DNA was quantitated and 50 ng/µl was used in subsequent PCR reactions with gene specific primers designed based on known cDNA sequences (Uknes *et al*., 1992). The primers also contained upstream 5' *Hind*III and downstream 5' *EcoR*1 linkers as follows:

PR-1	upstream	5'CCCAAGCTTGGGTGTAGCTCTTGTAGGTGCTC
	downstream	5'CCGGAATTCCGGCATCCTGCATATGATGCTCC
PR-2	upstream	5'CCCAAGCTTGGGATGCTACGGGATGCTAGGCG
	downstream	5'CCGGAATTCCGGGATCTGGATGAAACAGTCCCC
PR-5	upstream	5'CCCAAGCTTGGGGCGGCATTGCTGTTATGGCC
	downstream	5'CCGGAATTCCGGCAGCTGAGTGTAACAACTGAC

PCR conditions were: (1) 94°C, 1 minute; (2) 49°C, 1 minute; (3) 72°C, 2 minutes, 49 cycles. 25 ng of each PCR product was then labeled using RTS RadPrime DNA labeling System and 5µl α-^{32}P (Invitrogen, Carlsbad, CA). A 517 bp *PR-1*, a 864 bp *PR-2*, and a 814 bp *PR-5* PCR product was obtained. Probes were sequenced to ensure accuracy.

IV.3.3 Extraction and Northern Analysis of Total RNA

Blots containing tobacco RNA were prepared and hybridized at 60˚C with an 800 bp *PR-1a* dig probe as described in Chapter III. Blots containing Arabidopsis RNA were pre-hybridized at 60°C in 5X SSC, 5X Denhardt's solution, 1% SDS (v/v), and 100 µg/mL denatured salmon sperm. Hybridization to random primed ^{32}P-labeled DNA was performed under the same conditions using either a *PR-1*, *PR-2*, or *PR-5* DNA clone from Arabidopsis and incubation was for 24 hr. After hybridization, the blots were washed twice in 2X SSC, 1 % SDS at room temperature, and then twice in 0.1X SSC, 0.1 % SDS at 60°C for 20 min. Blots were exposed to x-ray film at -80°C. After exposure, the membrane was stripped of the hybridized probe by boiling twice for 20 min in 0.1X SSC, 0.5 % SDS and then rinsed with 5X SSC. The membrane was placed on film to check for any remaining radioactivity and then hybridized with a second gene specific probe as described above.

IV.3.4 SA Treatment

Tobacco plants (var. Coker) were infected with *O. aegyptiaca* seeds as previously described in Chapter III. Plants were grown under 100 μmol/m^2/sec light, 12 hr days at 25 ± 3°C. After 20 d, plants were separated into three replicate groups that were treated with a concentration of SA (50 mM) known to induce SAR. Plants received one of two treatments: 1) injection of 50 mM SA into the back of the polyethylene bag to directly treat roots or 2) application of 50mM SA directly to leaf tissue by spraying plants to saturation. Plants were placed back under light as described earlier and *O. aegyptiaca* attachments were examined under the microscope at 3, 6, 14, and 21 d after SA treatment. *Orobanche* growing on the treated plants were compared to those on tobacco plants not receiving SA.

IV.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 *Orobanche* induces genes involved in isoprenoid metabolism (Westwood *et al*., 1998; Chapter III). Evidence also indicates that *Orobanche* induces *PRB-1b* in parasitized host plants (Joel and Portnoy, 1998). To further study the SAR defense response in *Orobanche* infected plants, the expression of additional PR genes was analyzed.

To determine if parasitization induces tobacco *PR-1a*, northern analysis was performed on non-inoculated control, *O. aegyptiaca* parasitized, SA, and cellulase-treated tobacco root tissue (Fig. IV.1 A). Results indicated that tobacco *PR-1a* was highly induced in the SA treated root tissue, expressed only at trace levels in the untreated control, cellulase, and *O. aegyptiaca* infected samples, and undetectable in wounded tissue. These results support previous data from Eyal *et al*. (1992) which indicated that *PR-1a* was inducible by fungal elicitors or SA and not by wounding or other elicitors. However, the lack of *PR-1a* expression in *O. aegyptiaca* infected tissue contrasts with results obtained from Joel and Portnoy (1998) in which *O. aegyptiaca* parasitization induced *PRB-1b*. The differential expression between the acidic and basic PR-1 proteins is logical given that *PR-1a* is SA dependent, and not wound inducible whereas *PRB-1b* is JA dependent, wound inducible, and expressed at only low levels in SA treated tissue.

Thus *O. aegyptiaca* exhibits differential induction of PR proteins similar to that reported in tobacco plants infected by *Erwinia carotovora* or treated with SA in which *E. carotovora* infection induced expression of *PR-2b* and *PR-3b*, but not *PR-1a* whereas SA treatment induced *PR-1a* but not *PR-2b* or *PR-3b* (Vidal *et al*., 1997; Vidal *et al*., 1998).

To further study the effects of SAR induction on *O. aegyptiaca*, infected tobacco plants were treated with known elicitors of SAR. Plant leaves were sprayed to saturation or roots were directly treated with SA and then harvested at 3, 6, 14, or 21 d after treatment. *O. aegyptiaca* attachments present on treated plants were then compared to those on *O. aegyptiaca* plants not receiving SA treatment. There were no observable differences between tubercle size, color, or overall health in the SA treated plants as compared to plants not treated with SA. Results indicated that *O. aegyptiaca* attachments were not affected by SA treatments (data not shown), however, leaf necrosis, which is indicative of the HR response, was apparent 24 h after SA leaf and root treatment. Although SAR was induced, *O. aegyptiaca* attachments were not affected as compared to untreated *O. aegyptiaca* infected plants. These results are surprising given that the induction of SAR is known to provide protection against several pathogens such as TMV and *E. carotovora* (Van Loon, 1997; Vidal *et al*., 1998; Hammerschmidt, 1999). The results from these SA treatments may indicate an ability of *Orobanche* to inhibit the local expression of genes involved in SAR or that *O. aegyptiaca* possesses a physical resistance to enzymes such as glucanases and chitinases activated during SAR.

Arabidopsis has become a model system for studying PR gene expression and SAR (Uknes *et al*., 1992; Mauch-Mani and Slusarenko, 1993; Hu and Reddy, 1997; Reymond and Farmer, 1998; Hammerschmidt, 1999). In order to further study the SAR-mediated response to *O. aegyptiaca* parasitization, *PR-1*, *PR-2*, and *PR-5* genes from Arabidopsis were analyzed by northern hybridization. The PR-1 acidic gene from Arabidopsis shares less than 60% homology with *PR-1a* from tobacco. Previous reports indicated that *PR-1*, *PR-2*, and *PR-5* are induced by INA treatment, pathogen infection, and SA treatment (Uknes *et al*., 1992) and thus represent a cross-section of SA-mediated PR genes.

O. aegyptiaca parasitization did not induce *PR-1*, *PR-2*, or *PR-5* expression (data not shown). In fact, data from *PR-2* indicated that *O. aegyptiaca* parasitization repressed

expression as compared to control tissue. No mRNA was detected in the *O. aegyptiaca* infected tissue, whereas *PR-2* mRNA was present at low levels in control and wounded samples, and induced in SA treated tissue (Fig. IV.2 A). The high level of expression of *PR-2* in response to SA provided a positive control and correlated with the reported induction of *PR-2* (Uknes *et al*., 1992). The expression in the untreated control tissues was not unusual because the presence of PR-type proteins such as PR-2 in healthy plant tissues appears to be common in dicots (Van Loon and Van Strien, 1999). However, the detection of *PR-2* in wounded tissue does contrast with results obtained from Moran and Thompson (2001) in which *BGL2*, an apoplastic form of β-1,3-glucanase with 94% homology to *PR-2* at the DNA level, was not expressed in wounded tissue 72-96 hours after wounding. However, *BGL2* gene induction was only studied in leaf tissue and mRNA levels in root tissue were not analyzed. Previous studies (Westwood *et al*., 1998;

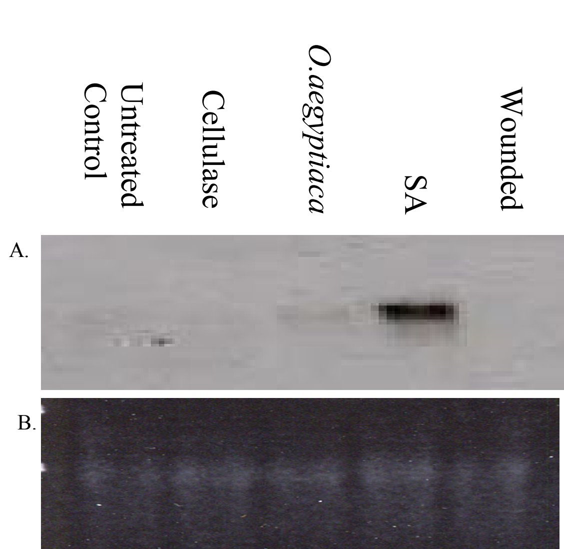

Figure IV.1:A) Northern hybridization of tobacco root RNA with a *PR-1a* probe. Treatments were untreated control, cellulase, parasitized by *O. aegyptiaca*, salicylic acid (SA), and wounded. B) Ethidium bromide stained gel demonstrating equal sample loading.

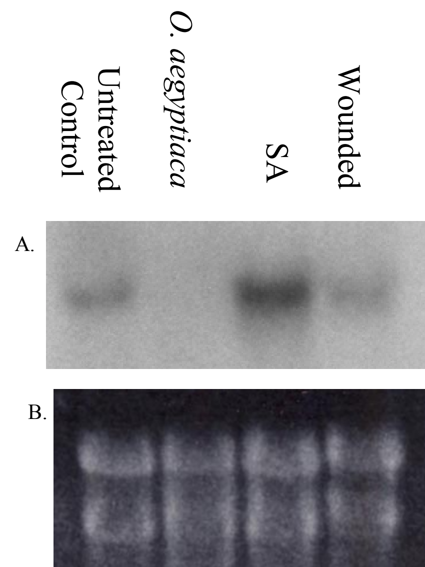

Figure IV.2:A) Northern hybridization of *Arabidopsis* root RNA with a *PR-2* probe. Treatments were untreated control, parasitized by *O. aegyptiaca*, salicylic acid (SA), and wounded. B) Ethidium bromide stained gel demonstrating equal sample loading.

Chapter III) suggest that *O. aegyptiaca* induces genes associated with wound responses, so these results indicating that the infected tissue has less *PR-2* mRNA than the wounded tissue raises the possibility that the parasite can somehow inhibit the synthesis or enhance the degradation of *PR-2* mRNA.

Northern analyses of Arabidopsis *PR-1* and *PR-5* indicated no expression of these genes in control, *O. aegyptiaca* parasitized, SA treated, or wounded tissue samples (data not shown). The lack of induction in the *O. aegyptiaca* treated tissue was not surprising based on our previous results from *PR-1a* from tobacco, and *PR-2* from Arabidopsis. However, the lack of expression in the SA treated lane was unexpected and led to the conclusion that the six hr of SA treatment used in this study did not allow enough time for the accumulation of *PR-1* or *PR-5* mRNA. Most studies in Arabidopsis involving analysis of *PR-1*, *PR-2*, or *PR-5* gene expression in SA treated tissue allowed 2-5 days for mRNA accumulation before RNA isolation (Dong *et al*., 1991; Datta *et al*., 1999; Cameron *et al*., 1999; Hu and Reddy, 1997; Moran and Thompson, 2001). To confirm the expression pattern of *PR-1* and *PR-5* in plants parasitized by *O. aegyptiaca*, experiments in which Arabidopsis plants are treated with SA and harvested over a longer time course (ie. 24-96) hours are needed.

This variation in PR gene induction times is supported by studies on the role of *PR-1*, *PR-2*, and *PR-5* expression in pathogen defense signaling related to SAR. For instance, Mauch-Mani and Slusarenko (1994) found that Arabidopsis *PR-2* gene expression was induced within one day following inoculation with *Fusarium oxysporum*, a fungus that causes vascular wilting, while *PR-1* induction did not occur until four days after inoculation. Additionally, Moran and Thompson, (2001) found that in Arabidopsis shoots, *BGL2* was clearly induced by aphid feeding after 24 hours while *PR-1* mRNA accumulation was not detectable until 48 hours after infestation.

The mechanism by which *Orobanche* invades the host root tissue may provide a method of attack that avoids induction of PR genes and SAR. Unlike necrotizing pathogens, which often use proteases or cutinases to degrade plant cell wall components, *Orobanche* uses a less disruptive mechanism of penetration involving enzymes such as PME and PGA which allow the pathogen to loosen the adhesions holding the host cells together and physically push itself in between the host cells (Ben-Hod *et al*. 1993, Joel *et*

al. 1994). By using this method of penetration, *Orobanche* may decrease the number of host cell fragments produced that might trigger additional defense responses within the plant. Additionally, it is well known that plants possess pathogen resistance genes (R genes) that are specific for certain pathogens that contain corresponding avirulence (avr) genes. It appears that *Orobanche* does not possess avr genes that are recognized by tobacco or Arabidopsis and therefore can invade the host plant without triggering a HR response.

Although *O. aegyptiaca* parasitization of susceptible plants induces defense genes related to wounding and pathogen infection, these plants are unable to defend themselves against the parasite. *O. aegyptiaca* parasitization induced the localized expression of *hmg2* and other defense related genes involved in the isoprenoid pathway (Westwood *et al*., 1998; Chapter III). *Orobanche*-triggered defense signaling appears to be dependent on the wound response/ JA pathway and does not seem to involve the expression of PR proteins associated with SAR. The fact that JA-mediated defense responses are not effective in deterring *O. aegyptiaca* suggests that the parasite has a mechanism to avoid, inhibit the synthesis of, or detoxify the phytoalexins produced by this pathway (see Chapter III). The products of the SA-mediated pathway may prove more effective in defense against *O. aegyptiaca*, but induction of this pathway has yet to be demonstrated. Thus the success of *O. aegyptiaca* may be explained by the ability of the parasite to avoid inducing genes involved in SAR. Additionally, since the products of SAR are not toxic to the host plant, and are usually aimed at fungal or bacterial pathogens, *O. aegyptiaca* may be resistant to these effects based on the simple fact that it is an angiosperm. To better understand the role of PR proteins in defense responses to parasitization, and to verify these hypotheses, additional acidic and basic PR genes, as well as other genes involved in SAR signal transduction, need to be studied.

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Appendix I

A.I Introduction

A.I.1 Microarray Technology

One method recently developed to study the interaction of thousands of genes involves the use of microarray technology. This method requires a probe with a known identity that can be fluorescently labeled and then hybridized to a chip containing thousands of spots representing different DNAs. If the probe contains a cDNA whose sequence is complimentary to a spot on the chip, then the cDNA will hybridize to the spot and later be detected. This technique provides a means of analyzing thousands of genes simultaneously.

For our purposes, we would like to utilize this technology to discover additional genes of interest. We are currently collaborating with the Bioinformatics Institute at Virginia Tech to prepare an Affymetrix chip. We have isolated total and polyA RNA from control and *O. aegyptiaca* infected Arabidopsis plants and are now awaiting preparation of the chip.

A.I.2 Materials and Methods

Isolation of PolyA RNA for Use in Microarray Technology

Arabidopsis plants were infected with *O. aegyptiaca* seeds as described in Chapter III. Untreated control plants were also treated as described in Chapter III, however, to ensure equal treatment conditions, GR-24 was also added to the control plants 14 days after transplantation into polyethylene bags. Total RNA was isolated from roots using the following modified Trizol method suggested by Dr. Maureen Dolan, Virginia Tech: 1) 1.5-2.0 g of frozen root tissue was ground in liquid nitrogen 2) frozen samples were immediately added to ice cold oak ridge Teflon tubes containing 14 mL Trizol Reagent (Invitrogen, Carlsbad, CA) (14 mL Trizol/ 1.5 g tissue) and tubes were placed laying sideways on a shaker and gently rocked for at least 10 minutes 3) 2.8 mL chloroform (0.2 mL/ 1 mL Trizol) was added to each sample, and tubes were mixed gently in a back-and-forth motion 4) tubes were placed at room temperature for 10 minutes and then centrifuged at 12,000 rpm for 15 min at 4˚C 5) centrifugation caused the samples to separate into three layers and the top colorless layer was removed and placed in a clean chloroform treated oak ridge tube 6) 3.5 mL isopropanol (0.25 mL/ 1

mL Trizol) was then added to each sample, and tubes were mixed gently in a back-and-forth motion 7) 3.5 mL of 0.8M DEPC treated NaCl/NaCitrate (0.25 mL/ 1 mL Trizol) solution was added to each sample. Tubes sat at room temperature for 30 min at which time they were centrifuged at 12,000 rpm for 15 min at 4-25˚C 8) after centrifugation, the supernatant was removed from each sample and the remaining pellet was washed with 75 % EtOH (DEPC treated water + 100 % EtOH). Each pellet was then flicked loose from the wall of the tube and all samples were placed at –20˚C overnight 9) samples were spun at 12,000 rpm at room temperature, the supernatant was removed from each, and all samples were placed under the hood to dry the RNA 10) each dried pellet of RNA was then resuspended by adding 300 µl DEPC treated water and heating at 50˚C. Next, 30 µl 3M DEPC treated Na-acetate, pH 5.2, and 660 µl 100 % EtOH was added to each sample and all samples were gently mixed and stored overnight at –20˚C 11) the next day, the samples were centrifuged, the supernatant was removed from each, and pellets were rinsed in 75 % EtOH. Pellets were then dried as in step 9 and resuspended in DEPC treated water by heating at 50˚C. PolyA RNA was then isolated from total RNA (Qiagen, Valencia, CA).