# **Study and Manipulation of the Salicylic Acid-Dependent Defense Pathway in Plants Parasitized by *Orobanche aegyptiaca* Pers.**

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ABSTRACT

The parasitic angiosperm *Orobanche aegyptiaca* (Pers.) (Egyptian broomrape) is a root holoparasite that causes severe losses in yield and quality of many crops. Control of *Orobanche* is extremely challenging, in part because the parasite is hidden underground for most of its life cycle. However, the dependence of the parasite on the host suggests that broomrape-resistant hosts could be an ideal control method. Genetic engineering strategies may facilitate realization of this goal, but require an understanding of host defense responses to parasitism. Previous studies with tobacco indicated that broomrape parasitism induces host genes associated with jasmonic acid (JA)-mediated defenses such as wound responses and localized production of phenylpropanoid and isoprenoid phytoalexins. However, the gene for the pathogenesis-related (PR) protein, PR-1a, was not induced by parasitism in tobacco. Expression of *PR-1a* is correlated with the salicylic acid (SA)-mediated defense pathway that leads to systemic acquired resistance (SAR). The objective of this research was to extend the characterization of PR gene expression in order to define the scope of host defense response. Analyses of gene expression using RNA hybridization and RT-PCR in broomrape-parasitized *Arabidopsis thaliana* roots indicated that *PR-1*, *PR-2*, *PR-5*, as well as the JA-associated *PDF1.2*, were slightly induced by parasitism. Expression of *PR-1*, *PR-5*, and *PDF1.2* in parasitized roots was not detectable by RNA hybridization analysis, but was demonstrated by RT-PCR. Interestingly, shoots of the parasitized plants showed greater PR gene expression levels than roots, indicating that *O. aegyptiaca* induced a response in the host that was systemic and amplified in shoots. Microarray analysis of parasitized *Arabidopsis* roots demonstrated a broad range of host gene expression changes including both defense- and non-defense-related genes. Genes induced were consistent with *O. aegyptiaca* preferentially stimulating JA-mediated responses.

The failure of *O. aegyptiaca* to elicit SA-mediated defenses in host roots suggested that exogenous induction of this signaling pathway could enhance host resistance to parasitism. Treatment of *O. aegyptiaca*-inoculated tobacco with BTH, a SA analog that activates SAR, caused a 49% reduction in *O. aegyptiaca* numbers. Analysis of PR-1a using RNA hybridizations and protein immunoblots in treated plants showed the expected induction in shoots, but not in roots, confirming the organ-specific differences in defense response observed in *Arabidopsis*. Experiments using a strategy to engineer the hypersensitive response via the gene-for-gene interaction confirmed previous findings that parasite-specific activation of an *R/Avr* interaction in tobacco reduced parasitism by approximately 50%. This research suggests that approaches to stimulate SAR in susceptible host plants may be useful for reducing *Orobanche* parasitism

# Dedication

This work is dedicated to my family, friends and to the memory of my beloved mother, Cristina

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# CHAPTER ONE

# Literature Review

## I.1 *Orobanche*

### I.1.1. Introduction

*Orobanche* spp. are root holoparasitic plants that cause severe losses in yield and quality in a wide range of dicotyledonous crops. The genus *Orobanche* has more than 100 species, but the most economically important are *O. aegyptiaca* (Pers.), *O. ramosa* (L.), *O. cernua* (Loefl), *O. minor* (Sm.), *O. crenata* (Forsk.) and *O. foetida* (Poir.) (Parker and Riches, 1993). Because most methods for controlling *Orobanche* either have limited effectiveness or are prohibitively expensive, it has become increasingly important that we understand mechanisms of crop resistance to *Orobanche*. The best long-term strategy for limiting damage by *Orobanche* is the development of resistant crops, but traditional plant breeding has generally failed to produce resistance that is durable across time, location, and varying parasite pressure (Cubero, 1991). Knowledge of the molecular events in host defense response to parasitic weeds will increase our understanding of the interaction between the host and parasite, and lead to strategies for engineering novel parasite-resistance mechanisms into crops.

### I.1.2. Distribution, economic impact, and host range of *Orobanche*

*Orobanche* spp. have been reported in 58 countries around the world, primarily eastern Europe, the Mediterranean, the Middle East, eastern Africa, southern and west Asia. In the Mediterranean area and western Asia approximately 16 million ha of

cropland are threatened by *Orobanche* spp. representing about 1.2% of world's arable land (Sauerborn *et al*., 2002).

Yield reductions caused by *Orobanche* depend on the severity of the infestation, with yield losses ranging from 5% to 100%, and at high levels can force farmers to abandon fields. In the former Yugoslavia, the area of cultivated sunflower was reduced by 37% following an *O. cumana* infestation (Sauerborn *et al*., 2002). Garcia-Torres et al. (1994) reported that *Orobanche* spp. infested about 100,000 and 350,000 ha in the central and southern parts of Spain, respectively.

*Orobanche* spp. parasitize only dicotyledonous hosts. The most economically devastating *Orobanche* species attack a broad range of hosts, including members of families Solanacae (tomato, tobacco, potato, eggplant), Fabaceae, (broadbean, pea, lentil, alfalfa, clover, trefoil) Cucurbitaceae (cucumber, melon, water melon, pumpkin and squash), Compositae (lettuce, sunflower), Cruciferae (cabbage, mustard, rapeseed) and Umbelliferae (carrot, celery, parsnip) (Parker and Riches, 1993).

#### I.1.3. Physiology of *Orobanche* parasitism

The lifestyle of *Orobanche* requires close communication and physiological interaction with the host. The initial interaction between parasite and host is the perception by the parasite seed of a specific chemical germination signal produced by the host root. The most common class of germination stimulants produced by hosts of *Orobanche*. and other parasitic species such *Striga* are sesquiterpene lactones, which are collectively referred to as strigolactones (Wigchert and Zwanenburg, 1999). The parasite

seed germinates after detecting these stimulants, and the radical must then contact the host root. The radicle tip subsequently enlarges, forming a haustorium that is the specialized organ that enables the parasite to penetrate the host root. Vascular connections with the host root are formed by the haustorium.

The process of host attachment has been studied by light and electron microscopy. The radicles of *O. cumana and O. aegyptiaca* exhibit a conical root apex with a thin cuticle layer covering the entire radicle (Joel and Losner-Goshen, 1994). The apical cells have large nuclei and dense cytoplasm, rich in ribosomes and endoplasmic reticulum (ER) cisternae. The Golgi apparatus in these cells is composed of many dictyosomes and vesicles. Root elongation is the result of both cell division and cell elongation at the root apex, but the root meristem of *Orobanche* does not resemble that of a typical dicot root, as it lacks a well-defined root cap and does not produce procambium and a typical meristem (Joel and Losner-Goshen, 1994). Later in radicle development, two to four vacuoled cells become visible at the tip of the root apex. Cell elongation ceases after the radicle reaches the host, and then starch accumulates along with the production of a mucilaginous secretion. The external layer of the radicle then differentiates into a papillate cell layer that serves as an adhesion surface forming a crown around the apical cells, which remain nonpapillate, but which differentiate to form the central part of the intrusive body (Joel and Losner-Goshen, 1994).

Following attachment, cells of the parasite haustorium grow intrusively between cells of the host epidermis and cortex. The production of pectin methylesterase (PME), polygalacturonases and endocelullase apparently facilitate this process (Shomer-Ilan, 1993; Ben-Hod *et al.,* 1993 and Losner-Goshen *et al*., 1998). The parasite then forms

connections to the host vascular system via the haustorium, which serves as a bridge for water and nutrient uptake. The parasite captures host resources by establishing a low osmotic potential relative to the host through the biosynthesis of polyhydric alcohols such as mannitol. (Harloff and Wegmann, 1993; Robert *et al*., 1999). After the connection between the parasite and the host is established, the parasite grows rapidly and develops roots that are also capable of forming new attachments to the host. Ultimately, the parasite produces shoots that emerge above the ground to flower and set seeds.

### I.1.4. Effects of *Orobanche* parasitism on the host

A parasitic plant starts to influence its host from the moment of contact. The effects of *Orobanche* parasitism on the host may range from no visible injury to host death. However, common visible effects can include wilting, reduction in plant size, lower yield, and decreased quality of the crop. As a general rule, a combination of three factors determines the impact of *Orobanche* parasitism on the host: the size of the parasite, the rate of growth and metabolic activity of the parasite, and the stage of development of the host when the parasitism occurs (Press and Graves, 1995).

Some physiological observations on specific host-*Orobanche* pathosystems have demonstrated that *Orobanche* parasitism produces diverse effects on host nutrient status. In the tobacco-*O. ramosa* interaction, a depletion of potassium in the host was proposed to be responsible for the damage to the host (Ernst, 1986). Other studies have demonstrated a parasite-induced decrease in host potassium and phosphorous and an increase in calcium (Abou-Raya *et al*., 1973). In the carrot-*O. crenata* system, one third of total host sugar (primarily sucrose) was lost (Schaffer *et al*., 1991). Stewart and Press

(1990) proposed that the impact from *Orobanche* parasitism may reduce the host's ability to absorb water from the soil, leading to the closing of stomata similar to that caused by drought stress, and thus indirectly impacting the host's capacity for photosynthesis. In this way, changes in the host nutrient budget incurred by *Orobanche* parasitism were considered to be responsible for up to 30% reduction in host growth.

### I.1.5. Control of *Orobanche*

#### I.1.5.1. Cultural, mechanical and biological methods

A wide variety of methods have been tested for *Orobanche* control. Among these are cultural methods such as hand-pulling, trap-crops (crops that induce *Orobanche* germination, but are not attacked by the parasite), and catch-crops (crops susceptible to parasite attack but which are killed prior to maturity of the weed) (Parker and Riches, 1993). Other cultural practices such as flooding, time of planting, method of planting, fertilization, and solarization have demonstrated some positive results, but more studies need to be done to establish the best conditions for maximum effectiveness (Parker and Riches, 1993). Currently, none of these methods alone is reliably effective, and many are either not economically justified or are laborious. Biological control of *Orobanche* via mycoherbicides or insects offers some potential. For example in Syria, applications of *Phytomyza orobanchia* to a field naturally infested with *Orobanche* reduced the production of seeds by 30%. However, effectiveness depended on climate factors favorable for the insect and the absence of natural predators (Sauerborn, 1998).

The most effective method used currently for controlling *Orobanche* are soil sterilants (e.g., methyl-bromide), which can kill parasite seeds in the soil. However, these methods are expensive and hazardous to the environment. Other chemical methods include the use of herbicides (such as glyphosate, imazethapyr, etc.) applied pre- or post-emergence of the parasite (Foy *et al*., 1989). However, most herbicides lack selectivity, producing injury not only to the weed, but also to the crop.

The development of crops resistant to chemicals such as glyphosate, chlorsurfuron, and the imidazoline family of herbicides, overcomes the lack of herbicide selectivity and provides excellent opportunities for *Orobanche* control. These methods require that crops have herbicide resistance based on alteration of the target site rather than herbicide metabolism so that herbicide moves from the host to the parasite in its herbicidally active form (Joel *et al*., 1995). Recombinant DNA technologies, as well as the selection of herbicide-resistant plants from mutagenized populations, will provide new resources for this approach. Despite the advantages offered by herbicide-resistant crops, their use could accelerate the development of herbicide-resistant *Orobanche* populations (Gressel *et al*., 1996). Also, this technology is largely controlled by private industry and is not being made available in the relatively low value crops that are most impacted by parasites.

#### I.1.5.2. *Orobanche*-resistant crop varieties

Despite efforts to identify sources of resistance to *Orobanche* species in germplasm of crops and wild relatives (sunflower, faba bean, vetch, tomato, tobacco,

etc.), few examples of stable resistance are known. A particularly interesting example is sunflower, for which new races of the parasite have been reported to overcome each new resistant variety (Cubero, 1991). Studies were started in the former USSR in 1906, by introgressing resistance genes from Jerusalem artichoke (*H. tuberosus* L.) into cultivated sunflower (Vranceanu *et al.,* 1980). Initial varieties with resistance to *O. cumana* were found to be parasitized by new races. The most widely used sources of resistance, in the case of sunflower, have been based on single dominant genes (Lu *et al*., 2000). However, there is a lack of resistant germplasm in other most affected crops and much screening has been conducted in vain. In cases where resistant varieties have been identified, the trait is often quantitative and difficult to breed into commercial varieties. In addition, the existence of physiological races of the parasite has further complicated and limited the effectiveness of breeding programs.

## I.2. The plant defense system

### I.2.1. Introduction

Plants are sessile and cannot escape from the diverse range of biotic agents that prey on them. Therefore, plants have evolved elaborate defense strategies to detect and deter pathogens. Plants are generally able to protect themselves, so disease is an exception to the norm (Loake, 2001). To be successful, pathogens have to breach several lines of defense, the first of which includes passive defenses such as the leaf wax layer, cell wall, and preformed antimicrobial compounds. Those pathogens able to penetrate beyond this barrier will try to establish a relationship with the plant. For some pathogens,

this will be limited to molecular signals released from outside the plant cell wall, such as fungal-cell wall constituents (e.g., chitin, glucan, glycoproteins), flagellin, and bacterial lipopolysaccharides, but for others it includes penetration of the cell wall and the delivery of signal molecules directly to the plant cytosol (Jones and Takemoto, 2004). Within minutes of contact between the pathogen and the host, reactive oxygen intermediates (ROS) are produced that activate defense gene expression in adjacent cells, for example by causing the accumulation of transcripts of oxidation stress-protective glutathione *S*-transferases (Levine *et al*., 1994), or by initiating the hypersensitive response (HR). HR is a form of programmed plant cell death which results from a reprogramming of metabolism in cells surrounding the infection in order to stop the spread of the pathogen (Lamb *et al*., 1989). This localized response can trigger a long-lasting systemic response, systemic acquired resistance (SAR), which has been shown to be effective against a broad range of pathogens (Uknes et a., 1992). SAR is associated with accumulation of salicylic acid (SA) and a number of pathogenesis related (PR) proteins (Ryals *et al*., 1996).

Plant defense against pathogens is regulated by multiple signal transduction pathways through a complex network that allows the plant to have a fine-tuned response (Kunkel and Brooks, 2002). These signaling pathways involve three major endogenous plant signal molecules: SA, jasmonic acid (JA), and ethylene (ET). The defense pathways are not isolated, but instead are all part of a complex metabolic network as Scheideler et al. (2002) demonstrated using cDNA microarrays in a time-course experiment during the incompatible interaction between *Arabidopsis thaliana* and *Pseudomonas syringae* pv *tomato*. Over the course of the infection, changes were observed across functional

categories of genes including those involved in metabolic enzymes, cellular organization, signal transduction, control of gene expression, stress response-related and other unknown genes. Data revealed significant changes in the steady state transcript levels of ~650 genes within 10 min and a massive shift in gene expression patterns by 7h involving ~2000 genes representing many cellular processes.

### I.2.2. Pathogen recognition

#### I.2.2.1. R gene-mediated resistance

The *R*-gene mediated resistance concept was first introduced by Flor (1956) while conducting genetic studies in the flax-flax rust interaction. This concept is based on the capability of the plant (through an *R* gene product) to recognize specific factors from the pathogen (the product of an *avr* gene, which is called the effector). The recognition activates inducible plant defense responses (Dangl and Jones, 2001). Since this model was conceived, *R* genes have been shown to govern plant-pathogen interactions in several host plants with specificity to a variety of phylogenetically diverse pathogens including bacteria, fungi, oomycetes, nematodes, viruses, and insects. *R*-mediated recognition triggers a highly effective resistance leading to a hyper-activation of basal defense responses in the challenged and surrounding cells, accompanied by a programmed host cell death or HR (Lamb *et al*., 1989).

The hallmark of *R*-mediated resistance is specificity; most *R* genes recognize one, or in limited cases, two specific pathogen-derived molecules encoded by a corresponding *avr* gene (Nimchuk *et al.,* 2003). This simple mechanistic interpretation of the genetics of

the *R-avr* interaction lacks sufficient evidence. Rather, it has been suggested that R proteins may not recognize pathogen virulence molecules directly, but recognize the cellular changes of their actions (i.e., products of enzymatic activity produced by Avr proteins) in the host cell. This is the "guard hypothesis" (Dangl and Jones, 2001), which still lacks essential proof (identification of host proteins for pathogen virulence, or "guardees"), but it is a useful guide for interpretation of experiments.

Some recent approaches have demonstrated the role of other proteins in the function of R proteins, specifically that *R* genes form a multimeric complex for an indirect recognition of pathogen effectors. When members of the heat shock protein family (Hsp90 and Hsp70) were silenced in tobacco, this resulted in the absence of HR (Kanzaki *et al.,* 2003 and Yu *et al*., 2004).

Genetic studies have demonstrated that plants devote a considerable percentage of their genome to *R* gene families (Nimchuk *et al*., 2003). It is estimated that there are approximately 125 *R*-genes in *Arabidopsis* and about 600 in rice, however the functions for a large number of them are still unknown. The largest class of *R* genes encodes a nucleotide-binding site plus a leucine–rich repeat sequence (NB-LRR). NB-LRR proteins have distinct N-terminal domains, with either a putative coiled coil (CC) or a domain that shares homology with the Drosophila TOLL and mammalian IL-1 receptor (TIR). Their most striking structural feature is a variable number of carboxy-terminal LRR in which some residues are subject to diversifying selection (Ellis and Dodds, 2000). In addition, mutational analysis and domain-swapping experiments have demonstrated that recognition specificity is governed largely by the LRR domain (Dangl and Jones, 2001). The LRR domain has also been found to mediate protein-protein interaction in other

proteins. If in fact R gene products interact directly with Avr products, the LRR domain may be the feature responsible.

Several NB-LRR genes have been found to undergo alternative splicing, generating variants that encode proteins similar to truncated *R* genes. This is the case for the TIR-NB-LRR type N gene in tobacco that recognizes the tobacco mosaic virus (TMV). Changes in the abundance of splice variants have been observed during TMV infection (Dinesh-Kumar and Baker 2000). The abundance of a certain type of transcript in a specific ratio resulted in an effective response against pathogen infection (Dinesh-Kumar and Baker 2000). Thus, the ability of *R* genes to encode more than one transcript appears to play a role in plant disease resistance.

#### I.2.2.2. Gene activation and signaling pathways

After pathogen recognition, a cascade of gene activation occurs. Genetic dissection of local and systemic signaling networks has been possible mainly due to the exploitation of *Arabidopsis* mutants and transgenic plants. Several studies demonstrate the complex interplay between defense molecules such as SA, nitric oxide (NO), ROS, JA and ET (Hammond-Kosack and Parker, 2003).

The structural similarity among R proteins suggests the existence of common or related signal transduction mechanisms downstream of pathogen perception (Shirasu and Schulze-Lefert 2000). What determines which pathway is used by a particular *R* gene is not entirely clear, but some generalizations can be made. R-protein–dependent activated signaling cascades may be correlated with two different classes of R proteins: CC-NB-

LRR and TIR-NB-LRR. A possible convergence point in a signaling pathway engaged by multiple *R* genes is at RAR1/SGT1, both of which operate upstream of the HR and the oxidative burst (Hammond-Kosack and Parker, 2003).

After R-gene activation occurs, there are two separable R-dependent signaling branches involved. The first is the *EDS1*/*PAD4* (*ENHANCED DISEASE SUSCEPTIBILITY1*/*PHYTOALEXIN DEFICIENT4*) dependent signaling pathway which is generally associated with TIR-NB-LRR. The second is associated with CC-NB-LRR *R*-genes that requires the *NDR1* (*NON-RACE SPECIFIC DISEASE RESISTANCE1*) protein (Glazebrook, 2001; Loake 2001). Despite this generalization, there are some exceptions that do not use any of these signaling regulators, for example *RPP*7 (class CC-NB-LRR), which encodes resistance to *Peronospora parasitica* and functions independent of either *EDS1* or *NDR1* (McDowell *et al*., 2000).

#### I.2.2.2.1 Salicylic acid signaling in defense response

SA has long been recognized as being required for local resistance and SAR (Durner *et al*., 1997). SA concentration increases at pathogen infection sites in tobacco (Ryals *et al*., 1996), and transgenic plants unable to accumulate SA were compromised in their ability to establish local resistance and SAR (Delaney *et al*., 1994). In contrast, transgenic tobacco plants encoding bacterial genes isochorismate synthase (*ICS*) and pyruvate lyase (*PIL*) that overproduced SA showed constitutive expression of defense genes and enhanced resistance to various pathogens (Verberne *et al*., 2000). Exogenous application of SA or synthetic analogs such as BTH (benzo (1,2,3)-thiadiazole-7-

carbothioic acid *S*-methyl ester) (Friedrich *et al*., 1996) have been demonstrated to restore SAR.

Previous studies suggested that SA was synthesized from the phenylpropanoid pathway, but recent investigations indicate that SA is synthesized from ICS in *Arabidopsis* (Wildermuth *et al*., 2001). Characterization of an *Arabidopsis* defense-related gene *SID2* (*SALICYLIC-ACID-INDUCTION DEFICIENT*2), showed that it encodes a putative chloroplast-localized ICS that is activated in infected tissues and in tissues exhibiting SAR. Moreover, plants with the mutant allele (*sid2*) were defective in SA synthesis and had enhanced susceptibility to pathogens (Wildermuth *et al*., 2001).

Mutation in *EDS1* and *PAD4* genes leads to a reduction in SA production, which suggests that these important *R*-gene mediated regulators act upstream of SA (Nimchuck *et al*., 2003). It has also been shown that SA activates *EDS1* and *PAD4* genes (Feys *et al*., 2001), indicating that these proteins act in a positive feedback loop (Jirage *et al*., 1999). The presence of a positive feedback loop ensures the amplification of the weak input created during initial pathogen invasion.

The SA biosynthesis pathway is regulated at several levels, and SA-signaling is mediated by at least two mechanisms: *NPR-1* (*NON-EXPRESSOR OF PR-1*)- dependent and *NPR*-1-independent models (Shah, 2003). NPR-1 protein appears to act as a key regulator of SAR (Maleck *et al*., 2000). The application of SA or its analogs promotes the translocation of NPR1 into the nucleus (Kinkema *et al*., 2000), indicating a role for this protein in transcriptional regulation during plant defense response.

The mechanism by which NPR-1 regulates transcription of SAR has been studied extensively. It has been shown that members of the TGA–element binding protein family

of basic-leucine-zipper (bZIP) interact physically with NPR-1 in yeast-two hybrid assays (Despres *et al*., 2000). This interaction has also been demonstrated to be enhanced by SA (Fan and Dong, 2002). These TGA factors can bind to the *as-1* elements that are present in the *PR*-1 promoter (Lebel *et al*., 1998). Recently, Shonglin et al, (2003) have shown that NPR-1 oligomers present in the uninduced state convert to a monomeric form under a cellular redox change that can be induced by SA analogs. The monomeric NPR-1 protein then moves into the nucleus to control SAR-related gene expression (Shonglin *et al*., 2003). Lately, Despres et al. (2003) showed that Cys residues from TGA1 factor are reduced by SA and this enables TGA to bind to NPR-1. Thus, NPR-1 stimulates the DNA binding activity of the reduced form of TGA1. Hence, SA affects NPR-1 activity at three stages: first, it activates NPR-1 expression; second, it stimulates the translocation of NPR-1 into the nucleus, and third, it reduces Cys residues from TGA that are able to interact with NPR-1.

Recently, it has been shown that SA perception appears to be modulated by phytochrome signaling (Genoud *et al*., 2002). An intact phytochrome pathway is required for full expression of SA-mediated responses and HR (Genoud *et al*., 2002).

Members of another transcription family, WRKY, have been shown to be involved in plant defense response (Eulgem *et al*., 2000). WRKY family proteins bind W-boxes, and a mutation in a W-box element in the NPR-1 promoter caused WRKY to be unable to bind, thereby compromising the effectiveness of NPR-1 (Yu *et al.,* 2001).

The oxidative burst is one of the earliest events after pathogen recognition, and the primary product appears to be superoxide, which is then converted to hydrogen peroxide (Lamb and Dixon, 1997). Studies conducted by blocking the production of NO

in *Arabidopsis* infiltrated with *P. syringae* pv *maculicola* demonstrated that NO potentiates the induction of HR (Delledonne *et al*., 1998). A positive feedback loop involving production of ROS, NO and SA appears to play a central role in the activation of the defense program (Nimchuck *et al*., 2003).

##### I.2.2.2.2 Jasmonic acid- and Ethylene-dependent defense signaling pathways

Many studies have demonstrated the involvement of JA as a wound response regulator during pathogen defense response (Wang *et al*., 2002; Lorenzo *et al*., 2003 and reviewed in Dong, 1998). Moreover, ET is also clearly involved in disease symptom expression, although the role of ethylene varies with the type of pathogen and plant species (Wang *et al.,* 2002). Interactions between JA and ET signaling pathways have also been demonstrated during plant defense responses, and they appear to interact downstream of their biosynthetic pathways, possibly at the level of the specific defense gene promoters in the pathway (Penninckx *et al*., 1998). One of the best-characterized final products of the JA/ET-signaling pathway during pathogen attack is a small cysteine-rich polypeptide that has antifungal properties, called PDF1.2, which belongs to the plant defensins (Penninckx *et al*., 1996).

## I.3. Host Response to *Orobanche* parasitism

Previous investigations of *Orobanche* parasitism have suggested that several mechanisms may be involved in host resistance. Resistant hosts have been shown to undergo cell wall deposition, vessel occlusion, accumulation of phenolic compounds, induction of genes associated with phytoalexins, and necrosis at the site of parasite penetration (Goldwasser *et al*., 2002).

In vetch (*Vicia* spp.), microscopic observations of resistant and susceptible genotypes demonstrated that *O. aegyptiaca* parasitism triggered the resistant genotype "Popany" to produce necrotic lesions (with the appearance of HR) surrounding the contact point of the parasite radicle, thus preventing further development of the parasite (Goldwasser *et al*., 1997). Additionally, biochemical analysis of resistant and susceptible host roots correlated higher concentrations of phenolic compounds and lignin with resistant genotypes, and greater peroxidase activity with the susceptible genotypes (Goldwasser *et al*., 1999). An increase in phenylalanine-ammonia lyase (PAL) activity was also demonstrated to correlate with the degree of resistance in three resistant *Vicia* species (Goldwasser *et al*., 1997). These previous studies suggest that resistance may be due to the combined actions of several genes, making this a quantitative trait in defense responses (Goldwasser *et al*., 2002). Anatomical studies of *Vicia* spp. during *Orobanche* parasitism showed that in the resistant genotype, the parasite haustorium was blocked at the root endodermis layer (Goldwasser *et al*., 2000).

Another factor in determining successful parasitism is virulence of the parasite. Research conducted by Antonova and Ter Borg (1995) showed that greater virulence of *O. cumana* races correlated with lower levels of peroxidase in cells of the parasite radicle.

Their observations suggested that peroxidase secreted by the parasite may be used by the sunflower for protective lignification, perhaps by causing the polymerization of phenolic compounds into lignin in host cells. Thus, the more aggressive parasite races showed less extracellular peroxidase, thereby minimizing host cell wall lignification and facilitating invasion of the host. Observations of a continuous layer of lignin compounds in the host sunflower roots at the haustorium interface supported this hypothesis. More recently, Labrousse et al. (2001) reported that a resistant sunflower genotype showed enhanced cell wall deposition, vessel occlusion, and parasite cellular disorganization during infection by *O. cumana.*

At the level of gene expression only a few studies have focused on host defense. Westwood et al. (1998) using transgenic tobacco plants bearing a chimeric construct [promoter fused to the β-glucuronidase (GUS) reporter gene] demonstrated the expression of the tomato (*Lycopersicon esculentum*) *HMG*2 promoter, an isogene of 3-hydroxy-3-methyglutaryl CoA reductase (HMGR), in transgenic tobacco plants within one day of host root penetration by *O. aegyptiaca*. *HMG2* has been demonstrated to be activated predominantly during defense responses associated with the production of sesquiterpene phytoalexins (Cramer *et al*., 1993; Chappell, 1995). The induction of *HMG2* during *Orobanche* infection was among the first studies to indicate that the host is able to recognize the parasite as an invader (Westwood *et al*., 1998).

Later, Griffitts et al. (2004), using the same reporter gene strategy, studied the expression of the bean (*Phaseolus vulgaris*) chalcone synthase (*CHS*) promoter in response to *Orobanche* parasitism. The blue precipitate indicative of GUS activity was observed surrounding the sites of penetration by the parasite. The *CHS8* promoter has

been previously shown to be strongly induced locally by pathogen attack (Schmid *et al*., 1990). Chalcone synthase catalyzes a step in the phenylpropanoid pathway that leads to the production of secondary metabolites, and in leguminous species gives rise to isoflavonoid–derived phytoalexin whose production is inducible by wounding, pathogen infection, biotic and abiotic elicitors (Ryder *et al*., 1987). The *PAL* (phenylalanine ammonia lyase) gene was also induced during *Orobanche* parasitism (Griffitts *et al*., 2004).

In contrast to genes associated with phytoalexin synthesis, Griffitts et al. (2004) demonstrated that the gene for the tobacco *PR-1*a protein (a SA-dependent gene) was not induced by *Orobanche*. On the other hand, Joel and Portnoy (1998) used a GUS reporter gene fusion in transgenic tobacco plants to study expression of the promoter of the basic *PRb-1b* gene, which is involved in JA-dependent defense responses (Xu *et al*., 1994). This gene was also found to be induced following *O. aegyptiaca* penetration.

Sauerborn et al. (2002) tested the efficacy of inducible resistance against *Orobanche* by adding benzothiadiazole (BTH), an analog of SA, to sunflower seeds. The resulting plants showed a reduction in infection by *O. cumana.* This reduction was attributed to the induction of SAR in sunflower roots, as indicated by the accumulation of an acid-chitinase enzyme, the presence of reactive oxygen intermediates, and the accumulation of a phytoalexin (scopoletin).

Recently, Vieira Dos Santos et al. (2003) used the *Arabidopsis-O. ramosa* interaction to study the expression of several host genes involved in defense response (e.g., those associated with phenylpropanoid, isoprenoid, JA, and SA pathways). RT-PCR analyses over the first hours to days after attachment suggested an induction of genes

associated with the JA signaling pathway (*ACC2* and *PDF1.2*) but not the SA pathway (*PR-1*, *PR-2* and *PR-5*). In further studies using suppression subtractive hybridization (SSH) in the same pathosystem, Vieira Dos Santos et al. (2003) demonstrated the expression of genes involved in cell wall reinforcement, defense response, signal transduction and JA biosynthesis during the first hours of attachment.

Despite of the activation of defense mechanisms in hosts such as tobacco and *Arabidopsis*, *Orobanche* is still successful in establishing a parasitic relationship. However, susceptibility in host-pathogen interactions is becoming valued as a necessary component of understanding plant defense responses (Panstruga, 2003). Thus, progress in characterizing compatible *Orobanche* interactions contributes to the understanding of resistance mechanisms.

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# CHAPTER TWO

## Expression of salicylic acid-mediated defense genes in *Arabidopsis*-parasitized by *Orobanche aegyptiaca*

## II.1. Abstract

The parasitic angiosperm *Orobanche aegyptiaca* Pers, (Egyptian broomrape) is a root holoparasite that causes severe losses in yield and quality of many crops. Control of *Orobanche* is extremely challenging, in part because the parasite is hidden underground for most of its life cycle. However, the dependence of the parasite on the host suggests that broomrape-resistant hosts would be an ideal control method. Genetic engineering strategies may facilitate realization of this goal, but require an understanding of host defense responses to parasitism. Previous studies in tobacco indicated that broomrape parasitism induces host genes associated with wound responses and production of phytoalexins from phenylpropanoid and isoprenoid pathways. However, the gene for the pathogenesis-related (PR) protein, *PR-1a*, was not induced by parasitism. The objective of this research was to characterize the expression of *PR* genes in order to define the scope of host defense response. Analyses of gene expression in broomrape-parasitized *Arabidopsis thaliana* roots indicated that *PR-1*, *PR-2*, *PR-5*, and *PDF1.2* were slightly induced by parasitism. Expression of *PR-1, PR-5,* and *PDF1.2* in parasitized roots was undetectable by RNA hybridization analysis, but RT-PCR demonstrated their induction. In addition, shoots of parasitized plants showed much greater *PR* gene expression levels than those observed in roots, indicating that *O. aegyptiaca* induces a systemic response in the host. Microarray analyses also demonstrated a broad range of host gene expression changes including both defense- and non-defense-related genes; these data were consistent with signatures from other classes of pathogens and biological process.

## II.2. Introduction

*Orobanche* spp. (broomrapes) are holoparasitic plants that depend on their hosts for water, minerals, and photoassimilates, often causing a reduction in host crop growth and yield. *Orobanche* species have been reported in 58 countries around the world, having the most devastating effects in the Mediterranean region, Middle East and western Asia. In these regions *Orobanche* spp. parasitize many economically important crops (e.g., sunflower, potato, tomato, eggplant, faba bean, etc.) and threaten 16 million ha of crop land (Sauerborn *et al.,* 2002).

Strategies currently used to control *Orobanche* species include cultural and physical methods (host denial, flooding, solarization, etc.), parasite-resistant hosts (only available in a few crop varieties such as sunflowers) (Tang *et al.,* 2003), and chemicals. Chemical control has relied on toxic, soil sterilants such as methyl-bromide, to destroy *Orobanche* seeds in the soil. Herbicides have been extensively tested for control of *Orobanche*, but until recently most herbicides did not provide sufficient selectivity. The development of crops engineered for resistance to herbicides may facilitate selective control of *Orobanche* in these crops (Joel *et al.,* 1995). Nevertheless, the best strategy for *Orobanche* control remains the development of parasite-resistant crops. Unfortunately, most affected crops lack resistant germplasm on which to base breeding programs and much unsuccessful screening has been conducted. In most cases where resistance has been identified, the specific mechanisms plants use to defend themselves from *Orobanche* are not well elucidated. Understanding how plants respond to parasitism – either in

resistant or susceptible interactions - will be important in producing resistant crops by recombinant DNA techniques.

Previous attempts to understand the physiological events underlying the parasite-host interaction in *Orobanche*-resistant crops have demonstrated that multiple mechanisms may act to deter parasite invasion. Such mechanisms include cell wall deposition, vessel occlusion, accumulation of phenolic compounds, induction of genes associated with phytoalexin production, as well as necrosis at the site of parasite penetration (Goldwasser *et al.,* 1997; Goldwasser *et al.,* 2002). Biochemical analyses have also demonstrated high phenylalanine-ammonia lyase (PAL) activity correlated with resistance in different *Vicia* species (Goldwasser *et al.,* 1997).

To examine molecular events important in the host-parasite interaction, Westwood et al. (1998) studied tobacco plants expressing a *HMG2* promoter:β-glucoronidase (GUS) gene fusion and demonstrated that *O. aegyptiaca* parasitism induced expression of the host *HMG*2 gene, a defense-related isogene encoding 3-hydroxy-3 methylglutaryl Coenzyme A reductase (HMGR). Joel and Portnoy (1998) used the same approach to demonstrate that *Orobanche* parasitism induced the expression of the basic *PRb-1b* gene in tobacco. Further studies of *Orobanche*-responsive promoters have demonstrated that other defense- and wound-responsive members of the phenylpropanoid pathway (PAL and chalcone synthase) are induced during parasitism (Griffitts *et al.,* 2004). These local defense genes are regulated predominantly by the jasmonic acid (JA)-mediated defense signaling pathway in plants. In contrast, a defense gene regulated through the salicylic acid (SA)-mediated pathway, *PR-1a*, was not induced after *O. aegyptiaca* attack (Griffitts *et al.,* 2004). In *Arabidopsis* parasitized by

*O. ramosa*, RT-PCR analyses of several defense-related genes supported these findings, showing transient induction of JA-dependent genes (*PDF*1.2, *ACC*2 and *Thi*2.1), but not SA-dependent genes (*PR-1*, *PR-2* and *PR-5*) (Vieira Dos Santos *et al.,* 2003). In further studies using suppression subtractive hybridization (SSH) in the same pathosystem, Vieira Dos Santos et al. (2003) demonstrated induction of genes involved in cell wall reinforcement and jasmonic acid biosynthesis during the first hours of parasite attachment.

With the exception of Vieira Dos Santos et al. (2003), efforts to dissect the molecular events occurring during the *Orobanche*-host interaction have identified relatively few components of the plant defense machinery involved in the response. Evidence to date suggests that parasitism by *Orobanche* selectively induces host defenses correlated with JA/ethylene (ET)-mediated pathways, but not SA-mediated responses. Such a distinction in defense pathway activity has implications for how the host perceives parasitism and what constitutes an effective defense against an angiosperm parasite. Our objective was to specifically evaluate the expression of host *PR* genes indicative of systemic acquired resistance (SAR) (Uknes *et al.,* 1992). To this end, we have used RNA hybridization and RT-PCR analyses on the *Arabidopsis*-*O. aegyptiaca* interaction to monitor gene expression. Results were supported and expanded by microarray analysis of *Arabidopsis* gene expression in response to *Orobanche* parasitism. This is the first example of microarray application to a host attacked by an angiosperm parasite, and contributes new insight into our understanding of parasitism.

## II.3. Materials and methods

### II.3.1. Plant material and growth conditions

*Arabidopsis thaliana* ecotype Columbia plants were grown for three weeks in potting media (Metromix 360, Scotts-Sierra Co, Marysville, OH), and were then transplanted to a polyethylene bag growth system (six plants per bag) as described by Westwood (2000). Seedlings were allowed to recover and grow roots for two weeks, at which time bags were randomly assigned to five different treatment groups with twelve bags per group. Growing conditions were 9 h light (150 $\mu$E /$m^2$/sec), 15 h dark at 20°C ± 2°C. Plants were watered as needed by adding half strength Hoagland solution (Hoagland and Arnon, 1950) to the bags. Bags were suspended in boxes to exclude light from the root systems.

### II.3.2. Treatments

Five different treatments were applied in order to compare patterns of gene expression during *O. aegyptiaca* infection with known defense response elicitors (JA, SA, and wounding). The *O. aegyptiaca*-parasitized group was inoculated by applying surface-sterilized *O. aegyptiaca* seeds (described in Westwood 2000) to filter paper around host roots. After seven days of pre-conditioning, *O. aegyptiaca* seeds were stimulated to germinate synchronously by adding 10 ml of a 10 ppm solution of GR-24, a synthetic germination stimulant (Mangnus *et al.,* 1992), to each bag. Ten days after stimulation, host root and shoot tissues were harvested. Control samples consisted of non-

inoculated roots that did not receive treatments, except for GR-24 in the same concentration as the *O. aegyptiaca*-inoculate plants. Three additional sets of plants received treatments of either 5 mM SA or 0.5 mM JA (Sigma, St Louis, MO) (10 ml of each solution was applied directly to the root systems after draining the Hoagland solution), or wounding by cutting tissue (roots as well as leaves) into approximately 1 cm sections with a scalpel. SA- and JA-treated roots were harvested 24 h after treatment, and wounded tissues were harvested after 8 h at room temperature. Harvest of all treatments was coordinated to occur on the same day, and tissues were frozen with liquid nitrogen and stored at -80ºC until RNA isolation. *O. aegyptiaca*-parasitized tissue contained a small proportion of *O. aegyptiaca* tissue because *Orobanche* haustoria are embedded within the host root.

### II.3.3. RNA isolation, RNA hybridization and probes

Tissue samples were ground to a fine powder in liquid $N_2$ and added to a Corex tube containing 14 ml of Trizol reagent (Invitrogen Corp. Carlsbad, CA) per g of tissue. The solution was gently shaken for 5 min at room temperature. Chloroform was added at 0.2 ml per ml Trizol, mixed, and placed at room temperature for 10 min. The solution was centrifuged (12,000g) for 15 min at 4ºC. The aqueous phase was transferred to a new tube and precipitated with isopropanol (0.25 ml per ml Trizol) and an equal volume of a sodium chloride/sodium citrate solution (1.2M/0.8M). Samples were stored at -20ºC for one h, then centrifuged (12,000g) for 15 min at 4ºC. The pellet was washed with 75% cold ethanol, using 1 ml for each ml Trizol used and centrifuged as above. The pellet was

air-dried, resuspended in 300 µl of RNAase-free water plus 30 µl of 3 M Na-acetate (pH 5.2; final concentration of 90.9 mM) and 660 µl of 100% ethanol, and stored overnight at -20ºC. The pellet was collected by centrifugation as above, washed with 70% ethanol, air-dried, and dissolved in 100 µl of RNAase-free water. RNA was quantified spectrophotometrically. Total RNA (20 µg) of each sample was fractionated by electrophoresis in a formaldehyde-agarose gel and blotted onto nylon membrane (Hybond-XL, Amersham Pharmacia Biotech Piscataway, NJ) as described in Sambrook et al. (1989). Gene-specific probes were obtained from BAC clones T6B13, F28O9, F9E10 (from the Arabidopsis Biological Resource Center, ABRC) containing *Arabidopsis PR-1*, *PR-2*, and *PR-5,* respectively. BAC clones were grown overnight in selective media (kanamycin 100 mg/ml) and DNA was isolated using a standard alkaline procedure (Sambrook *et al.,* 1989). PCR amplification using specific primers were performed. *PDF1.2* probe was produced by direct amplification from *Arabidopsis* genomic DNA using specific primers (Table 2.1) and the identification of the PCR product was confirmed by sequencing (Virginia Bioinformatics Institute, DNA Sequencing Facility, Blacksburg, VA). Primer design was performed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

**Table 2.1** Sequence specific primers for *PR-1* (M90508.1), *PR-2* (M90509), *PR-5* (M90510), *PDF1.2* (AY063779), and actin (AY087348) genes.

| Primer Name | Sequence |
|---|---|
| *PR-1* Forward | 5'-TTCTTCCCTCGAAAGCTCAA-3' |
| *PR-1* Reverse | 5'-CGTTCACATAATTCCCACGA-3' |
| *PR-2* Forward | 5'-TACGGGATGCTAGGCGATAC-3' |
| *PR-2* Reverse | 5'-GTCTCCGACACCACGATTTC-3' |
| *PR-5* Forward | 5'-CACATTCTCTTCCTCGTGTTCA-3' |
| *PR-5* Reverse | 5'-AGCTCCGGTACAAGTGAAGG-3' |
| *PDF1.2* Forward | 5'-TCATGGCTAAGTTTGCTTCC-3' |
| *PDF1.2* Reverse | 5'-GGTAGATTTAACATGGGACG-3' |
| Actin Forward | 5'-ATGAAGATTAAGGTCGTGGCAC-3' |
| Actin Reverse | 5'-GTTTTTATCCGAGTTTGAAGAGGC-3' |

Probes were labeled with $^{32}$P-dCTP (50 µCi) (PerkinElmer Life Sciences, Boston, MD) using the Prime-It® RmT Random Primer kit (Stratagene La Jolla, CA) and 50-100 ng of each specific PCR products. After labeling, probes were separated from unincorporated radioactive nucleotides using a Sephadex G-25 TE spin column according to the manufacturer's instructions (Millipore, Bedford, Massachusetts).

The membrane was pre-hybridized for 1 h at 65 ºC in 5X SSC, 1% SDS, 5X Denhardt's solution (Denhardt, 1966), and 2 mg of salmon sperm DNA (Sigma, St Louis, MO). Probes were added to fresh buffer and hybridized at 65ºC for 16 h. Washes were performed using a solution of 1X SSC and 0.1 % SDS at room temperature for 30 min, with a final wash in 0.1 X SSC and 0.1% SDS solution for 30 min at 65ºC. Radioactivity was detected using Kodak X-OMAT AR-5 Scientific Imaging Film (Eastman Kodak Co., Rochester, NY) exposed at -80ºC.

### II.3.4. Reverse Transcriptase-PCR (RT-PCR)

Total RNA (4 µg) was treated with DNAase (Ambion Inc., Austin TX) following manufacturer's instructions. cDNA was synthesized using SuperScript II Reverse transcriptase (Invitrogen Corp. Carlsbad, CA) following manufacture's guidelines. Resulting cDNA (10 ng) was used as template for PCR with gene-specific primers (Table 1). PCR conditions were standardized for each pair of primers. Conditions for *PR-1* and *PR-2* probes were: 4 min at 94 ºC, then 29 cycles of 1 min at 94 ºC, 50 sec at 50 ºC and 1 min at 72ºC. Conditions for *PR-5 PDF1.2* and actin were the same except that the annealing temperature for *PR-5* and *PDF1.2* was 53 ºC and for Actin was 55 ºC. All PCR reactions were performed using a RoboCycler Gradient 96 (Stratagene, La Jolla, CA). PCR product identities were confirmed by sequencing (Virginia Bioinformatics Institute DNA Sequencing Facility, Blacksburg, VA). RT-PCR was conducted three times for each sample, and using tissue from two biological replicates for a total of six reactions per treatment.

### II.3.5. Microarray experiment

RNA samples (5 µg) from control and *O. aegyptiaca*-parasitized root tissue used in previous experiments were also used for microarray analysis. The Affymetrix Genechip platform ATH-1 (22,810 genes) was used and all steps were conducted by the Core Laboratory at the Virginia Bioinformatics Institute (Virginia Tech Blacksburg, VA). Prior to microarray hybridizations, RNA samples were analyzed to verify quality and purity using Bioanalyzer 2100 (Agilent Technologies Inc.,). Preparation and labeling of

the samples were performed according to manufacturer's instructions (Affymetrix Inc.,). Hybridizations were performed using tissue from two biological replicates with a total number of 4 chips (2 controls and two parasitized tissues).

#### II.3.5.1. Data analysis

Data analysis was performed using GeneSpring v 6.2 software (Silicon Genetics, Redwood, CA) in collaboration with Dr. Hanna Craig (Virginia Bioinformatics Institute). The results from four hybridizations were considered for the analysis. Probes with absence readings across the four different hybridizations were discarded. Per gene normalizations were also performed and utilized the median expression values for all genes in all samples as a baseline prior to statistical comparison. One-way ANOVA comparisons were performed using treatment and biological replicate as variables to determine significantly different genes (significantly up-regulated or down-regulated).

Functional categorization of genes was performed for the up-regulated and down-regulated genes based on the functional categories in the *Expresso*® database (Dr. Grene, Virginia Tech, Blacksburg, VA). Additional categorization for genes not included in the *Expresso* database was done using annotations found in the Plant Science Database (http://genomics.msu.edu/cgi-bin/plant_specific/family_search.cgi) from Michigan State University, and the TAIR database (http://www.arabidopsis.org).

## II.4. Results

### II.4.1. RNA hybridization and RT-PCR analyses

Our research is aimed at understanding the parasite-host interaction at the molecular level in order to improve host resistance. Analysis of well-characterized gene markers for the two major plant signaling defense responses (*PR-1*, *PR-2*, *and PR-5* for SA and *PDF1.2* for JA) was initially performed on parasitized *Arabidopsis* root tissue. RNA hybridization analysis showed no expression of *PR-1*, *PR-5*, and *PDF1.2* in parasitized root tissue (Fig. 2.2). Similar results were observed in the positive control SA- and JA-treated and wounded root tissue (Fig. 2.2). A low level of *PR*-2 was detected in the *O. aegyptiaca*-parasitized roots as well as in the SA- and JA-treated tissues for the same gene. Shoots of the same plants showed a very different pattern, responding to positive controls and *O. aegyptiaca*. Shoots of parasitized plants showed expression of all *PR* genes analyzed, but in most cases (*PR-1*, *PR-5* and *PDF1.2*), the expression was weak. In the case of *PR-2*, the level of gene expression in parasitized plants was higher in comparison to its control (Fig. 2.2). Subsequent analysis of the same RNA samples using the more sensitive RT-PCR approach demonstrated that these genes were in fact induced in the host roots by *O. aegyptiaca* parasitism (Fig. 2.2). The absence of PCR product of *PR-1* and *PR-2* in non-treated tissues suggested induction of these genes by parasitism, whereas *PR-5* and *PDF1.2* were present in controls but may have been slightly induced by *O. aegyptiaca*. Amplification of actin across the treatments served as a standard to verify RNA quality and an effective PCR amplification.

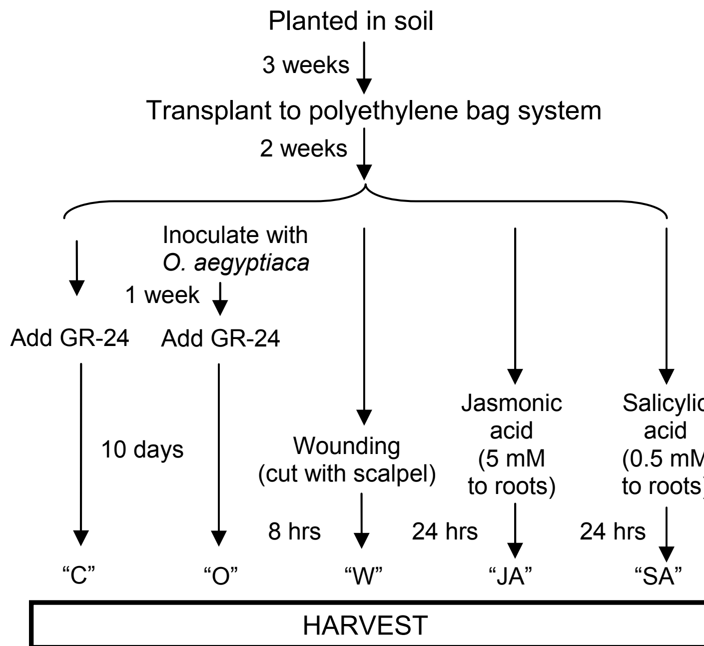



**Fig. 2.1** Schematic diagram showing the treatments made to *Arabidopsis* plants to generate the tissues used in gene expression analyses. Note that chemical treatments (SA and JA applications) were applied only to roots, while wounding was also done to shoots.

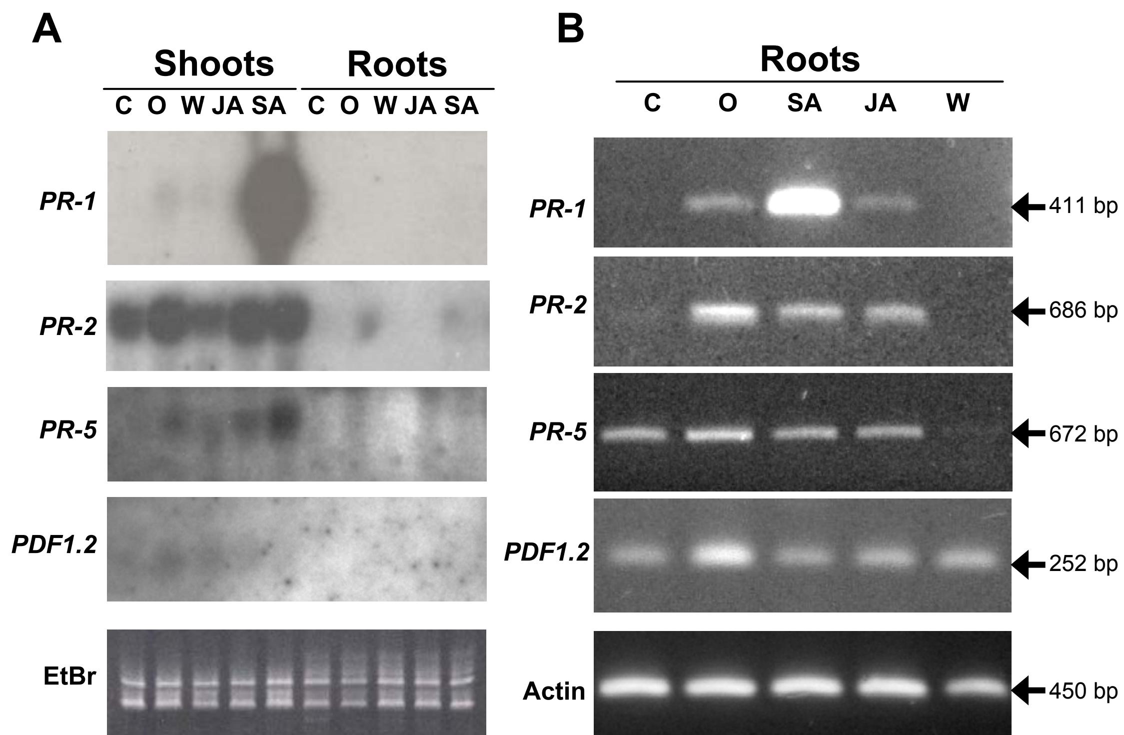



**Fig. 2.2** Expression of *PR-1*, *PR-2*, *PR-5*, and *PDF1.2* genes in *Arabidopsis* in response to *O. aegyptiaca* parasitism and control treatments. **(A)** RNA hybridization analyses of *Arabidopsis* shoots and roots. **(B)** RT-PCR analyses using RNA from roots. RNA was generated from **n**on-treated control **(C)**, *O. aegyptiaca*-parasitized **(O)**, wounded **(W),** jasmonic acid treated **(JA),** and salicylic acid-treated **(SA)** plants. Ethidium bromide (ETBr)-stained RNA gel and PCR of *Arabidopsis* are included to indicate equal loading and amplification.

## II.4.2. Microarray data

### II.4.2.1. Reproducibility of hybridization

Scatter plot analyses in the four different hybridizations using GeneSpring version 6.2 software showed that hybridizations were reproducible.

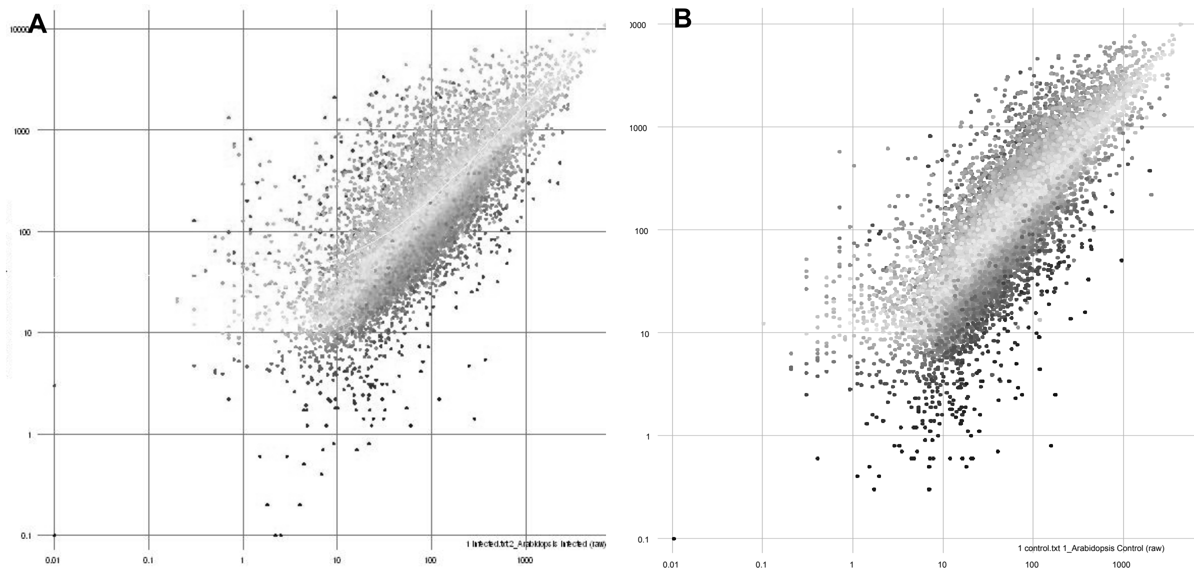



**Fig. 2.3** Scatter plots of the expression levels of ~22,000 *Arabidopsis* genes, showing reproducibility between experiments. A high correlation observed in comparisons of experiments 1 and 2 for parasitized (A) and non-parasitized (B) tissues indicates that the results were reproducible.

### II.4.2.2. *O. aegyptiaca*-induced changes in host gene expression

We examined the effect of *O. aegyptiaca* parasitism on the abundance of transcripts corresponding to approximately 22,000 genes in the *Arabidopsis* genome.

Total RNA samples from non-treated control and parasitized root tissues were the same as those used for RNA hybridization and RT-PCR analyses. For microarray analysis, only changes in mRNA abundance that were significantly different in both experiments were accepted. Analyses of these data revealed that 757 *Arabidopsis* genes showed significant differential expression in response to *O. aegyptiaca* parasitism. From this group of genes, 427 genes were significantly up-regulated and 330 were down-regulated. (Fig. 2.4) Differentially-regulated genes were assigned to functional categories using the databases described in the methods section.

From the up-regulated group of genes, 238 genes (55%) had functions classified into defined categories (i.e., defense, signal transduction, stress, cell wall related, etc.) and 189 genes (44%) resulted with unknown function and were defined in the database as putative protein, hypothetical protein or expressed protein. From the down-regulated group, 187 genes (56%) were classified into known categories, and 147 genes (43%) were classified as unknown function.

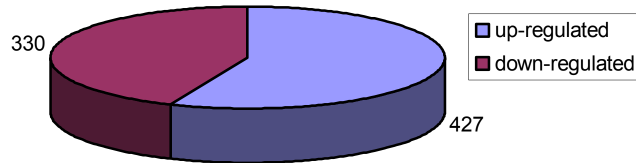



**Fig. 2.4** Breakdown of differentially-regulated *Arabidopsis* genes in response to *O. aegyptiaca* parasitism.

Those genes to which a function could be assigned are shown in Fig 2.4. Genes from many categories were up- and down-regulated in response to parasitism, but some of the major trends included genes implicated in protein biosynthesis being up-regulated during *O. aegyptiaca* parasitism (Fig 2.4). Several defense related genes were up-regulated during parasitism (Table 2.2). However defense related genes such as those belonging to the TIR-NB-LRR *R*-genes were down-regulated (Table 2.3). Genes involved in cell wall and lignin biosynthesis were found to be up-regulated during parasitism. Genes involved in respiratory electron transport were also up-regulated in response to *O. aegyptiaca* parasitism. A large number of genes involved in carbon metabolism were observed to be up-regulated as well as genes involved in chloroplast function. Specific transcription factors were induced as well as repressed during *O. aegyptiaca* parasitism. A number of genes involved in producing trafficking proteins was also up-regulated when *O. aegyptiaca* parasitism occurs. A summary of the categories that were differential regulated is presented in Fig 2.5.

**Table 2.2** Selected examples of *Arabidopsis* genes that were significantly induced by *O. aegyptiaca* parasitism.

| PUTATIVE FUNCTION | TAIR | p-value | fold change |
|---|---|---|---|
| **OXIDATIVE BURST/STRESS/DEFENSE/CELL DAMAGE and DEATH** | | | |
| Resistance response protein-related | At1g07730 | 0.0365 | 1.78 |
| Isoflavone reductase homolog P3 | At1g75280 | 0.0197 | 1.87 |
| putative vacuolar ATP synthase | At1g75630 | 0.0445 | 1.12 |
| vacuolar H+-transporting ATPase 16K chain P2 | At4g38920 | 0.0456 | 1.11 |
| mitochondrial ATP synthase beta chain 2 | At5g08690 | 0.0121 | 1.25 |
| thaumatin-like protein | At5g40020 | 0.0334 | 2.01 |
| phosphoribulokinase precursor | At1g32060 | 0.0385 | 3.00 |
| phosphoribulokinase/uridine kinase family protein | At2g01460 | 0.0064 | 3.20 |
| putative peroxidase | At2g39040 | 0.0011 | 1.77 |
| putative glutathione transferase | At5g41240 | 0.0355 | 1.30 |
| putative iron superoxide dismutase | At5g51100 | 0.0485 | 1.99 |
| UMUC-like DNA repair family protein | At1g49980 | 0.0026 | 1.04 |
| putative SNF2/RAD54 family DNA repair | At2g18760 | 0.0222 | 1.16 |
| similar to Dehydration-responsive protein RD22 | At1g49320 | 0.0257 | 1.46 |
| similar to ERD3 dehydration responsive | At5g06050 | 0.0171 | 1.18 |
| major latex protein (MLP)-related | At1g14950 | 0.0293 | 1.84 |
| **CELL WALL BIOSYNTHESIS/LIGNIN/FLAVONOIDS** | | | |
| shikimate kinase-like protein | At4g39540 | 0.0498 | 1.63 |
| polygalacturonase, putative | At1g05650 | 0.0167 | 3.27 |
| glycosyl hydrolase family 9 (endo-1,4-beta-glucanase) | At1g64390 | 0.0331 | 2.09 |
| UDP-galactose 4-epimerase, putative | At1g64440 | 0.0077 | 1.20 |
| putative polygalacturonase | At1g80170 | 0.0493 | 5.73 |
| glycosyl hydrolase | At5g45300 | 0.0371 | 1.14 |
| Sialyltransferase family | At1g08660 | 0.0178 | 1.36 |
| pectin methylesterase | At1g41830 | 0.0321 | 1.57 |
| glycosyl transferase family 2 protein, | At4g31590 | 0.0062 | 1.97 |
| UDP-glucuronyltransferase-like protein | At4g36890 | 0.0113 | 1.05 |
| glycosyl transferase family 2 protein, similar to beta-(1-3)-glucosyl transferase | At5g03760 | 0.0343 | 1.75 |
| N-acetylglucosaminyltransferase | At5g15050 | 0.0042 | 2.25 |
| putative cinnamoyl-CoA reductase | At2g33600 | 0.0072 | 1.07 |
| 4-coumarate-CoA ligase-like protein | At3g48990 | 0.0224 | 1.57 |
| putative laccase (diphenol oxidase) | At5g01190 | 0.0467 | 3.56 |
| putative xyloglucan fucosyltransferase | At2g15370 | 0.0074 | 2.54 |
| **SIGNAL REGULATION/TRANSCRIPTION FACTORS** | | | |
| putative Ser Thr protein kinase | At1g16900 | 0.0113 | 1.05 |
| putative response regulator 5 | At1g19050 | 0.0475 | 1.33 |
| auxilin-like protein | At1g21660 | 0.0297 | 1.11 |
| elongation factor Tu family protein | At1g62750 | 0.0127 | 1.24 |
| putative two-component response regulator protein | At2g25180 | 0.0411 | 1.05 |
| putative ADP ribosylation factor 1 GTPase activating protein | At2g37550 | 0.0255 | 1.12 |
| ADP-ribosylation factor-like protein | At3g62290 | 0.0448 | 1.03 |
| F-box protein family | At5g21040 | 0.0093 | 1.14 |

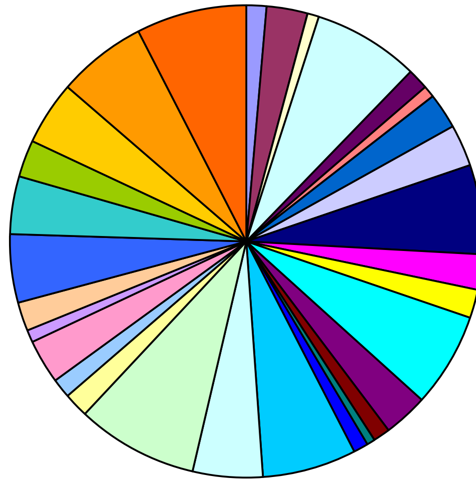
| | | | |
|---|---|---|---|
| Rac-like GTP-binding protein (ARAC2) | At5g45970 | 0.0308 | 2.07 |
| myb family transcription factor | At5g12870 | 0.0289 | 2.26 |
| myb family transcription factor, similar to CCA1 | At5g17300 | 0.0388 | 2.17 |
| DP-E2F-like 2 | At5g14960 | 0.0467 | 1.07 |
| NOL1/NOP2/sun family protein | At5g26180 | 0.0000 | 1.48 |
| transcription regulator NOT2/NOT3/NOT5 family protein | At5g59710 | 0.0482 | 1.51 |
| DREB-like AP2 domain transcription factor | At2g38340 | 0.0062 | 2.11 |
| Homeodomain-like protein | At4g35550 | 0.0096 | 1.89 |
| WRKY family transcription factor | At5g28650 | 0.0196 | 2.00 |
| putative receptor-like protein kinase(ATR1) | At5g60890 | 0.0018 | 1.85 |
| **PLANT GROWTH/DEVELOPMENT** | | | |
| no apical meristem (NAM) family protein | At1g25580 | 0.0308 | 1.05 |
| apical meristem formation protein-related | At1g60380 | 0.0246 | 2.46 |
| BEL1-like homeobox 4 protein (BLH4) | At2g23760 | 0.0116 | 1.04 |
| Homeodomain-like protein | At4g35550 | 0.0096 | 1.89 |
| apetala2 protein | At4g36920 | 0.0059 | 1.15 |
| homeobox-leucine zipper protein, HAT7 | At5g15150 | 0.0113 | 1.36 |
| abscisic acid responsive elements-binding factor | At3g19290 | 0.0034 | 1.11 |
| **MAINTENANCE/ BASIC METABOLISM/ AMINOACID BIOSYNTHESIS** | | | |
| putative trehalose-phosphatase | At1g35910 | 0.0339 | 2.38 |
| putative uridine diphosphate glucose epimerase | At1g63180 | 0.0037 | 2.55 |
| glycosyl hydrolase family 9 (endo-1,4-beta-glucanase) | At1g64390 | 0.0331 | 2.09 |
| putativeUDP-galactose 4-epimerase | At1g64440 | 0.0077 | 1.20 |
| glycosyl hydrolase family 31 (alpha-xylosidase) | At1g68560 | 0.0394 | 1.60 |
| putative polygalacturonase | At1g80170 | 0.0493 | 5.73 |
| digalactosyldiacylglycerol synthase | At3g11670 | 0.0156 | 1.14 |
| glycosyl hydrolase family 35 (beta-galactosidase) | At3g52840 | 0.0156 | 2.28 |
| glycosyl hydrolase family 9 | At4g39010 | 0.0032 | 1.05 |
| glycosyl hydrolase family 14 (beta-amylase) | At5g45300 | 0.0371 | 1.14 |
| putative polygalacturonase | At1g05650 | 0.0167 | 3.27 |
| putative branched-chain amino acid aminotransferase | At3g05190 | 0.0040 | 1.21 |
| lysine decarboxylase - like protein | At5g03270 | 0.0108 | 1.72 |
| ornithine aminotransferase | At5g46180 | 0.0264 | 1.16 |
| protein-methionine-S-oxide reductase | At4g25130 | 0.0100 | 1.10 |
| glucose-6-phosphate dehydrogenase | At5g35790 | 0.0185 | 4.39 |
| putative phosphate/phosphoenolpyruvate translocator protein | At2g25520 | 0.0091 | 1.41 |
| **PROTEIN SYNTHESIS AND PROCESSING** | | | |
| 60S ribosomal protein L18A | At2g34480 | 0.0178 | 1.15 |
| 60S ribosomal protein L7 | At2g44120 | 0.0048 | 1.10 |
| putative pre-mRNA splicing factor RNA helicase | At2g47250 | 0.0405 | 1.03 |
| putative 60S ribosomal protein L13A | At3g07110 | 0.0030 | 1.12 |
| 60S ribosomal protein L37a (RPL37aC) | At3g60245 | 0.0492 | 1.24 |
| putative arginine/serine-rich splicing factor (atSRp34) | At4g02430 | 0.0080 | 1.85 |
| putative ribosomal protein L9, cytosolic | At4g10450 | 0.0154 | 1.33 |
| 40S ribosomal protein S10 (RPS10C) | At5g52650 | 0.0235 | 1.33 |
| YbaK/prolyl-tRNA synthetase family protein | At1g44835 | 0.0128 | 1.46 |
| chloroplast RNA-binding protein cp33 | At3g52380 | 0.0419 | 1.01 |

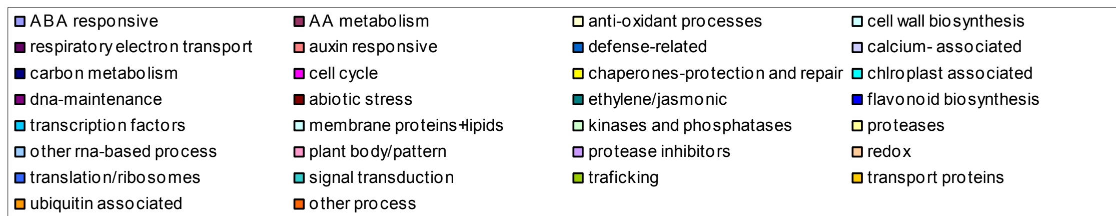
**Table 2.3** Selected examples of *Arabidopsis* genes that were significantly down-regulated by *O. aegyptiaca* parasitism.

| PUTATIVE FUNCTION | TAIR | p-value | fold change |
|---|---|---|---|
| **OXIDATIVE BURST/STRESS/DEFENSE/CELL DAMAGE and DEATH** | | | |
| leucine rich repeat protein family | At1g17250 | 0.0266 | 1.22 |
| band 7 family protein, strong similarity to hypersensitive-induced response protein (Zea mays) | At1g69840 | 0.0196 | 1.61 |
| putative disease resistance protein (TIR-NBS class) | At1g72870 | 0.044 | 1.21 |
| quinone reductase-like protein | At3g56460 | 0.039 | 1.01 |
| putative disease resistance protein (TIR-NBS-LRR class) | At4g16890 | 0.016 | 1.13 |
| disease resistance protein RPS4 (TIR-NBS-LRR class) | At5g45250 | 0.0295 | 1.02 |
| putativedisease resistance protein (TIR-NBS-LRR class) | At5g46470 | 0.0159 | 1.53 |
| putative disease resistance protein (TIR-NBS-LRR class) | At5g58120 | 0.0194 | 1.79 |
| glutathione S-conjugate ABC transporter (AtMRP1) | At1g30400 | 0.00565 | 1.04 |
| putative peroxidase | At3g03670 | 0.0431 | 1.04 |
| **CELL WALL BIOSYNTHESIS/LIGNIN/FLAVONOIDS** | | | |
| UDP-glucose glucosyltransferase, putative | At1g07240 | 0.0469 | 1.12 |
| pectinesterase (pectin methylesterase), putative | At1g76160 | 0.0233 | 1.05 |
| pectinesterase family protein, contains Pfam | At4g02330 | 0.0107 | 1.22 |
| glycine-rich protein | At1g27090 | 0.0271 | 1.12 |
| glycine/proline-rich protein | At5g07570 | 0.0455 | 2.28 |
| **SIGNAL REGULATION/TRANSCRIPTION FACTORS** | | | |
| receptor-like protein kinase, putative | At1g11330 | 0.0126 | 1.14 |
| AIG1-like protein | At1g33910 | 0.0328 | 2.41 |
| ABC transporter family protein | At1g53390 | 0.0241 | 1.21 |
| WD-40 repeat family protein,G-beta repeat | At1g64610 | 0.0062 | 1.20 |
| putative receptor-like protein kinase | At2g41890 | 0.00585 | 1.16 |
| receptor like protein kinase | At3g45440 | 0.014 | 1.23 |
| KOW domain-containing protein / D111/G-patch domain-containing protein | At4g25020 | 0.0186 | 1.23 |
| S-receptor kinase-like protein | At4g32300 | 0.0284 | 1.50 |
| receptor like protein kinase | At5g01540 | 0.0108 | 1.19 |
| lectin-like protein kinase | At5g06740 | 0.0465 | 1.88 |
| transducin family protein / WD-40 repeat family protein | At5g14530 | 0.0287 | 1.06 |
| G-protein beta family | At5g15550 | 0.0359 | 1.09 |
| ABC transporter family protein | At5g60740 | 0.000422 | 1.29 |
| pseudo-response regulator 1 | At5g61380 | 0.00606 | 1.39 |
| GTP binding protein Arac10 | At5g62880 | 0.0251 | 1.07 |
| G protein-coupled receptor-like protein | At5g65280 | 0.0371 | 1.29 |
| myb family transcription factor | At1g14350 | 0.00214 | 1.08 |
| transcription factor-related, similar to enhancer of polycomb | At1g16690 | 0.00272 | 1.23 |
| bHLH protein | At1g26260 | 0.0193 | 1.03 |
| **SIGNAL REGULATION/TRANSCRIPTION FACTORS** | | | |
| AP2 domain-containing transcription factor, putative, Similar to DREB1A | At1g77640 | 0.0471 | 1.89 |
| transcription factor jumonji (jmjC) domain-containing protein, | At1g78280 | 0.0253 | 1.95 |
| putative transcription factor IIIB 70 KD subunit (TFIIIB) | At2g45100 | 0.0439 | 1.11 |
| GATA zinc finger protein | At3g54810 | 0.00407 | 2.01 |
| transcription factor TINY, putative | At4g32800 | 0.00273 | 1.72 |
| SCARECROW-like protein | At4g36710 | 0.00403 | 2.36 |
| mitochondrial transcription termination factor-related | At5g06810 | 0.0348 | 1.16 |
| AP2 domain transcription factor-like | At5g67000 | 0.0419 | 1.20 |
| WRKY family transcription factor | At4g01250 | 0.0279 | 1.32 |
| WRKY family transcription factor | At4g23810 | 0.0384 | 1.33 |

| | | | |
|---|---|---|---|
| **PLANT GROWTH/DEVELOPMENT** | | | |
| homeobox RRM-containing protein | At1g27050 | 0.0123 | 1.33 |
| floral homeotic protein (HUA1), identical to floral homeotic protein HUA1 | At3g12680 | 0.0223 | 1.08 |
| NAM/NAP like protein | At4g10350 | 0.00534 | 1.30 |
| NAC2-like protein | At5g24590 | 0.0346 | 1.80 |
| axi 1 (auxin-independent growth promoter)-like protein | At5g35570 | 0.0168 | 1.00 |
| IRE (root hair elongation) | At5g62310 | 0.0248 | 1.24 |
| MYB family transcription factor | At2g23290 | 0.0349 | 1.04 |
| DNA-binding bromodomain-containing protein | At2g42150 | 0.0237 | 1.10 |
| MYB transcription factor like protein | At4g17785 | 0.0364 | 1.62 |
| ABA-responsive protein–like | At5g13200 | 0.0136 | 2.14 |
| **MAINTENANCE/ BASIC METABOLISM/ AMINOACID BIOSYNTHESIS** | | | |
| pyruvate decarboxylase | At5g54960 | 0.00381 | 1.44 |
| glycosyl hydrolase family 32 | At1g12240 | 0.0255 | 1.11 |
| glycosyl hydrolase family 17 | At3g46570 | 0.0238 | 1.70 |
| pyruvate decarboxylase | At5g54960 | 0.00381 | 1.44 |
| **PROTEIN SYNTHESIS AND PROCESSING** | | | |
| transformer serine/arginine-rich ribonucleoprotein | At1g07350 | 0.00869 | 1.28 |
| translation initiation factor IF-2, chloroplast precursor | At1g17220 | 0.00653 | 1.05 |
| Eukaryotic translation initiation factor 5, putative | At1g36730 | 0.0359 | 1.27 |

**A**






**B**

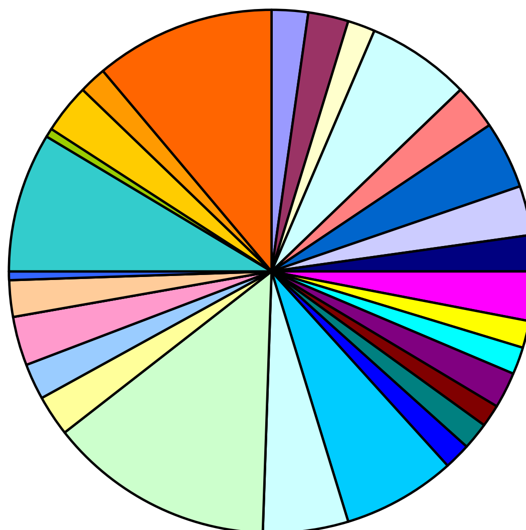


**Fig 2.5** Functional categorization of *Arabidopsis* genes differentially up-regulated (**A**) and down-regulated (**B**) in *response to O. aegyptiaca* parasitism.

## II.5. Discussion

This research was aimed at studying changes in host defense gene expression, particularly PR genes, following parasitism by *O. aegyptiaca*. The analysis focused on the study of classical gene markers for the major plant defense pathway mediated by SA. In contrast to previous results (Griffitts *et al*., 2004; Vieira Dos Santos *et al*., 2002), we found evidence that genes belonging to this pathway were slightly induced by *O. aegyptiaca* parasitism (Fig. 2.2).

As part of this analysis we also included shoot tissues from parasitized *Arabidopsis* plants, and results demonstrated that *O. aegyptiaca* parasitism provokes a systemic defense response in the host. Plants under attack by certain pathogens can trigger SAR (Ryals *et al*., 1996), and RNA hybridization analysis showed that the SA-dependent genes (*PR*-1, *PR*-2 and *PR*-5) were slightly up-regulated in shoots of parasitized plants. In addition to this activation of SA-dependent genes, *O aegyptiaca* also induces the JA-signaling-associated gene *PDF1.2* (Fig 2.2), which is consistent with previous reports of *Orobanche* stimulating JA-associated defense genes (Vieira Dos Santos *et al*., 2002).

The fact that *PR* gene expression was not detected in root tissue using Northern blots agrees with previous findings (Uknes *et al*., 1992, Penninckx *et al*., 1996). Although the root tissue has a low concentration of *PR*-gene transcripts, PCR techniques are able to detect the transcripts. When SA is applied to the roots, the translocation of the signal to the leaves stimulates a large production of *PR* gene transcripts (Fig 2.2).

Our results agree with observations that other root pathogens are able to trigger a systemic response in the host. Thus, nonpathogenic, rizhosphere-colonizing bacteria such as *Pseudomonas fluorescens* stain WCS417r trigger a form of resistance, called rizhobacteria-mediated induced systemic resistance (ISR) (Van loon *et al*., 1998). ISR functions independently of SA and *PR* gene activation but requires JA and ethylene signaling (Pieterse *et al*., 1998). Van Wess et al. (2000) demonstrated that SAR and ISR are compatible, resulting in an additive effect on the level of induced protection against *P. syringae* pv. tomato (*Pst*). A similar effect may be occurring during *Orobanche* parasitism, although it may be masked by the detrimental effect of parasitism.

Genome-wide gene expression analyses using microarrays offers a powerful approach to study plant-parasite interactions. We conducted a microarray analysis of root tissue from *Arabidopsis* plants subject to *O. aegyptiaca* parasitism and observed that several genes previously characterized as being involved in response to different pathogens such as nematodes and rizhobacteria were up-regulated as identified by an analysis of variance (Table 2.2). Among these genes we found a thaumatin like protein (At5g40020) similar to *PR*-5 (Van Loon and Van Strien, 1999). Genes involved in the oxidative burst were also induced, such as putative peroxidase (At2g39040), and a glutathione transferase gene (At5g41240). This indicates that oxidative burst may be occurring during parasite invasion. Genes involved in lignin and cell wall biosynthesis and modification were also up-regulated, perhaps as a consequence of the host trying to reinforce its cell walls in response to *Orobanche* penetration and haustorial expansion. Vieira dos Santos et al. (2003) also found genes related to cell wall induced during parasitism of *Arabidopsis* by *O. ramosa*. A gene encoding a major-latex protein

(At1g14950) and genes involved in secondary metabolism were also induced, indicating an increase in secondary metabolism that may be part of a mechanism for deterring the parasite. Genes involved in dehydration and drought stress were also found to be induced during parasitism (Table 2.2), and this would be consistent with *Orobanche* parasitism and the withdrawal of water and nutrients from the host. It is important to note that *Arabidopsis* tissue used in these experiments consisted of segments of whole root systems. Despite abundant inoculation and parasitism, most of the tissue harvested was not parasitized, so mRNAs from cells around haustorium were likely diluted in a large proportion of mRNA from more distant cells. Refinements in microarray technology to enable use of smaller quantities of mRNA will provide better detection of altered genes.

*O. aegyptiaca* parasitism induces a large number of genes encoding transcription factors (Table 2.2). Among them are members of the AP2, MYB, and WRKY families. AP2 gene families have been reported to be induced by SA, JA, ethylene and pathogen attack (Maleck *et al*., 2000, Schenk *et al*., 2000). Members of the same transcription family were also up-regulated in response to wounding in *Arabidopsis* (Cheong *et al*., 2002), which is in agreement with previous studies in which wound-related genes (chalcone synthase, HMGR, etc.) were induced by *Orobanche* parasitism (Griffitts *et al*., 2004, Westwood *et al*., 1998). Other genes involved in signaling were involved in calcium-signaling. Among them, a calmodulin-binding protein (At4g16150) related to ethylene signaling was also up-regulated during parasitism. Vieira Dos Santos et al. (2003) also observed the overexpression of a calmodulin gene during *O. ramosa* parasitism. Other similarities with Viera Dos Santos et al. 2003) were found in the

category of cell wall related genes, stress related (glutathione) and secondary metabolism (latex).

*Orobanche* may also take an active role and specifically down-regulate plant gene expression by an unknown mechanism (Table 2.3). These genes include serine/threonine kinases, calmodulin-related protein, calcium-dependent protein kinase and several transcription factors that could be elements of signal transduction cascades that regulate plant responses. The same phenomenon was observed in a microarray analyses during cyst-nematodes infection in *Arabidopsis* (Puthoff *et al*., 2003). An interesting question is whether the down-regulation is part of a general metabolic shift from cell maintenance to defense (Scheideler *et al*., 2002), or whether *Orobanche* specifically represses host processes that are disadvantageous to parasitism.

*Orobanche* parasitism is complex. Defense response is only one aspect and it will be interesting to use more detailed microarray analyses to further refine our understanding of parasitism. Promoter studies in specific groups of genes (up or down regulated) could reveal the existence of regulons that may play a crucial role in the response against parasitism. In addition, detailed studies of transcription factors could uncover signaling events previous to a final response (production of PR proteins) in the host.

In summary, our data are consistent with previous studies conducted on *Orobanche*–host interactions. We observed the induction of genes related to defense and cell wall modification. This suggests that our microarray analyses are sensitive enough to explore the *Arabidopsis*–*O. aegyptiaca* interaction using whole roots, and also that many genes identified here might be directly related to the response to parasitism providing in

this way, new candidate genes for further characterization in this pathosystem. The statistical analyses of our data accounted for the inherent variability of our biological system and led to the identification of significant gene expression changes without relying on fold changes. Due to the nature of our pathosystem, a synchronize infection providing established series of events can not be monitored and therefore the resulting particular overexpressed genes during our analyses is the result of a combination of states in the process of parasitism. This is the first report of a global gene expression analysis in a host-parasitic plant relation providing valuable data for further research.

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# CHAPTER THREE

# Induction of SAR in tobacco as a method to increase host defense against parasitism

## III.1. – Abstract

The parasitic angiosperm *Orobanche aegyptiaca* Pers, (Egyptian broomrape) is a root holoparasite that causes severe losses in yield and quality of several crops. Because of the close association of the parasite with its host, control of *Orobanche* is extremely challenging. Recent reports suggest that sunflower resistance to *O. cumana* can be enhanced by treating seeds with benzothiadiazole (BTH), a chemical inducer of systemic acquired resistance (SAR). The objective of the current research was to study the effect of SAR as a mechanism of protection against *O. aegyptiaca* in susceptible tobacco, a species for which SAR is well characterized. Tobacco plants receiving a soil drench of 200 ml BTH (300 µM) showed a 49% reduction in dry weight of *O. aegyptiaca* tubercles in comparison to non-treated plants. Levels of *PR*-1a protein (a SAR-related gene marker) were only slightly induced in roots following BTH application, although shoots of the same plants showed large increases. These results indicate that BTH treatment can partially protect tobacco roots against *O. aegyptiaca* parasitism despite the lack of a strong correlation with SAR.

### III.2. - Introduction

O*robanche* spp. are obligate root parasitic plants that rely completely on their hosts for water and nutrients. The diversion of resources from the host to parasite can lead to significant host yield losses (Parker and Riches 1993). *Orobanche* spp. attack crops such as potato, tomato, eggplant, sunflower, celery and many other economically important dicotyledonous crops. *Orobanche* infestation areas are found mainly in the Mediterranean, Eastern Europe and Middle East regions, but have spread around the world.

Control of *Orobanche* is challenging because of the close association of the parasite with its host and because the parasite is hidden underground for most of its life cycle. Methods currently used for control may include a variety of approaches. Toxic sterilants such as methyl-bromide can kill *Orobanche* seeds in the soil. Herbicides are effective for a few crops, but generally lack the selectivity to control the parasite without injuring the crop. The availability of crops engineered for resistance to herbicides may facilitate selective control (Joel *et al.,* 1995), but this is dependent on the generation and commercialization of herbicide-resistant crop varieties, and may be countered by the development of herbicide-resistant populations of *Orobanche* (Gressel *et al.,* 1996). Parasite-resistant hosts are an ideal solution, but breeding programs to produce resistant crop varieties have only been successful in a few species, such as sunflower and faba bean, and in these cases the resistance has been repeatedly overcome by new physiological races of *Orobanche* (Alonso, 1998). Without effective control alternatives, many farmers are forced to grow non-host crops or abandon their fields completely

(Parker and Riches, 1993). Therefore, new control strategies are needed to protect crops against *Orobanche* parasitism.

An intriguing approach to the control of *Orobanche* was recently reported by Sauerborn et al. (2002). They induced the systemic acquired resistance (SAR) response in sunflower (*Helianthus annus*) by imbibing seeds in a solution of benzothiadiazole (BTH), a chemical inducer of SAR. The resulting plants showed a decrease in *Orobanche* attachment by 80%. Markers of SAR such as the accumulation of chitinase protein and reactive oxygen species (ROS) in sunflower roots were correlated with the decreased *O. cumana* parasitism.

The induction of SAR is associated with effective defense against a broad spectrum of pathogens (Ryals *et al.,* 1996). SAR in tobacco has been extensively characterized and has been shown to protect plants from pathogens such as tobacco mosaic virus, *Pseudomonas syringae* pv *tabaci*, *Cercospora nicotianae*, *Phytophthora parasitica*, *Peronospora tabacina*, and *Erwinia carotovora* (Vernooij *et al.,* 1995; Friedrich *et al.,* 1996). SAR is associated with the coordinate expression of a set of genes, broadly classified as encoding pathogenesis-related (PR) proteins (Ward *et al.,* 1991). Some of these SAR-related genes play an active role in the resistance process, although the functions of the majority of PR proteins have not been determined *in vivo*. *PR-1a* gene is the most widely used indicator of SAR (Ward *et al*., 1991).

Activation of SAR requires the signaling molecule salicylic acid (SA) (reviewed in Durrant and Dong, 2004). Plants accumulate high levels of SA after pathogen infection (Metraux *et al.,* 1990), and SA treatment induces disease resistance and SAR gene expression (Ward *et al.,* 1991). SA analogs have also been shown to induce SAR, and

include 2,5-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) (Lawton *et al.,* 1996), which activate the same spectrum of SAR genes observed during pathogen-induced SAR (Ward *et al.,* 1991; Friedrich *et al.,* 1996; Lawton *et al.,* 1996). Several investigations have confirmed that BTH is effective in enhancing resistance. For example BTH systemically protected wheat from powdery mildew (Gorlach *et al.,* 1996), tomato from bacterial canker (Baysal *et al.,* 2003) and apple from *Erwinia amylovora* (Brisset *et al.,* 2000). BTH also stimulated soybean seedlings to resist parasitism by nematodes (*Rotylenchulus renifmmis* and *Meloidogne javanica*) (Chinnasri *et al.,* 2003).

Studies of host defense genes induced by *Orobanche* parasitism indicate that the host activates defense-related genes primarily associated with the jasmonic acid (JA)-mediated plant defense pathway. Such genes include the defense-related isogene of 3-hydroxy-3 methylglutaryl Coenzyme A reductase (*HMG*2) (Westwood *et al.,* 1998), *PRB*-1b (Joel and Portnoy, 1998), phenylalanine ammonia lyase (PAL), and chalcone synthase (CHS) in tobacco (Griffitts *et al.* 2004). In addition, *O. ramosa* parasitism induced JA-mediated genes in *Arabidopsis* (Vieira Dos Santos *et al*., 2003). Hurtado et al. (Chapter 2, manuscript in preparation) observed that *PR* gene members of the SA-dependent pathway were only slightly induced during *O. aegyptiaca* parasitism in *Arabidopsis.* Thus, it appears that JA-mediated defenses are induced in response to parasitism, but that the SA-mediated pathway is only slightly induced. Based on these results, SA-defense response are not induced at the site of penetration, however it would be interesting to know if BTH-induced defenses would be sufficient to deter *Orobanche* of tobacco.

The activation of SAR-related defense mechanisms before parasitism may enable plants to resist *Orobanche*. In this study, we evaluated whether treatment with BTH could be effective in protecting tobacco roots against *O. aegyptiaca* parasitism. Our results show that the application of BTH to the soil confers partial resistance to parasitism. However, only a slight increase in accumulation of *PR*-1a was observed in roots of the treated plants. This work suggests that the induction of SAR is partially effective against *O. aegyptiaca* parasitism and may represent an effective component of an integral control strategy.

## III.3. Materials and methods

### III.3.1. Plant growth and treatments

Tobacco (*Nicotiana tabacum* L. cv. Xanthi-NN) was sown initially in a mixture of Promix BG/PGX media (2:1 v/v) (Wetsel, Harrisonburg, VA). Four-week-old plants were transplanted to a combination of the Promix BG/PGX mixture and Profile (PROFILE Products LLC, Buffalo Grove, Illinois) in a 1:1 (v/v) ratio. A slow-release fertilizer (Osmocote, 15-9-12) was added at 3 g per kg of medium. Seeds of *O. aegyptiaca* were mixed thoroughly into the potting medium at the rate of 50 mg per kg of medium. Pots were randomly assigned to three treatment groups with six replicates per group: 1) controls that did not receive BTH; 2) BTH applied as a foliar spray, and 3) BTH applied as a soil drench. Tobacco plants were watered as needed.

BTH was prepared according to manufacturer's guidelines (125 mg/L, equivalent to 300 µM BTH), formulated as 50% active ingredient of the commercial product

*Actigard 50WG* (Syngenta, Basel, Switzerland) in water. BTH was applied every-other week beginning the third day after transplanting tobacco into inoculated soil. BTH was applied to the foliage of tobacco by spraying the leaves until saturated, and to the soil by watering pots with 100 ml of BTH solution. The experiment was repeated to confirm results. Plants were grown under greenhouse conditions (25ºC ± 4ºC with relative humidity from 70% to 80%). Harvest of all tissues was performed 40 days after transplanting to the inoculated soil. Parameters analyzed included host shoot dry weight, host root dry weight, number of parasites, and parasite dry weight. Dry weights were determined after drying for 24 h at 80ºC. Statistical analyses (Student's t-test) were performed using SAS version 8.1 (Statistical Analysis System, Cary, NC).

Tissues for gene expression and protein accumulation analyses were collected from tobacco plants growing under the conditions and treatments described above. Harvesting of tissues for gene expression analysis was done 4 days after BTH applications. Tissues for protein analysis were collected 10 days after BTH application. Foliar tissue was collected from young leaves (the first 5 visible leaves starting from the apical bud of the plant), middle leaves (after the 5 young leaves, continuing until the sixth leaf starting from the bottom), and bottom leaves (the lowest six leaves). Root tissue was collected by gently rinsing off potting media in water.

### II.3.2. Effect of BTH on *O. aegyptiaca* seed germination

In order to determine whether BTH directly effects *O. aegyptiaca* seed germination, an *in vitro* assay of seed germination was conducted. Approximately 200

mg of *O. aegyptiaca* seeds were surface-sterilized (as described in Westwood, 2000) with an additional step of rising in 0.01 M of HCl for 10 min, prior to water rinses. Under aseptic conditions, approximately one hundred seeds per treatment were spread on a sterile glass fiber filter paper disk (Whatman GF/A Whatman International Ltd., Maidstone, England) moistened with 2.5 ml of either sterile water or BTH (300µM). The plates were then sealed with parafilm, and incubated at 20ºC in darkness. After four days, 0.5 ml of distilled water or BTH (300 µM) were added to control and treated plates respectively, to maintain moisture. On day seven, *O. aegyptiaca* seeds were stimulated to germinate by adding 3 ml of 2 ppm GR-24, a synthetic germination stimulant (Mangnus *et al.,* 1992), to each plate. On day ten, additional water or BTH solution was added to each treatment to maintain moisture. On day fourteen, germination was rated by counting seeds with visibly emerged radicles under a stereomicroscope. Treatments were replicated five times and means of the germination rates were compared using Student's t-test.

### III.3.3. Evaluation of BTH in tobacco plants

#### III.3.3.1. RNA isolation and hybridization analysis

Tissue samples were ground to a fine powder in liquid $N_2$ and added to a Corex tube containing 14 ml of Trizol reagent (Invitrogen Corp. Carlsbad, CA) per g of tissue. Total RNA was isolated as described in chapter II. Total RNA (20 µg) of each sample was fractionated by electrophoresis in a formaldehyde-agarose gel and blotted onto nylon

membrane (Hybond-XL, Amersham Pharmacia Biotech Piscataway, NJ) as described in Sambrook et al. (1989).

The *PR*-1a probe (750 bp) was synthesized from a cDNA clone. The pGEM-3Z$_{PR\text{-}1a}$ vector was used as a template for amplification with universal primers (SP6 and T7). PCR conditions were: 94ºC for 4 min, then 30 cycles of 94ºC for 1 min, 50ºC for 1 min, 72ºC for 1 min, and a final step of 72 ºC for 4 min.

The probe was labeled with $^{32}$P-labeled dCTP (50 μCi) (PerkinElmer Life Sciences, Boston, MD) using the Prime-It® RmT Random Primer kit (Stratagene La Jolla, CA) and 50-100 ng of the *PR-1a* PCR product. After labeling, the probe was separated from unincorporated radioactive nucleotides using a Sephadex G-25 TE spin column according to the manufacturer's instructions (Millipore, Bedford, Massachusetts). The membrane was pre-hybridized for 1 h at 65 ºC in 5X SSC, 1% SDS, 5X Denhardt's solution (Denhardt, 1966), and 2 mg of salmon sperm DNA (Sigma, St Louis, MO). The *PR*-1a probe was added to fresh buffer and hybridized 65ºC for 16 h. Washes were performed using a solution of 1X SSC and 0.1 % SDS at room temperature for 30 min, with a final wash in 0.1 X SSC and 0.1% SDS solution for 30 min at 65ºC. Radioactivity was detected using Kodak X-OMAT AR-5 Scientific Imaging Film (Eastman Kodak Co., Rochester, NY) exposed at -80ºC.

#### III.3.3.2. Protein isolation and western blots

Tissue samples (0.3 g) from plants described in Section III.3.1 were ground to a fine powder in liquid $N_2$ and added to a centrifuge tube containing 1 ml of protein

extraction buffer (100 mM sodium acetate, 100 mM sodium formate, pH 4.2). Plant material was mixed by vortexing for 1 min and centrifuged at 8,000g at 4°C. The supernatant was filtered through two layers of Miracloth (Calbiochem-EMD Biosciences, San Diego, CA), and transferred to a fresh tube. Total protein content was quantified using the Bradford assay (Bradford 1976) with BSA as a standard.

. Protein samples were analyzed by SDS-PAGE gel. Total protein (5 μg per sample) was electrophoresed under denaturing conditions in 10% Bis-Tris gels (Invitrogen Corp. Carlsbad, CA) using MES running buffer, and transferred to 0.2 μm Trans-Blot® nitrocellulose membrane (Bio-Rad, Hercules, CA) using an X-Cell IITM blot module and NuPAGE transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, 0.05 mM chlorobutanol). Membranes were incubated for 1 h at room temperature with blocking solution (PBS containing 0.1% Tween-20, 3% BSA) (BSA Fraction V, Roche Diagnostic Corp., Indianapolis, IN), followed by incubation with primary antibody (rabbit anti-*PR-1a*) at 1:3,000 in blocking solution for 1 h. Membranes were washed three times 15 min in PBS, 0.1% Tween-20 and incubated with secondary antibody (goat anti-rabbit-IgG whole molecule) AP-conjugated (Sigma, St. Louis, MO) at 1:3,000 for 45 min, and washed 3 times as above. Chemiluminescence detection was performed using Immun-Star™ AP substrate (Bio-Rad) and Nitro-Block Enhancer II™ (Tropix, Bedford, MA) following manufacturer's procedures.

## III.4. - Results

### III.4.1. *O. aegyptiaca* seed germination was not affected by BTH

In order to determine whether BTH effects on parasitism were mediated through host responses or via direct impacts on the parasite, we treated *O. aegyptiaca* seeds directly with BTH. The *in vitro* germination of *O. aegyptiaca* seeds in the presence of BTH demonstrated that this compound did not significantly affect germination. The germination rate of the non-treated control seeds was 88.7% (SE=1.5) while that of the BTH-treated seeds was 88.3% (SE=2.4). Radicles of BTH-treated and control seedlings were also similar in appearance and length of the radicle. Therefore, BTH does not have a substantial phytotoxic effect on *O. aegyptiaca* seeds or seedlings.

### III.4.2. Application of BTH reduced *O. aegyptiaca* parasitism in tobacco

In tobacco, BTH has been proven to increase resistance pathogens such as powdery mildew (Achuo *et al.,* 2004). Our objective was to determine whether activation of SAR in tobacco using BTH was capable of protecting the plant against *O. aegyptiaca* parasitism. BTH treatments had a significant effect ($p<0.05$) on the dry weights of associated parasites in comparison to those growing on non-treated plants (Table 3.1). The reduction was consistent in two separate experiments, despite a difference among the experiments in the magnitude of parasite weights that was likely due to different growth rates caused by environmental effects. BTH applied to the leaves reduced parasite dry weights by 18.8% and 43.1% on the first and second experiment respectively as

compared to their respective controls (Table 3.1). BTH applied as a soil drench was generally more effective, reducing dry weights by 49.7% and 48.9% in the two experiments.

**Table 3.1** Effect of BTH application on dry weights of *O. aegyptiaca* growing on tobacco.

| **Experiment** | **BTH application method** | ***O. aegyptiaca* dry weight (g/host plant)** | **% Reduction** |
|---|---|---|---|
| I | Control | 37.2 (2.64) | |
| | BTH foliar | 30.2 (2.07) * | 18.8 |
| | BTH soil | 18.7 (2.24) ** | 49.7 |
| II | Control | 4.5 (1.77) | |
| | BTH foliar | 2.5 (1.48) * | 43.0 |
| | BTH soil | 2.3 (0.94) * | 48.9 |

SE of the means are indicated in parentheses. * and ** indicate significant differences from the control group as determined by Student's t-test with $\alpha = 0.05$, and $\alpha = 0.01$ respectively.

The number of parasites on BTH treated plants was also reduced as compared to non-treated controls, but the difference was only significant in the first experiment (Table 3.2). The total number of parasites in the first trial was reduced by 25% and 34% for BTH foliar and soil applications, respectively, when compared to untreated plants.

**Table 3.2** Effect of BTH application on of *O. aegyptiaca* number of parasites growing on tobacco.

| Experiment | BTH application method | *O. aegyptiaca* number of parasites (No./host plant) | % Reduction |
|---|---|---|---|
| I | Control | 29.8 (5.96) | |
| | BTH foliar | 22.3 (0.85) * | 25.4 |
| | BTH soil | 19.7 (4.09) * | 34.0 |
| II | Control | 40.8 (12.02) | |
| | BTH foliar | 37.3 (10.49) | 1.8 |
| | BTH soil | 38 (12.0) | 6.9 |

SE of the means are indicated in parentheses. * indicates significant differences from the control group as determined by Student's t-test at α= 0.05.

### III.4.3. BTH does not affect tobacco growth

BTH applications had no effect on growth of the tobacco host when compared to untreated plants. Neither shoot nor root dry weights were significantly affected (Figs. 3.1 and 3.2).

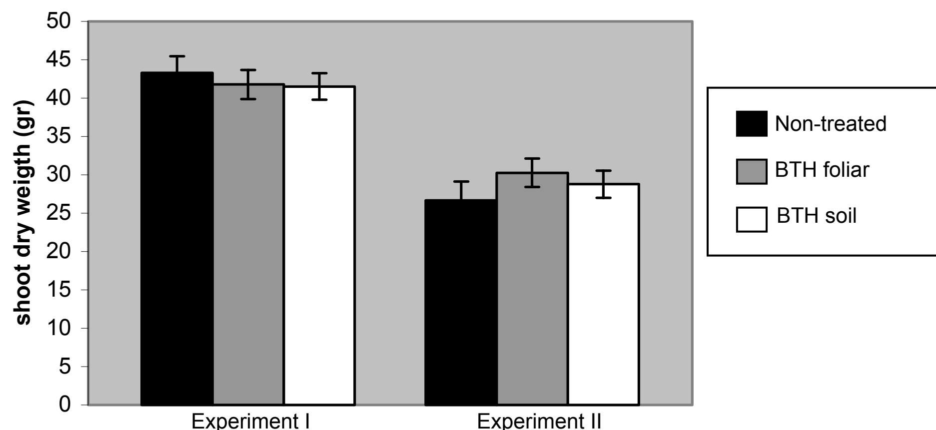


**Fig. 3.1** Effect of BTH on shoot dry weights of tobacco plants parasitized by *O. aegyptiaca*. Bars represent means of 6 plants with vertical lines indicating SE.

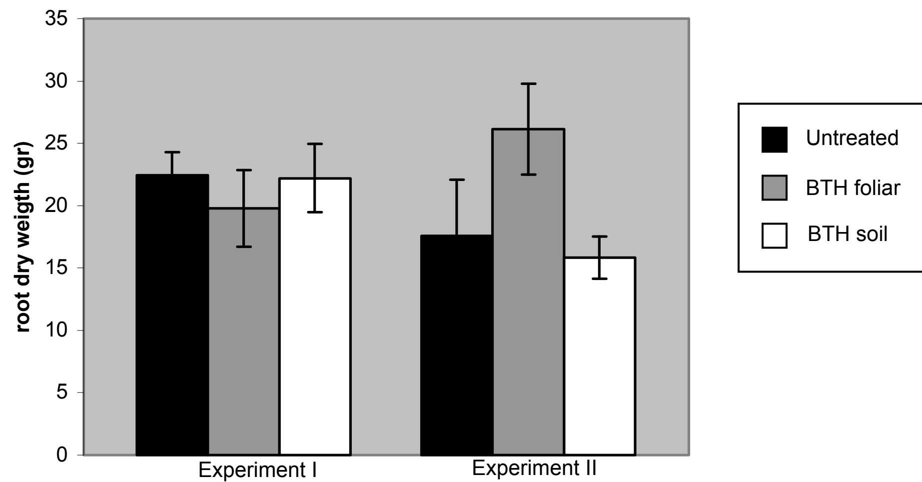


**Fig. 3.2** Effect of BTH on root dry weight of tobacco plants parasitized by *O. aegyptiaca*. Bars represent means of 6 plants with vertical lines indicating SE.

### III.4.4. BTH slightly alters the accumulation of *PR*-1a in tobacco roots

In order to confirm that BTH induced SAR in tobacco, a SAR-related marker gene (*PR-1a*) was evaluated in tobacco root and leaf tissues. RNA hybridization analysis of BTH-treated plants showed no expression of *PR-1a* in tobacco roots. In contrast, *PR-1a* expression was strongly induced in the leaves of plants receiving either foliar or soil BTH treatments, with a more intense signal observed following BTH applied to the soil (Fig. 3.3).

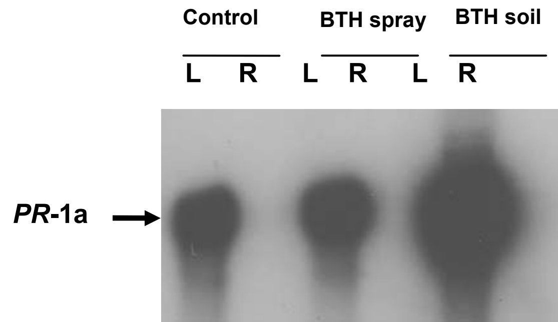


**Fig. 3.3** RNA blot hybridization showing expression of *PR-1a* in middle leaves (L) and root tissues (R) 4 days after treatment with BTH.

PR-1a protein accumulation was also evaluated in several locations on the BTH-treated plants. Protein immunoblot analysis showed that PR-1a accumulated at low levels in roots when compared shoots, where PR-1a was strongly detected (Fig. 3.4). No major differences in accumulation of PR-1a were observed when the concentration of BTH in soil drenches increased from 300 µM to 450 µM.

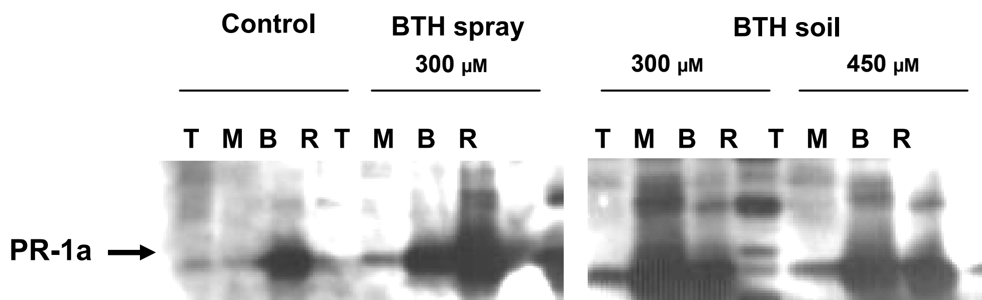


**Fig. 3.4** Immuno blot analysis showing the accumulation of PR-1a protein in leaves and roots of tobacco plants ten days after treatment with BTH. T: top leaves, **M**: middle leaves, **B**: bottom leaves and **R**: roots

## III.5. Discussion

Several studies have demonstrated that parasitism by *Orobanche* is recognized by the host's defense system (Westwood, *et al*., 1998, Joel and Portnoy 1998, Goldwasser *et al.,* 2002, Vieira Dos Santos *et al.,* 2003a, 2003b, Griffitts *et al*.*,* 2004), but despite this recognition and activation of specific defense mechanisms, the parasite is still successful in invading the host. The observation that BTH can protect sunflower from *O. cumana* parasitism is intriguing because our studies of *Arabidopsis* suggest that *O. aegyptiaca* does not significantly induce SAR in its host. Using tobacco, which is also an excellent model for studying plant defenses, we have found that application of BTH reduced *O. aegyptiaca* parasitism (Table 3.1).

The reduction in parasitism from BTH applied to soil appeared to be equal to or better than that applied to foliage (Table 3.1). The systemic effect of BTH was reported by Metraux et al. (1991), showing that cucumber plants drenched with INA had increased resistance to a foliar pathogen, *Colletotrichum lagenarium*. BTH was observed to be translocated and its systemic activity is most likely due to translocation of the BTH itself (Metraux *et al.,* 1991, Friedrich *et al.,* 1996). Our data confirm that SAR was stimulated (Figs. 3.3 and 3.4) by BTH drenches at least as well as by foliar treatments.

BTH-treated plants were also shown to have fewer numbers of parasites (Table 3.2) when compared to non-treated controls. Similar observations were found during a study performed by Chinnasri et al. (2003) in a nematode-host experiment. Soybean seedlings treated with BTH showed a reduction in development and fecundity of the nematode *Meloigdone javanica*.

Sauerborn et al. (2002) reported that imbibing sunflower seeds in BTH reduced parasitism of the resulting plants by *O. cumana*. This treatment resulted in a reduction on sunflower biomass. In contrast, we did not observe any significant effect on biomass of tobacco in response to either of the BTH treatments (Figs. 3.1 and 3.2). It is also important to note that we did not observe direct phytotoxic effects of BTH on *O. aegyptiaca* seeds or seedlings. This agrees with results from *O. cumana* (Sauerborn *et al*., 2002) and indicates that reductions in parasite growth are due to effects of BTH on the host rather than the parasite.

In order to try to attain the maximum effects of an enhanced SAR against *O. aegyptiaca* parasitism, we wanted to ensure that SAR was stimulated early and continuously during the exposure to parasites. BTH was applied three days after transplanting tobacco into infested soil. Considering that a maximum induction of *PR-1a* was detected at day 7 post-treatment (Friedrich *et al.,* 1996), BTH applied to the roots of tobacco in the current studies should have produced its maximum induction of SAR by the time *O. aegyptiaca* started to penetrate the root tissue because *Orobanche* takes between three to five days to germinate and reach the host tissue. In comparison, Sauerborn *et al.* (2002) induced sunflower only once by priming seeds in a BTH solution.

*PR-1a* expression is tightly correlated with induction of SAR in tobacco shoots, but *PR-1a* is expressed in low amounts in tobacco roots (Memelink *et al.,* 1990). The absence of *PR-1a* message in tobacco root (Fig. 3.3) is consistent with this, but the utility of *PR-1a* as an indicator of activation of SAR was useful because it demonstrated plant-wide induction of SAR (Fig. 3.3).

Foliar application of BTH can induce cytological changes in host roots as was demonstrated by a study in which cucumber and tomato root tissue were attacked by the soil-borne pathogens *Fusarium oxisporum* and *Pythium ultimum* (Benhamou and Belager, 1998a, 1998b). Massive deposition of phenolic-enriched occluding material and the formation of structural barriers that appear to prevent pathogen ingress were observed in cucumber and tomato root tissue treated with BTH. It was proposed that BTH prepared susceptible hosts to react more rapidly and more efficiently to pathogen attack through the formation of protective layers at sites of potential pathogen entry (Benhamou and Belager, 1998a).

Similar cytological observations have been made in the roots of parasite-resistant vetch when attacked by *O. aegyptiaca* (Goldwasser *et al*., 2000). Obstruction of the intruding haustorium was attributed to a chemical and/or mechanical barrier activated at the endodermis cell layer in vetch root. A similar mechanism of resistant was found in resistant sunflower against *O. cumana* (Labrousse *et al*., 2001). In spite of the success of the parasite to develop on BTH-treated plants, we may conclude that defenses activated by BTH could be responsible for enhancing resistance in susceptible tobacco roots. To understand why resistance was not stronger, it may be important to consider the relative defensive capacities of sunflower and tobacco. Sunflower produces well characterized phytoalexins such as scopoletin, which are abundantly secreted in seedling roots (Gutierrez-Mellado *et al*., 1996). These phytoalexins have been implicated in defense against *O. cernua* (Serghini *et al*., 1996). Furthermore, sunflower has a long history of producing *Orobanche*-resistant varieties (Alonso, 1998). Tobacco, in contrast, has no such history of resistance to *Orobanche*. Although BTH treatments can increase

resistance to parasitism, the ability of tobacco to respond may depend largely in its innate defense capacity.

This research has shown that parasitism of a susceptible host such as tobacco may be reduced when plants are stimulated to a state of heightened defensiveness by activating SAR. This technique offers an additional option for the farmer to supplement other approaches currently used for controlling *O. aegyptiaca*. It is an approach worth exploring in other parasitized crop species.

## III.6. Acknowledgments

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# CHAPTER FOUR

# Characterization of an engineered R-Avr interaction as a strategy for resistance to *Orobanche* parasitism

## IV.1. Abstract

The parasitic angiosperm *Orobanche aegyptiaca* Pers, (Egyptian broomrape) is a root holoparasite that causes severe losses in yield and quality of many crops. *Orobanche* live directly on their hosts by attaching haustoria to their roots and absorbing water and photosynthates directly from the host. Because most current methods aimed at controlling *Orobanche* have limited effectiveness, the search for resistant host crops has become increasingly important. The objective of this research is testing the hypothesis that an engineered *R-Avr* interaction will trigger the hypersensitive response (HR) in the host root at the site of parasite penetration, leading to a severing of the living continuum and starvation of the parasite. Winston (2003) engineered tobacco plants fusing an *Orobanche*-inducible promoter (*CHS8*, chalcone synthase from *Phaseolus vulgaris*) with the replicase protein of the tobacco mosaic virus (TMV). The recognition of the replicase by tobacco *N* gene product should trigger the host defense machinery leading to HR. Transgenic tobacco plants were challenged with *O. aegyptiaca* and parasitism was reduced by 40 %. These observations agree with Winston's results. Efforts to confirm the induction of HR-associated necrosis in host roots using trypan blue staining produced ambiguous results because parasite tubercles retained the stain more than host roots. Nevertheless, no specific accumulation of blue pigment was observed in host roots adjacent to attached parasites. The precise mechanism by which these transgenic plants deter *Orobanche* remain unclear.

## IV.2. Introduction

*Orobanche aegyptiaca* Pers. (Egyptian broomrape) is an obligate root parasite that affects many economically important crops, causing severe losses in yield and quality. Control of *Orobanche* is extremely challenging, in part because the parasite is hidden underground for most of its life cycle and is intimately associated with the roots of its host. *Orobanche* species currently have greatest economic impact in countries from the Mediterranean region, Middle East and western Asia where they parasitize important dicotyledonous crops such as sunflower, potato, tomato, eggplant, and faba bean. Because most methods for controlling *Orobanche* either have limited effectiveness or are prohibitively expensive, the best long-term strategy is the development of parasite-resistant crops.

Plants defend themselves from pathogens by producing resistance responses that inhibit pathogen progression. In certain interactions the pathogen is specifically recognized by the plant, triggering signal transduction cascades that lead to rapid defense mobilization, and ultimately, cell death in the area immediately surrounding the point of pathogen entry (Greenberg, 1997). This cell death effectively separates a biotrophic pathogen from its source of nutrition and limits its spread. This process is called the hypersensitive response (HR) and is controlled by interactions of specific molecules produced by the pathogen and host plant in the gene-for-gene interaction (Greenberg, 1997). Recruiting a similar mechanism for the engineering of a host resistant to *Orobanche* could be an effective strategy, and this was proposed by Winston (2003).

HR is known to be induced by the coexpression of resistance (R) genes in the host and avirulence (Avr) genes by the pathogen. For this strategy Winston and coworkers selected the tobacco N gene and the tobacco mosaic virus (TMV) replicase gene as the R and Avr genes, respectively (Padgett et al., 1997). The induction of HR in plants requires tight regulation because inappropriate cell death would be detrimental or lethal to the plants. Therefore, Identification of *Orobanche*-inducible promoters (Westwood et al., 1998; Griffitts et al., 2004) played a crucial role in the strategy so that the defense mechanism would be activated as specifically as possible. The full strategy consisted of the introduction of the *TMV-replicase* gene under the regulation of the *Orobanche*-inducible chalcone synthase promoter (CHS8) from bean (*Phaseolus vulgaris*) (Schmid et al., 1990). The tobacco N gene was fused to the tomato *HMG2* promoter (Westwood et al., 1998). Simultaneous induction of these genes should lead to the triggering of HR at the site of parasite penetration.

Because of difficulties involved in making the N gene construct, Winston (2003) developed a system for testing the fundamental hypothesis by using a tobacco line Xanthi-NN that carries the *N*-gene. This line was transformed with the *CHS8:TMV* replicase construct, and transgenic tobacco plants were shown to be tolerant to *O. aegyptiaca* parasitism. The objectives of the current work were to (1) confirm the presence of the transgene in transgenic plants by PCR and genomic DNA hybridization; (2) confirm the response of transgenic plants to *O. aegyptiaca* parasitism, and (3) assay for HR at the site of infection using trypan blue staining.

## IV.3. Materials and methods

### IV.3.1. Plant growth and inoculation

Tobacco transgenic plants (lines *CHS8:TMV 1*, *CHS8:TMV 5*, *CHS8:TMV 8*, and *CHS8:TMV 9*, including Xanthi-NN wild type) were vegetatively propagated in vitro from parent plants originally generated by Winston (2003). Four to eight replicate plants of each line were transplanted from media to a polyethylene bag growth system as described by Westwood (2000). Plants were allowed to recover and establish new roots for two weeks. Growing conditions were 9 h light (150 $\mu E/m^2/sec$), 15 h dark at 20°C ± 2°C. Plants were watered as needed by adding half-strength Hoagland solution (Hoagland and Arnon, 1950) to the bags. Roots were inoculated with surface-sterilized *O. aegyptiaca* seeds to filter paper around tobacco roots (described in Westwood 2000). After seven days for *O. aegyptiaca* seed pre-conditioning, seeds were germinated by adding 10 ml of a 10 ppm solution of GR-24, a synthetic germination stimulant (Mangnus *et al.,* 1992), to each bag. Parasitized plants were harvested approximately 4 weeks after addition of germination stimulant. Parameters studied included root mass, number of tubercles, and fresh weight of tubercles. Statistical analyses was performed using ANOVA and Duncan's multiple range test ($\alpha$=0.05) using SAS version 8.1 (Statistical Analysis System, Cary, NC).

### IV.3.2. Analysis *CHS8:TMV* transgenic tobacco plants by PCR

Leaf tissue (2 g) from *CHS8:TMV* transgenic plants was harvested, frozen in liquid nitrogen, and ground by mortar and pestle. Total genomic DNA was extracted by CTAB (hexadecyl trimethyl-ammonium bromide)-phenol/chloroform method as described by Murray and Thompson (1980). Presence of the transgene was established by PCR using 100 ng of genomic DNA. PCR was performed with gene-specific primers TMV-577 forward 5'-CGATAGCCTGGTA GCATCACT-3' and TMV-1332 reverse 5'-GAGTAGTTCTGCGTGTCTCCAC-3'. Primers were designed to anneal to the coding region of the TMV replicase gene, producing an expected fragment of 770 bp. The PCR parameters was conducted for 4 min at 94 ºC, 35 cycles of 1 min at 94 ºC, 50 sec at 55 ºC and 1 min at 72ºC using a RoboCycler Gradient 96 (Stratagene, La Jolla, CA).

### IV.3.3. Trypan blue staining

Root samples for trypan blue staining (McDowell et al., 2000) were obtained from transgenic plants parasitized by *O. aegyptiaca*. Samples from each transgenic and non-transformed line were transferred to 2 ml screw cap centrifuge tubes and 1.5 ml of trypan solution (50 g phenol, 50 ml lactic acid, 50 ml glycerin, 100 mg trypan blue and 50 ml of water) was added and incubated for 3 min at 90ºC, followed by an incubation of 5 min at room temperature. The staining solution was removed and 1.5 ml of chloral hydrate (2.5 g/ml of distilled water) was added to the samples and incubated at room temperature overnight. Chloral hydrate solution was replaced with fresh solution twice. Images were

captured using a digital imaging system (RT Color Diagnostic instrument, Inc., Sterling Heights, MI) coupled to a stereomicroscope (Leica G Z7).

## IV.4. Results

### IV.4.1. Transgenic tobacco were confirmed for the presence of the TMV replicase gene via PCR

Transgenic plants produced by Winston (2003) were confirmed to have the TMV replicase transgene by PCR amplification of a segment of the TMV replicase gene. A PCR product of approximately the expected 770 bp size was obtained from genomic DNA of the putatively transformed lines (Fig. 4.1). These transgenic plants were used for evaluating tolerance against *O. aegyptiaca*.

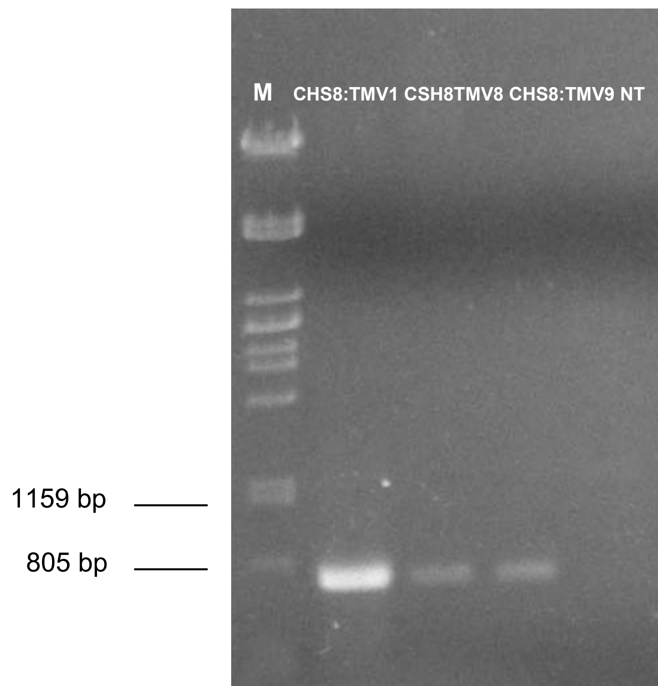


**Fig. 4.1** Presence of the TMV replicase gene in putatively transformed tobacco lines by PCR using TMV-specific primers. **M**, Lambda/*Pst* I size marker; *CHS8:TMV1*, *CHS8:TMV8*, and *CHS8:TMV*9 are putatively transformed tobacco lines. NT, non-transformed Xanthi-NN plant.

### IV.4.2. Transgenic *CHS8:TMV* replicase tobacco plants show partial tolerance against *Orobanche* parasitism

Winston (2003) observed that *CHS8:TMV* transgenic plant showed a high degree of reduction in *Orobanche* parasitism. In order to confirm the efficacy of the engineered HR strategy against parasitism found by Winston (2003), transgenic tobacco plants were challenged against *O. aegyptiaca*. Transgenic plants showed a reduction in parasitism when compared with non-transformed plants, which agrees with data obtained by Winston (2003). The number of tubercles per g of host root were statistically lower (ANOVA, $p<0.01$) in parasitism on transgenic plants with about half of the parasites of the non-transformed line (Fig. 4.2).

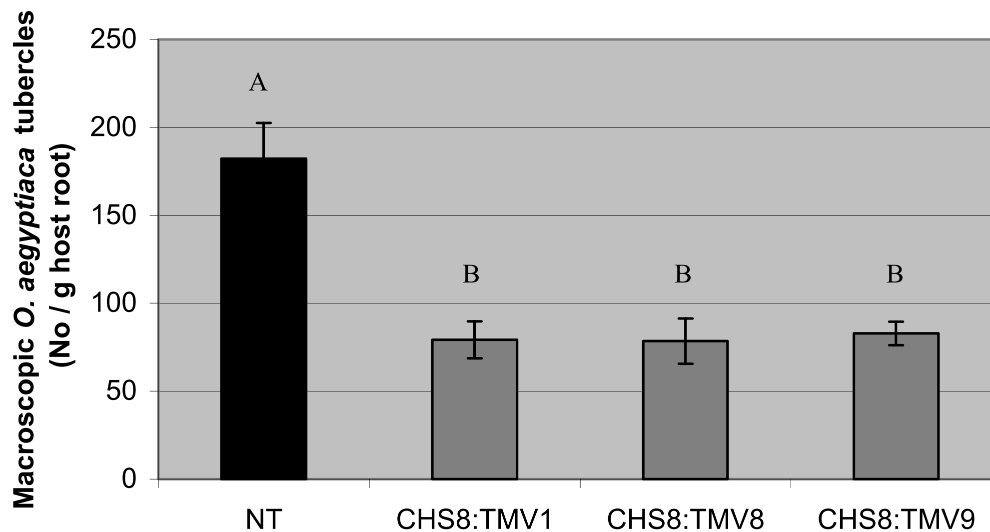


**Fig 4.2** Response of *CHS8:TMV* transformed tobacco plants to *O. aegyptiaca* parasitism. NT is the non-transformed plant (Xanthi-NN) while *CHS8:TMV1*, *CHS8:TMV8*, and *CHS8:TMV9* are transformed plants. Bars represent means of 4 to 8 plants with vertical lines representing standard errors. Columns with different letters are significantly different ($\alpha=0.05$) according to Duncan's multiple range test.

### IV.4.3 Trypan blue staining was not able to resolve HR at site of *O. aegyptiaca* penetration

Trypan blue staining is a classic procedure for distinguishing between dead cells (cells that will retain the blue coloration) and living cells. In order to study the occurrence of necrosis at the site of parasite penetration, trypan blue staining of parasitized transgenic tobacco roots was performed. Unexpectedly, *O. aegyptiaca* tubercles retained the staining solution, which obscured the observation of cells absorbing the stain at the site of infection (Fig. 4.2). Nevertheless, no specific accumulation of blue pigment was observed in host roots adjacent to attached parasites. Micrographs of parasitized transgenic hosts under light microscope showed a browning area surrounding the site of penetration (Figs 4.3. C, E, and G) which may be an indication of necrosis at the site of infection, although it is not clear whether it is necrosis of parasite or host tissues.

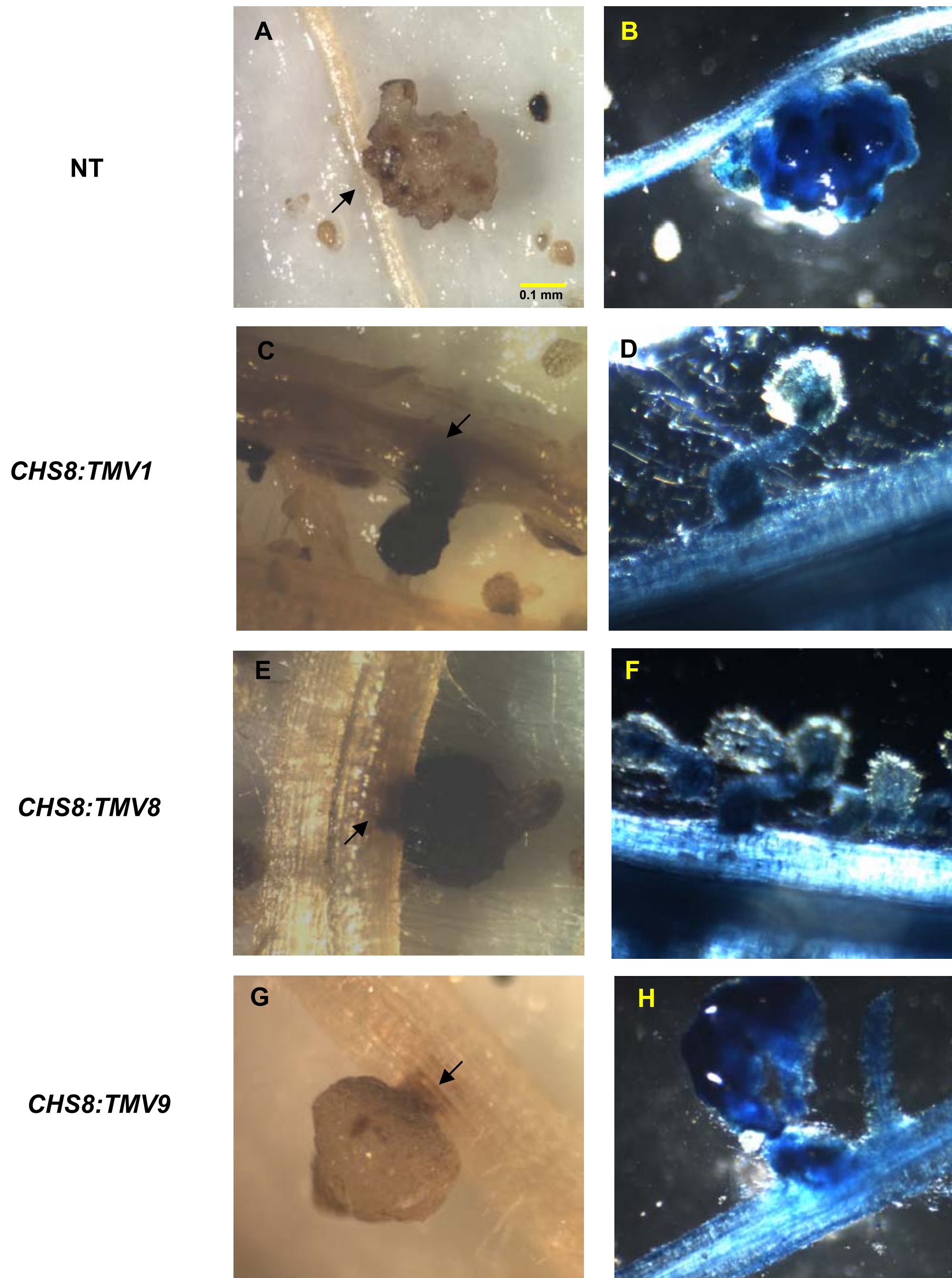


**Fig. 4.3** Trypan blue staining for evaluation of HR in roots of wild type (A-B) and *CHS8:TMV*-expressing (C-H) tobacco in response to *O. aegyptiaca*. A, C, E, and G show *O. aegyptiaca* attachments without trypan blue staining. B, D, F, and H micrographs showing different tubercles from the same host plants after trypan blue staining. Arrows indicate point of parasite attachment to host root. Xanthi-NN (NT) is the non-transformed line and *CHS8:TMV1*, *CHS:TMV8*, and *CHS8:TMV9* are transformed lines.

## IV.5. Discussion

Strategies for controlling *Orobanche* have been generally unsuccessful so the possibility of engineering crops that are able to resist *Orobanche* parasitism offers new possibilities to solve the problems of farmers who are affected by this parasite. Here we confirm the results of Winston (2003) that demonstrated that the *CHS8:TMV* replicase expressed in Xanthi-NN appears to confer enhanced host resistance to *O. aegyptiaca.* Results from an *O. aegyptiaca* challenge showed that transgenic lines had a reduced parasitism in about 40% when compared to the untransformed plant (Table 4.1). During the course of the experiment, we observed that the greatest number of dead tubercles were found in line 9. A transgene-dependent darkening in the root at the site of *O. aegyptiaca* attachment was observed in the different lines, and this darkening extended to the vascular system of the host. Trypan blue staining was not able to resolve whether parasite penetration induced HR. Macroscopic observations of attachment (Fig. 4.2) showed that even living tubercles readily take up the stain and obscure the observation of HR. Additional research on these plants will be needed to determine the exact mechanism of increased resistance to *O. aegyptiaca* parasitism.

### IV.6. References

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## VITA

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