Chapter I
LITERATURE REVIEW
I.1 INTRODUCTION
I.1 CYST NEMATODE PLANT DISEASE
I.1.1 Economic Importance

Plant parasitic nematodes are destructive pests that cause severe losses in agriculture. For example, losses of over 100 billion dollars per year world-wide can be attributed to infections caused by parasitic nematodes (Urwin et al., 1997). These tiny parasites obtain nutrition from the cytoplasm of living plant cells and comprise many species including ectoparasites and endoparasites (Williamson, 1999). The majority of species within 50 genera of plant parasitic nematodes feed on root tissue with diverse modes of parasitism (Lilley et al., 1999). The cyst (Heterodera spp. and Globodera spp) and root knot (Meloidogyne spp.) nematodes are obligate root parasites that are members of the family Heteroderidae. These genera cause approximately 80% of annual crop losses to nematodes, making the cyst and root-knot nematodes the most important species throughout the world. In addition to causing the most economic damage, both the cyst and root knot nematodes possess many similarities in their modes of parasitism, lifecycles, and host responses such as induction of defense related genes and symptoms produced. For the purposes of this paper we will focus on the cyst nematode in our review of literature and experimentation.

Of particular interest are the cyst-forming species within the family of Heteroderidae. The genera Heterodera and Globodera have comprised the most economically important species of the cyst nematode for several decades. When Franklin published her book on “The cyst-forming species of Heterodera” in 1951, the cyst nematodes were already a major concern and were known to cause serious yield losses in important food crops such as potatoes, cereals, brassicas, tomatoes, and sugar beet (Evans and Rowe, 1998). Major species of cyst nematodes, such as Heterodera schachtii (Schmidt 1871) on sugar beet, Globodera rostochiensis (Wolleneuber 1923; Behrens, 1975), and G. pallida (Stone 1983; Behrens, 1975) on potato, remain of major economic importance world-wide (Zunke and Eisenback, 1998). Infections caused by the cyst nematode can greatly reduce crop yields by as much as 30% to 75% (Agrios, 1988). Although nematode problems occur throughout the world, most damage due to cyst nematodes occurs in temperate, tropical, and subtropical regions, particularly in areas that have been cultivated for long periods of time (Mai, 1987). It is believed that members of the genera
Heterodera evolved on ancient orders of tropical plants. To date, there are at least 23 species of the cyst nematodes that have been found in the tropics. Major tobacco growing regions in the US endure substantial loss to the tobacco cyst nematode (TCN). In 1998, Virginia lost approximately 4 billion dollars of flue-cured tobacco to TCN infestations that reduced the crop by an estimated 3% (Crowder, 2000). Losses of such magnitude result in the continuous production of tobacco to compensate for TCN-induced crop loss, which leads to increased disease severity and greater crop loss.

I.1.2 The Globodera tabacum Complex

The genus Globodera can be divided into three separate groups, (1) the species G. pallida and G. rostochiensis that originate from regions of South America and parasitize potato and other closely related plant species within Solanaceae, (2) the species G. tabacum that also attacks members of Solanaceae, particularly tobacco, and (3) the three species of G. achillae, G. artemisae, and G. millifolii that exclusively parasitize members of Asteraceae (Evans and Rowe, 1998). The most damaging Globodera species are the potato cyst nematodes, which in extreme cases can cause total crop loss.

The tobacco cyst nematode has been described and categorized on numerous occasions. At one time, there were three individual species of the TCN, G. solanacearum, G. tabacum, and G. virginiae. Scientists classified them separately due to host range preferences and slight morphological differences. Lownsbery and Lownsbery (1954) reported the discovery of a cyst nematode in the early 1950’s in Hazardville, Connecticut on shade tobacco (Nicotiana tabacum L.) They concluded that the morphology of the recently discovered nematode resembled that of the potato cyst nematode, Heterodera rostochiensis, and it named the tobacco cyst nematode Heterodera tabacum (Rideout, 2000). The second cyst nematode was described in 1968 by Miller and Gray. They named it Heterodera virginiae because it was found in Tidewater, Virginia, parasitizing horsenettle (Solanum carolinense L.). Third, the Osborne’s cyst nematode was discovered by its namesake, W. W. Osborne in 1961, when it was found parasitizing flue-cured tobacco in Amelia County, Virginia. This nematode was later re-described as Heterodera solanacearum by Miller and Gray (1972). The morphology of these three cyst nematodes was re-evaluated by Behrens in 1975. He found that the gross morphology of the cysts were globular in shape and subsequently placed the three cyst nematodes in the genus Globodera. These
species were re-evaluated by Stone in 1983 and he concluded that they comprise the TCN complex and assigned each a specific subspecies. The Connecticut nematode originally designated *H. tabacum* is now known as *G. tabacum tabacum*, (c; Behrens, 1975), and Osborne’s cyst nematode became *G. tabacum solanacearum* (Miller and Gray, 1968; Behrens, 1975). Collectively, these species are known as the *G. tabacum* complex.

### 1.1.3 Morphological Characteristics of *Globodera* spp.

Morphology is the essential basis for the identification of cyst nematodes. Identification can be extremely difficult because morphological characteristics of the *G. tabacum* complex are subtle. The genus *Globodera* was first named by Skarbilovich in 1959 and renamed later by Behrens in 1975. It comprises less than a dozen species whose morphological characteristics are highly conserved. Stone (1983) believed that only *G. rostochiensis*, *G. pallida*, and *G. tabacum* could be consistently distinguished. However, he stated that the subspecies of the *G. tabacum* complex were difficult to separate (Baldwin and Mundo-Ocampo, 1991). Molecular studies have been performed to support the homology within the *G. tabacum* complex. Bossis and Mugni’ery (1993) performed two-dimensional gel electrophoresis to study the status of six *Globodera* spp. using a widely used distance matrix method to infer nematode phylogenies. Similarity clusters of *Globodera* spp. were computed by average linkage with root-mean-square genetic distances according to the group average unweighted pair group method (UPGMA). They found that *G. t. virginiae* and *G. t. solanacearum* are closely related, having a genetic distance of 0.5. Both of these subspecies have a 0.17 distance from *G. t. tabacum* has a 0.17 difference from the other two subspecies. They concluded that the subspecies of the *G. tabacum* complex should be considered as only one species (Bossis and Mugni’ery, 1993).

A general description of the major morphological characteristics among the *G. tabacum* complex is as follows. Adult female cyst nematodes have spherical shaped bodies that are 0.6 to 0.8 mm in length and 0.3 to 0.5 mm in width. Depending on the subspecies, the cyst color ranges from cream-colored to golden yellow to a dark reddish-brown. Adult females have a zig-zag pattern with many ridges between the vulva and the anus. Adult males have twisted, wormlike bodies that are approximately 1.3 mm in length by 30 µm in diameter. Males also have distally pointed spicules that are >30 µm. The infectious second stage juvenile or J2, possess conical, pointed tails with terminal regions partially hyaline (Baldwin and Mundo-

### I.1.4 Sedentary Endoparasitic Nematode Life Cycle

The sedentary endoparasitic nematodes have complex life cycles. These nematodes have evolved highly specialized and efficient interactions with their host plants (Lilley et al., 1999). The cyst and root knot nematodes are obligate, cross-fertile species with six life stages: the egg, four juvenile stages, and the sexually dimorphic adult stage (Opperman and Bird, 1998). The annual life cycle begins with the hatching of eggs in the soil. The egg develops into a first-stage juvenile that molts within the cyst and emerges as an infectious, motile second-stage juvenile. In a compatible interaction with a host plant, the second-stage juvenile invades the host root and migrates to the vascular cylinder in search of a permanent feeding site, but some aspects of this early infection process differ between the root knot and cyst nematodes. With respect to the root knot nematode, the second-stage juvenile or J2 penetrates the cell in the zone of elongation of the host root. It then migrates intercellularly and uses a combination of mechanical force and enzymatic secretions to separate cells at the middle lamella. Ultimately, the nematode migrates to the root tip and the zone of differentiation (Williamson and Hussey, 1996). Cyst nematode second-stage juveniles penetrate host roots through the epidermis and mechanically force their way through the cortex and endodermis by piercing and rupturing cell walls with their stylets (specialized feeding apparati).

Both parasitic nematodes initiate the establishment of a complex feeding site after migration. They use their stylet to puncture five to six procambial cells surrounding their head and begin to feed on the cytosolic nutrients. As they feed, the nematodes intermittently secrete saliva into the cells, which triggers a series of dramatic changes in the feeding cells and those surrounding the feeding cells. The saliva contains digestive enzymes, including proteases, which aid in the degradation and liquification of plant cell materials. Depending on the nematode genus, these cells are either stimulated to form a system of hypertrophial multinucleate cells or “giant cells” (root knot nematode) or the initial feeding cell develops into a syncytium or nurse cell (cyst nematode) (Gheysen et al., 1996). Giant cells result from repeated nuclear divisions, whereas syncyntia are produced from the degradation of cell walls and the subsequent fusion of adjacent cells. Mitosis is not stimulated but nuclei and nucleoli...
become considerably enlarged, consistent with an increased syncyntial metabolism (Wyss, 1997). The giant cells or syncyntia supply the nematode with nutrients necessary for growth by transferring nutrients from the vascular tissue to the feeding nematode (De Boer et al., 1996). These feeding cells must remain healthy and metabolically active throughout the nematodes’ life cycle to ensure their survival (Gheysen et al., 1996). Once feeding commences, the juvenile becomes immobile or sedentary and undergo three moults while developing into an adult male or female.

The adult female nematode (4th stage larvae) becomes sedentary and increases in thickness during feeding. The body of the female root knot nematode is entirely embedded in the root tissues, whereas the female cyst nematode’s body is almost entirely exposed outside the root surface. Adult males of either nematode regains their mobility after feeding and can either fertilize the females and/or migrate through the roots to become free-living in the soil. The female can produce approximately 500 eggs (root knot) or 300 to 600 eggs (cyst) after fertilization. After development, which usually takes three to five weeks, the root knot nematode can lay eggs (covered in a gelatinous matrix) either inside or outside the root. The eggs of the cyst nematode remain inside the female until her death, at which time the body becomes darkened and forms a cyst. In the absence of a suitable host or unfavorable environmental conditions, cysts can survive up to 28 yr in the soil (Baldwin and Mundo-Ocampo, 1991). Nematode eggs may hatch immediately and further develop into second-stage juveniles that can cause secondary infections in the same host root or new infections in other host roots in close proximity. Depending upon the host and soil temperature, the entire life cycle of the root knot nematode may be completed in 17 to 57 d. From hatching to adult requires 38 to 45 d and females mate within 50 d of root invasion for cyst nematodes under optimal conditions (24 to 29°C) (Baldwin and Mundo-Ocampo, 1991). Dissemination of nematodes within and among fields can be by irrigation water, vegetative plant parts, and soil infested with eggs or larvae that adhere to farm implements, animals, or humans.

I.1.5 Elucidation of Molecular Mechanisms Involved in Nematode Pathogenesis

Studies have been performed to elucidate the molecular mechanisms that are involved in both pathogenesis and the modification of plant cells into feeding cells. The stylet secretions produced in the esophageal glands of plant parasitic nematodes. These substances play a crucial
role in the initiation and maintenance of feeding sites (Jones and Robertson, 1997). There have been studies performed that confirm the changes that occur in the contents, morphology, and activity of the nematode esophageal gland during parasitism. However, until recently, no studies clearly demonstrated that nematode esophageal gland proteins are secreted into host plant tissue. Monoclonal antibodies with specific activity against gland contents of *H. glycines*, *G. rostochiensis*, and *M. incognita* have been synthesized and used to isolate the corresponding nematode genes (Lilley et al., 1999). Identification of these proteins will help elucidate the roles of endoglucanases during pathogenesis.

Wang et al (1999b) first illustrated the secretion of nematode esophageal gland protein into host plant tissue by using polyclonal antisera specific to two β-1,4-endoglucanases (*Hg-eng1* and *Hg-eng2*) that are synthesized in the subventral esophageal gland cells of the soybean cyst nematode (*Heterodera glycines*). To demonstrate the secretion of the protein into plant tissue, the polyclonal sera were used to isolate the proteins HG-ENG1 and HG-ENG2. These proteins were used to probe sections of soybean roots that were harvested 24 h after being inoculated with second stage juveniles of *H. glycines*. Using indirect immunofluorescence microscopy, it was shown that both HG-ENG1 and HG-ENG2 antisera localized to the subventral glands of *H. glycines*. HG-ENG2 antisera were also shown to bind to the endoglucanase secreted into cortical root tissue around the nematode’s head and to root tissues disrupted along the migratory path. Unlike HG-ENG2, HG-ENG1 was not detected in the host plant tissue.

Monoclonal antibodies were also used to immunopurify subventral esophageal gland secretory proteins from *G. rostochiensis*. Resulting amino acid sequences were used to generate oligonucleotide primers to amplify cDNAs that encode two different β-1,4-endoglucanases isolated from the nematode (Smant et al., 1998). Lilley et al. (1999) stated that nematode β-1,4-endoglucanases demonstrate remarkable homology to bacterial endoglucanases. It is believed that these enzymes facilitate intracellular migration of the nematode through the roots and may aid other enzymes in degrading the plant cell wall. Genetic evidence has established that cellulases are required for high virulence by microbial pathogens (Keen and Roberts, 1998). It is suspected that plant parasitic nematodes utilized cellulase for initial infection as migration
occurs. For a detailed description of nematode secretions see the review article by Jones and Robertson (1997).

I.1.6 Distribution and Host Range

The *G. tabacum* complex is found primarily in the USA and China, particularly in areas where tobacco has been grown continuously for many years and solanaceous weeds are present. In addition to these countries, TCN is equally important in countries such as Columbia, Greece, Italy, Korea, Peru, Morocco, Thailand, and Yugoslavia (Evans and Rowe, 1998). Depending on the subspecies and the region in the US, TCN is a serious pest on flue-cured or shade tobacco in Virginia, Maryland, North Carolina, Connecticut, and Massachusetts (Crowder, 2000; LaMondia, 1992). *Globodera t. tabacum* is restricted in distribution to Connecticut and Massachusetts, whereas *G. t. solanacearum* is only known to occur in Virginia and North Carolina (Baldwin and Mundo-Ocampo, 1991). *Globodera t. virginiae* is present in Virginia as well, but has also been reported in Mexico and Central America.

The host range of *G. tabacum* complex is restricted to plants of *Solanaceae* like tobacco and potato (Lownsbery and Lownsbery, 1954; Osborne, 1961; Miller and Gray, 1968; Behrens, 1975). All subspecies can reproduce on *Nicotiana tabacum* L., however, they have differential responses. For example, *G. t. tabacum* attacks shade tobacco while *G. t. virginiae* prefers burley tobacco as a host rather than flue-cured tobacco. *Globodera t. virginiae* can also reproduce on horsenettle. *Globodera. t. solanacearum* is particularly severe on flue-cured tobacco and can mature on tomato, sweet pepper, and eggplant as well. *Globodera t. tabacum* can reproduce on potato, while *G. t. solanacearum* does not.

I.1.7 Symptoms caused by *Globodera* spp.

Whole plants respond to infection by the cyst nematode by a reduction of photosynthetic rate, growth, and yield. The nematode affects the physiology of the plants by interfering with the synthesis and translocation of growth hormones produced in the host roots (Bird, 1974). These plants are stunted and have leaves that are not only wilted, but also chlorotic and that senesce prematurely. Above-ground symptoms can be misdiagnosed as a nutritional deficiency. Sometimes plants do not display clear symptoms and their economic effect tends to be underestimated by growers (Lilley et al., 1999). The plant may have a reduction in root biomass,
but the most typical sign of cyst nematode infestation is the presence of cysts attached to the plant roots.

I.2 GLOBODERA CONTROL METHODS

The control of cyst nematodes can be very challenging for numerous reasons. Under optimal conditions, the adult female has a high level of fecundity, possessing the ability to produce 300 to 600 eggs per generation. Cyst nematode eggs are encased in a hard protective covering that prevents invasion of potential predators and protects the eggs inside from rapid dessication (Riggs and Schuster, 1997). This protective covering also makes it difficult to destroy eggs with nematicides (Johnson, 1999). Eggs can remain dormant for several years and cysts can remain viable in the soil without a host for as long as 28 yr where soil type and temperature are ideal (Baldwin and Mundo-Ocampo, 1991).

Currently, the control of cyst nematodes is dependent upon four main approaches: resistant cultivars, chemical applications, cultural practices, and biological control (Urwin et al., 1997). These measures are often more effective in controlling plant-parasitic nematodes when used in combination. The proper control strategy depends on the availability of resistant cultivars and the value of the crop (Riggs and Schuster, 1997).

I.2.1 Resistant cultivars

Plants are defined as resistant to nematodes when the expression of host genes inhibits or reduces nematode reproduction. Of the current control strategies, the use of resistant cultivars is the most attractive approach because it is economical and environmentally safe and has the potential to provide long-term population control. The role of host resistance for nematode control has grown in importance due to increasing restrictions to chemical pesticides (Williamson, 1999). However, plant resistance is not available in many important crops and effectiveness is often restricted to a few races of a nematode genus. This may cause the cultivars to become prone to selection of virulent nematode biotypes or siblings present in field populations (Whitehead, 1998; Gheysen et al., 1996). A more broadly based resistance would be useful in alleviating problems associated with resistant cultivars.

Planting resistant cultivars in the field can potentially reduce TCN densities. Wang et al (1999a) investigated the influence of TCN on the growth of resistant (NC567) and susceptible (K326) flue-cured tobacco cultivars in the field. Infection by TCN reduced the growth of both
resistant and susceptible cultivar during the first 11 wk after transplanting. The susceptible host displayed severe stunting when compared to the resistant cultivar. Tobacco cyst nematode infection reduced the fresh leaf weight two folds for K326 as compared to NC567. Similarly, Wheeler et al. (1997) conducted a two year study to determine the effect of soybean cyst nematode (SCN) on the yield of resistant and susceptible soybean cultivars grown in Ohio. Soil samples were assayed for nematode densities before planting and during harvesting. They reported that the yield of resistant cultivars (Madison Experimental 131527 and Asgrow A3431) averaged from 0% to 18% higher than those of susceptible cultivars (AgVenture and Resnik) in fine-textured soils with average preplant nematode populations ranging from 462 to 14,330 SCN eggs/100 cm$^3$ soil. In coarse textured soils, yields of resistant cultivars were 21% to 56% higher than those of susceptible cultivars with average preplant densities ranging from 1,661 to 15,558 SCN eggs/100 cm$^3$ soil. Wheeler et al. (1997) concluded that nematode resistant cultivars were excellent alternatives to currently grown susceptible varieties for managing SCN.

When TCN infestation levels are high, the use of resistant cultivars alone may not sufficiently manage TCN. Yield loss can be significantly decreased when a resistant cultivar is used in combination with the application of a nematicide. Johnson et al. (1989a) studied the feasibility of alternating resistant and susceptible flue-cured tobacco cultivars with nematicide application to improve TCN control. Their study demonstrated that planting the resistant cultivar, NC567, and the application of fenamiphos to TCN-infested fields for one year, significantly reduced nematode populations. Nematode infestations were further reduced when fenamiphos was applied for two consecutive year rather than 1 year with NC567. Treatments involving a combination of fenamiphos and NC567 resulted in higher economic returns compared to use of susceptible cultivar without nematicide applications. However, long-term use of resistant cultivars is not recommended. Problems associated with long-term use of resistant cultivars include shifts in nematode races or species and the occurrence of multiple species of nematodes within the same field (Young, 1992). Shifts in races of a nematode in response to planting resistant cultivars occurred in the interaction of soybean and Heterodera glycines. In the southern United States, race 3 of H. glycines was prevalent when the resistant cultivar, Pickett was released. Within a few years, race 4 became predominant in this region. Subsequently a second resistant cultivar, Bedford, was released and race 5 became problematic.
in Tennessee (Young, 1992). Problems associated with race shifting can be lessened by rotating susceptible cultivars and non-host crops with resistant cultivars.

Resistance has been linked to a single dominant gene (Milligan et al., 1998). Several dominant and semidominant resistance genes have been identified and mapped to chromosomal location or linkage groups (Williamson and Hussey, 1996). The recent isolation of some plant resistance genes indicates that the respective disease resistance genes are a part of the signal transduction pathway leading to a hypersensitive response (HR) against bacterial, fungal, viral, and nematodal pathogens (Dangl and Jones, 2001; Ellis et al., 2000; Martin, 1999; Hammond-Kosak and Jones, 1997; Bent, 1996; Ganal et al., 1995; Mindrinos et al., 1994; Jones et al., 1994; Whitham et al., 1994; 1994; Martin et al., 1993). A strong resistance response is induced when a host resistance gene and pathogen avirulence (avr) gene of matched specificity are expressed (Bent, 1996). Nematode resistance genes are present in several crop species and are important components in many breeding programs including those for tomato, potato, soybeans, and cereals (Williamson, 1999). Many of these have been mapped to chromosomal locations or linkage groups (See Huang, 1998; Williamson and Hussey, 1996 for detailed reviews). Resistance genes can be categorized into five classes based on their structural motifs, which include extracellular leucine rich repeat (LRR) domains, nucleotide binding site (NBS), serine/threonine protein kinase domains, leucine zipper domains, and intercellular NBS-LRR proteins with a region similar to the Drosophila Toll and mammalian interleukin-1 receptor (TIR) proteins.

Few nematode genes have been cloned and characterized to date. One of the best characterized nematode resistance genes is Mi, which confers resistance to three root-knot nematode species that infect tomato: M. incognita, M. arenaria, and M. javanica (Hwang et al., 2000; Williamson and Hussey, 1996). This gene was first introduced into cultivated tomato (L. esculentum) from the wild species L. peruvianum by embryo rescue of the interspecific cross in the early 1940s (Williamson, 1998). Positional cloning was used to isolate the Mi gene (Milligan et al., 1998). Two potential genes, Mi-1.1 and Mi-1.2, were identified in the 52-kb region to which Mi had been localized. The proteins that these genes encode are 91% identical in the amino acid sequence and belong to the family of plant resistance genes characterized by the presence of NBS and LRR domains (Hwang et al., 2000). Resistance via the Mi gene is
characterized by the development of localized tissue necrosis or hypersensitive response (HR) in plant cells that surround the anterior of the invading nematode within a few days of infection. In incompatible interactions with resistant plants, nematodes that fail to establish feeding sites either die or leave the roots (Milligan et al., 1998). It has been suggested that the recognition of the nematode by the host may occur in the cytoplasm due to the lack of a signal sequence in the Mi encoded protein (Williamson, 1999). Transgenic plants that contain the Mi gene also confer resistance to the potato aphid (Macrosiphum euphorbiae) as well as the root-knot nematode (Dellarduya et al., 2001). The Mi gene is an effective nematode resistance gene and has been introduced into several commercial tomato varieties. Efforts are underway to clone other genes with the possibility of transferring them to hosts that do not contain the appropriate resistance loci. Engineering plants with resistance genes has great promise for enhancing plant resistance. However, there is a possibility that the gene may not function in heterologous hosts or that the nematodes may overcome resistance acquired through these genes, thus causing the effectiveness of this approach to be short-lived (Williamson and Hussey, 1996).

I.2.2 Chemical methods of nematode control

Chemicals that paralyze or kill nematodes are collectively referred to as nematicides (Whitehead, 1998). These chemicals are often referred to as the most unacceptable class of pesticides widely used in agriculture due to their cost, phyto-toxicity, and negative effects on the environment (Urwin et al., 1998). Nematicides are generally applied to the soil as a fumigant. Currently, all available fumigants are hazardous to the environment and phytotoxic as well. To prevent plant damage, the chemicals must be applied to the soil for days, weeks, or months before planting. This method may not be very effective in controlling nematode populations and can be very expensive to maintain. The most widely used compounds are either halogenated aliphatic hydrocarbons or methyl isothiocyanate precursor compounds or mixtures (Whitehead, 1998). These include methyl bromide, chloropicrin, ethylene dibromide, dazomet, and metham- sodium. The most effective nematicides are those that are a combination of methyl bromide and chloropicrin. Since the 1950s, methyl bromide has been used as a reliable broad spectrum soil fumigant for the control of plant parasitic nematodes, fungi, and weeds. However, due to its emissions to the atmosphere as well as potential toxic effect to farmers and the environment, its use in developed countries is to be phased out by the year 2005 (Giannakou et al., 2002).
Limited use is now made of chloropicrin and dibromochloropropane, which have been withdrawn from use in many countries (Whitehead, 1998). The discovery and development of nematicides that are less toxic but yet effective would be very helpful in the control of nematodes of perennial and annual crops. A range of non-fumigant, non-phytotoxic nematicides such as organophosphates and carbamates, especially oximecarbamates, have been used to control plant parasitic nematodes. Some of the current most widely used non-fumigant nematicides include aldicarb, oxamyl, carbofuran, fensulphothion, thionazin, disulphoton, phorate, ethoprophos, and fenamiphos. Ethrop and fenamiphos were primarily used to control TCN in the 1970s and 1980s. Fenamiphos is no longer available to manage TCN due to a decrease in control performance. Oxamyl is currently used in Connecticut to control G. t. tabacum and is the preferred non-fumigant nematicide (Rideout, 2000). Giannakou et al. (2002) investigated chemical alternatives to methyl bromide for the control of root knot nematodes on tomato and cucumber in the field. Experiments were performed to compare efficacy of three fumigants (chloropicrin, dazomet, and methan-sodium) and three non-fumigant nematicides (fenamiphos, oxamyl, and cadusafos) to applications of methyl bromide against nematode disease. A reduction of nematode juveniles in soil and roots was observed in all plots following soil fumigation with methyl bromide. In addition, a significant reduction of juveniles and root-galling index was observed in plots treated with metham-sodium, and dazomet, chloropicrin compared with the control and plots treated with non-fumigant nematicides. The reduction in nematode populations led to an increase in fruit yield. The data suggests that a single application of contact nematicides or fumigants other than methyl bromide cannot provide complete control of nematodes. However, application of alternative fumigants combined with root drench or broadcast application of contact nematicides can provide good nematode control (Giannakou et al., 2002). The phasing out of widely used nematicides such as methyl bromide and fenamiphos leaves growers and researchers searching for effective alternative control methods for sedentary parasitic nematodes. Improved plant resistance to parasitic nematodes is urgently required to reduce the need for nematicides.

I.2.3 Cultural methods

Cultural practices for nematode control such as crop rotation are extensively used, but are rarely effective alone and are often not preferred by farmers because the loss of large acreage of
crops for extended amounts of time, which causes a loss in profits (Urwin et al., 1997). Continuous planting of TCN-susceptible cultivars can increase TCN populations. The rotation of TCN-resistant cultivars with TCN-susceptible cultivars can significantly reduce nematode populations. Populations can be further reduced when a nematicide application is implemented with the planting of resistant cultivars (Johnson, 1999; Johnson et al., 1989). Rotations for managing cyst nematode populations should consist of three years, including one year of a non-host, one of a resistant cultivar if available, and one of a susceptible cultivar (Riggs and Schuster, 1997). It is not recommended to plant a particular resistant cultivar for several consecutive years. If a resistant cultivar is planted each year for three or more years, nematodes capable of parasitizing the resistant cultivar are likely to be selected (Riggs and Schuster, 1997). Unfortunately, crop rotation is not an option for long-term perennial crops (Whitehead, 1998).

Trap cropping is another cultural practice that may be used to reduce TCN densities. This method involves growing a particular host for enough time to allow nematode juveniles to hatch and invade plant roots. Once the plants are infested, they are uprooted and destroyed. Removing the infested plants before the juveniles can successfully mature to adult nematodes and complete their lifecycles, prevents the production of new nematode eggs, which in turn, reduces the numbers of nematodes in the soil (Whitehead, 1998). Trap cropping, through destruction of a crop prior to nematode maturation as a means to decreasing nematode populations, was demonstrated as early as 1939 in aiding in cyst nematode management (LaMondia, 1996). LaMondia (1996) grew tobacco, eastern black nightshade, and tomato for 3 to 13 wk to assess differences in invasion, development, and soil density of TCN. Population levels of TCN were reduced by 80% by destroying susceptible tobacco or eastern black nightshade crops 3 to 6 wk after planting. By destroying tomato or resistant tobacco grown for 3 to 6 wk, TCN populations were reduced up to 96%. One of the caveats of implementing trap crops is timing because the duration of trap cropping can influence nematode population dynamics. Timing must be precise, otherwise the trap crop will increase the nematode population (Riggs and Schuster, 1997; LaMondia, 1996).

Sanitation is a very important cultural practice that can reduce TCN spread. Cleaning machinery and other tools used in TCN infested fields can significantly reduce the spread of TCN to non-infested fields as well. Early root and stalk destruction can reduce the number of
generations that TCN can complete in a year by erradicating host tissue necessary for survival. Another strategy for managing nematode population levels is the manipulation of planting dates. This can be accomplished by either reducing the time for completion of the life cycle or reducing the number of cycles in a growing season (Riggs and Schuster, 1997). In addition, cyst nematodes do not hatch well in highly acid soils (pH 4) or alkaline soils (pH 8). They prefer soils with a near-neutral pH of 6. This information can be used to some advantage in managing cyst nematodes. For example, potatoes may be safest from nematode damage in an acid soil, while cabbage and beets can be planted in alkaline soil. Planting crops in soil that are not conducive to nematode hatching is ideal, however, most plants thrive at conditions at the pH that favors nematodes (Yepsen, 1984).

### I.2.4 Biological control of sedentary endoparasitic nematodes

New control strategies are needed because the Environmental Protection Agency has banned the use of most effective nematicides, resistant cultivars, lose resistance after continuous planting, and rotations and cultural practices are often not practical for growers (Riggs and Kim, 1990). Natural biological control using antagonistic organisms has been demonstrated for over a 100 yr for some plant parasitic nematodes (Whitehead, 1998). There are several fungi, bacteria, and mycorrhiza that have been shown to successfully control the root knot nematode in the field and greenhouse. These include *Pasteria penetrans*, *Paecilomyces lilacinus*, *Arthrobotys irregularis*, *Aspergillus niger*, *Verticillium dahlia*, *Bacillus penetrans*, *Streptomyces* spp., and *Glomus manihotis*. Certain species of the fungus *Verticillum* have been reported to control cyst nematodes. Large populations of cereal cyst nematode, *Heterodera avenae*, are controlled except in very droughty soils by *V. chlamydosporium* and *Nemaopthora gynophila*.

Dackman (1990) performed *in vitro* studies to examine the extent of parasitism to the potato cyst nematode (*G. rostochiensis*) by *V. suchlasporium*. The fungus was grown on corn meal agar plates until mycelia spread to the plate edges. One cyst was placed close to the advancing mycelium and incubated for 10 d at room temperature. It was reported that *Verticillum suchlasporium* infected eggs within cysts by as much as 93% when compared with untreated eggs. He concluded that *V. suchlasporium* has a high parasitic ability and that its infection ability depends upon the formation of enzymes such as chitinase and protease to aid in the penetration of the egg shell (Dackman, 1990).
A research team at the University of Arkansas discovered a fungal isolate in SCN infested field with potential for biological control of the cyst nematode. The new isolate, which was designated as “Arkansas Fungus 18 (ARF18)”, naturally suppressed SCN in the soil. In a greenhouse experiment, ARF 18 was added to the SCN or root knot nematode infested soil either in alginate pellets or as infested rice grains to determine if ARF18 reduced the amount of eggs produced by either nematode species. Eggs were counted sixty d after inoculation with ARF18. They found that the fungus readily parasitized eggs and developing young females of SCN races 2, 3, 5, 6, and 12. It was also reported that SCN populations were 90% lower 60 d after inoculations with ARF18 and that plants were larger when ARF18 was added to the SCN infested soil (Riggs and Kim, 1990). The soybean cyst nematode eggs were reduced by 98% when compared with controls and root knot nematode eggs were reduced by 50%.

There are many aspects to consider when using biocontrol to manage root-parasitic nematodes. The control achieved with these organisms can be very variable and may be limited by different soil conditions, especially soil moisture status and the presence of antagonists of the nematode (Whitehead, 1998). In addition, large quantities of inoculum may be required to establish an effective colony of the desired biocontrol agent.

I.3 PLANT DEFENSE MECHANISMS

I.3.1 Host Resistance Responses to Nematode Infection

Plants have evolved complex defense mechanisms that enable them to protect themselves against invading pathogens. Resistance to nematode infection may occur by interruption of any steps occurring during pathogenesis. Plant resistance to cyst nematodes can be divided into two categories: (1) preformed (passive) resistance, and (2) induced (active) resistance (Huang, 1998). Preformed resistance is a characteristic of uninfected plants that possess naturally occurring chemicals or physical barriers that are constitutive in the plant. Induced resistance occurs when plant compounds are synthesized in response to nematode attack. Zacheo et al. (1997) suggests that host resistance mechanisms are generally expressed after cyst nematode infection thus producing compounds postinfectionally rather than as preformed constitutive plant products. Upon recognition of the nematode, the plant initiates a cascade of signal transduction events activating defense-related genes and subsequent synthesis of proteins encoded by these genes. The activation of these genes directs the synthesis of phytoalexins, proteinase inhibitors, reactive
oxygen species, cell wall fortification materials, and pathogenesis-related (PR) proteins, which include hydrolytic enzymes such as chitinase and glucanase.

It has been suggested that phytoalexins play a significant role in nematode resistance (Veech, 1981; Rich et al., 1977). Phytoalexins are low-molecular weight antimicrobial compounds that are synthesized and accumulate in response to pathogen attack. The role of phytoalexins in plant-nematode interactions has been reviewed (Kuc, 1995, Kuc and Rush, 1985; Zacheo and Bleve-Zacheo, 1995; Veech, 1981). Glyceollins are phytoalexins that are produced in soybean. There is evidence that suggests the production of glyceollin plays a critical role in resistance of soybean to endoparasitic nematodes (Zacheo et al., 1997). Studies were performed by Huang and Barker (1991) to determine the spatial and temporal accumulation of glyceollin I in soybean roots following inoculation with H. glycines. They found that glyceollin I accumulated in tissues of a resistant soybean cultivar, ‘Centennial’, immediately adjacent to the head region of the nematode, but not in the susceptible cultivar ‘Ransom’. Glyceollin was detected 8 hr after nematode penetration and the concentration increased 24 hr after penetration. Transcription of genes encoding chalcone synthase (an enzyme involved in glyceollin synthesis), increased in resistant and susceptible soybean in response to root-knot and cyst nematode infection; the increase was greater in resistant cultivars (Huang, 1998). Phenylalanine ammonia lyase (PAL), a key regulatory enzyme in the phenylpropanoid pathway, which leads to the synthesis of lignin and other phenolic compound as well as isoflavonoid phytoalexins, was significantly induced in tomato roots 12 hr after inoculation with root-knot nematode (Brueske, 1980). The timing and intensity of phytoalexin accumulation in resistant hosts are key factors that determine the efficiency of inhibition of nematode development.

Host cell wall strengthening is an important induced resistance response that can be very effective against an invading plant parasitic nematode. Hydroxyproline-rich glycoproteins (HRGP) and lignin are major structural components of plant cell walls. Cell wall lignification and increased HRGP have been reported to be associated with enhanced disease resistance (Giebel and Stokbieka, 1974; Giebel et al., 1971). Zacheo et al. (1997) reported that the hydroxyproline content of resistant tomato plants increased by 46% following inoculation with M. incognita, while amounts remained unchanged in infected susceptible plants and in uninfected plants. It has been suggested that increased hydroxyproline and lignification reduce
cell wall permeability and degradability, thus preventing nematode invasion (Zhang, 1994). Neibel et al. (1993) performed an in vitro experiment involving the inoculation of tobacco roots with the root knot and tobacco cyst nematodes. Nematodes were allowed to parasitize the tobacco and root tissues were harvested during early infection and later stages up to four weeks after inoculation. Using RNA gel blot analysis and in situ hybridization, it was shown that the induction of extensin during early infection was very weak and transient in response to TCN. This was attributed to wounding during penetration and migration by TCN. High extensin induction was observed during the entire second larval stage of the root knot nematode and was observed in root knot nematode galls as well. They reported that extensin expression gradually decreased during the later stages of parasitism.

I.3.2 Pathogenesis-related genes

Plants produce a variety of proteins in response to pathogen attack. Collectively, these are referred to as pathogenesis-related (PR) proteins and are defined as host encoded proteins induced following pathogen invasion, stress, or elicitor treatment (Van Loon, 1997). PR proteins have been implicated in plant defense based on their ability to accumulate in the apoplasts of infected plants. It has also been demonstrated that these proteins accumulate in large quantities in uninfected plants during flowering and senescence (Lotan et al., 1989). The synthesis of PR proteins has been associated with systemic acquired resistance (SAR). This phenomenon involves the development of enhanced host resistance in distal, uninfected parts of a plant following primary challenge by a pathogen. Exogenous treatment with chemicals such as salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA), benzothiadiazole (BTH), ethephon, manganese chloride, and ionic silver can mimic pathogen-induced SAR. The accumulation of endogenous SA appears to be necessary for the expression of the SAR phenotype as well as the accumulation of PR proteins (Van Loon, 1997). The role of PR proteins in SAR has been well documented (Van Loon et al., 1998, 1994, Van Loon, 1994; Oka et al., 1997, and Ryals et al., 1996). PR proteins are usually 10 to 40 kDa in size and are categorized according to homologous amino acid sequences, serology, and enzymatic or biological activity (Van Loon et al., 1998). They can be further divided into acidic and basic forms based on signaling of SA (acidic PR proteins) or jasmonic acid (basic PR proteins) and their cellular localization. PR proteins have been recognized in eleven plant families and identified in at least nine families.
PR proteins were originally found in tobacco plants infected with *Tobacco mosaic virus* (*TMV*) and have since been discovered in both monocots and dicots (Oka et al., 1997). In addition to tobacco, they have been well characterized in tomato and *Arabidopsis* as well. It is now known that PR proteins comprise four families of chitanases (PR-3, -4, -8, and -11), one of β-1,3-endoglucanases (PR-2), the thaumatin-like proteins (PR-5), one family of proteinase inhibitors (PR-6), one specific peroxidase (PR-9), in addition to those identified as proteinases and chitanases (Huang, 1998; Van Loon, 1997). These proteins have a variety of functions that act against invading pathogens such as lysing cell walls of invading pathogens with chitanases or glucanases, liberating elicitors of defense reactions, hydrolyzing peptide phytotoxins and inactivating proteinases secreted from nematodes during feeding. The molecular events involved in the activation of defense related products and mechanisms have been reviewed in detail (Hammond-Kosak and Jones, 1997, 1996; Bent, 1996; Dangl et al., 1996; Lamb, 1994).

PR proteins have been studied in plants infected with plant parasitic nematodes (*M. javanica* and *H. avenae*). In 1997, Oka et al. investigated the induction of PR proteins in tomato plants inoculated with root-knot nematode (*M. javanica*), and barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*) infected by the cyst nematode (*H. avenae*). Tomato seedlings were grown for four weeks and the cereal plants were grown until the primary roots were approximately 1.5 cm in length. Plants were then inoculated with second stage juveniles of the respective nematode species. Proteins were extracted at 4, 7, 10, and 14 d after inoculation (DAI), fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with antisera raised against tobacco acidic glucanase and chitinase. PR proteins were not detected in the leaves or roots of tomato plants infected with the root knot nematode. In cyst nematode-infected roots of barley and wheat, PR protein induction was observed during the early stages of infection 4 DAI.

In contrast, Rahimi et al. (1993) identified and characterized β-1,3-endoglucanases and chitanases in leaves and roots of various potato cultivars infected with *G. pallida*. Potato plants were inoculated with second stage juveniles 2 weeks after planting, and proteins were extracted from 3 to 8 wk old infected plants. Using denaturing and non-denaturing PAGE techniques, it was demonstrated that the activity of β-1,3-endoglucanases increased significantly in the leaves and chitanases increased in the roots. Hammond-Kosak et al. (1989) observed similar results...
when they used denaturing SDS-PAGE to show the accumulation of PR proteins in the apoplastic spaces of potato leaves following inoculation with *G. rostochiensis*. Although these and other studies have clearly demonstrated the induction of PR proteins by plant parasitic nematodes, further investigations are needed to clarify their biological function.

**I.3.3 Isoprenoid biosynthetic pathway in plants**

In plants, the isoprenoid pathway gives rise to over 22,000 products (Bach, 1995) and there are very few physiological or biochemical processes in plants that do not require the involvement of isoprenoids. This pathway begins with the conversion of acetyl CoA to HMG CoA by HMG CoA synthase (Figure I.1). The committed step of the isoprenoid pathway is the conversion of HMG CoA (3-hydroxy-3-methylglutaryl-CoA) to mevalonic acid by the enzyme HMG CoA reductase (HMGR). In the following step, mevalonate is decarboxylated and phosphorylated to a five-carbon central molecule, isopentyl pyrophosphate (IPP) (Daraselia et al., 1996). A myriad of products are derived from this common isoprene unit, and thus the end products are referred to as isoprenoids. Isoprenoids are very important in many plant biological processes such as the membrane architecture (sterols), regulation of growth and development (cytokinin, abscisic acid, gibberellins, and brassinosteroids), defense against pathogen invasion (sesquiterpene phytoalexins), respiration (cytochrome a and ubiquinone), and prenylation of proteins involved in membrane targeting and cell cycle regulation (Kato-Emori et al., 2001; Bach, 1995). Several of the above functions occur in various tissues, cell types and organelles within the plant and the isoprenoid products responsible for those functions are produced in varying amounts at different developmental stages as well (Denbow, 1997). This pathway must be carefully regulated to support the continuous production of many isoprenoid products at various stages of growth and development.
Fig. I.1. Simplified version of the isoprenoid pathway
I.3.4 3-hydroxy-3-methylglutaryl-CoA-reductase (HMGR)

The key rate-limiting step of the isoprenoid pathway is the NADPH-dependent reduction of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) to mevalonic acid, a specific precursor of plant isoprenoid compounds, by the enzyme HMG Co-A reductase (HMGR, EC 1.1.1.34). Genes encoding HMGR were first cloned from Arabidiopsis thaliana in 1989 (Caelles et al., 1989). Since then, over 15 different plant genes have been isolated that encode HMGR. Some of these plant species include potato (S. tuberosum), tomato (L. esculentum), tobacco (N. sylvestris), rubber tree (Hevea brasiliensis), wheat (T. aestivum), cotton (Gossypium sp.), mulberry (Morus sp.), periwinkle (Catharanthus roseus), melon (Cucumis melo), radish (Raphanus sativus), and rice (Oryza sativa) (Kato-Emori et al., 2001). Unlike animal genomes where HMGR is encoded by a single gene, all plant HMGRs are encoded by a small gene family of two or more members (Ha et al., 2001). The occurrence of multiple genes encoding HMGR is a general feature of higher plants (Enjuto et al., 1995). Two genes reported in Arabidiopsis (Caelles et al., 1989), three in Hevea brasiliensis (Chye et al., 1992), three in potato (Oba et al., 1993), four in tomato (Cramer et al., 1993), at least two in wheat (Aoyagi et al., 1993), and larger gene families in pea (Monfar et al., 1990). The activity of different HMGR isoforms are regulated by different physiological and environmental stimuli, such as phytohormones, light, wounding, pathogen invasion, feedback mechanisms, and endogenous protein factors. These isoforms are differentially expressed in organs and tissues during development as well (Choi et al., 1992; Weissenborn et al., 1995; Yang et al., 1991). Studies were performed by Korth et al. (1997) to study the developmental expression of two potato isogenes, Hmg2 and Hmg3. Transcript for Hmg2 accumulated developmentally in young flowers and mature sepals and ovaries, while transcript for hmg3 accumulated in mature petals and anthers. HMGR mRNA accumulates at low levels in roots, stolons, stems, and mature leaves, whereas higher transcript levels accumulate in the meristematic regions of young tissues such as expanding leaves around the leaf apical meristem (Korth et al., 1997). It is also known that potato tuber transcript levels of Hmg isozymes can be up-regulated or down-regulated by wounding by biotic agents like bacterial and fungal pathogens, or by the application of chemicals such as arachidonic acid or methyl jasmonate. In contrast, Ha et al. (2001) reported the rice Hmg2 transcripts were constitutively expressed in all organs of the rice plant. Strong expression was also observed in
mature flowers, seedlings, and inflorescence during early development. Narita and Grussheim (1989) investigated the role of HMGR in developmental processes in tomato. They found that both HMGR activity and mRNA levels are high during early stages of fruit development such as rapid cell division and early stage cellular expansion. Reductase activity and mRNA are significantly reduced as the tomato fruit ripens. They concluded that final stages of fruit ripening are independent of reductase activity utilizing either a preexisting pool of pathway intermediates or salvage pathways in the cell (Gillaspy et al., 1993; Narita and Grussheim, 1989).

Plant HMGR genes are considerably different in terms of structure compared to animals and fungi. The HMGRs found in animals and fungi possess seven membrane-spanning domains in the N-terminal domain, whereas plant HMGRs usually have only two membrane-spanning domains (Kato-Emori et al., 2001). Regardless of the organism and number of membrane-spanning regions, HMGR is membrane bound.

I.3.5 Hmg2 promoter expression

Of particular interest is the small gene family that encodes HMGR in tomato. Of these four genes Hmg1 and Hmg2 are expressed and regulated during development in tomato (Daraselia et al., 1996). The expression of Hmg2 is tissue-specific, occurring throughout young cotyledons, trichomes, secondary root branch points, root tips, and pollen (Cramer unpublished). The isogene Hmg2, has been characterized as possessing an inducible defense-related gene promoter (Cramer et al., 1993) that is activated by wounding, fungal elicitors (arachidonic acid), and pathogens (viral, bacterial, and nematodal) but is not expressed during developmental processes such as fruit ripening and rapid cell growth (Weissenborn et al., 1995; Park et al., 1992). Yang et al. (1991) found that the levels of HMGR mRNA rapidly increased in potato tubers 30 min after wounding and was induced 8 h after being inoculated or elicited with the bacterial soft rot pathogen Erwinia carotovora subsp carotovora or with the fungal elicitor arachidonic acid. This activity continued 22 h following inoculation or elicitation. Similarly, Choi et al. (1992) found that inoculation of potato tuber tissue with either compatible or incompatible races of the fungus Phytophthora infestans or fungal arachidonic acid resulted in the strong induction of Hmg2 mRNA levels. In tobacco, the activity of Hmg2 was elevated in and around TMV (tobacco mosaic virus) lesions (Cramer and Tolin, unpublished). The expression of Hmg2 was also histochemically confirmed with the β-glucoronidase (GUS) reporter gene.
Transgenic plants expressing fusions of the Hmg2 promoter to the GUS reporter gene can be used to express the temporal and spatial regulation of Hmg2 during host-pathogen interactions (Westwood et al., 1998). The expression of these genes can be observed in specific plant tissues at the site of pathogen ingress and those surrounding tissues by the presence of blue pigment. In this reaction, the colorless substrate 5-bromo-4-chloro-3-indoyl-β D-glucoronide is catalyzed by the enzyme β-glucoronidase (Jefferson, 1987).

Induced defenses against nematodes include pathways resulting in phytoalexin biosynthesis (Gheysen and Fenoll, 2002). Certain phytoalexin compounds are synthesized from geranylpyrophosphate in the isoprenoid/mevalonate pathway. Studies were performed to determine the responsiveness of Hmg2 to the root parasites, root knot and cyst nematodes. In a preliminary experiment performed by Weissenborn and Cramer (unpublished data), transgenic tomato plants expressing Hmg2 promoter:GUS reporter fusions were germinated on agar medium and then inoculated with second stage juveniles of the root knot nematode (M. hapla and M. incognita). Forty-eight hr after inoculation, no transgene activity was observed in the root tips of uninoculated or infected seedlings. However, when feeding or galling was initiated, increased levels of GUS activity was localized to the galling tissue of transgenic plants (Cramer et al., 1993). In analogous experiments, transgenic tobacco expressing Hmg2:GUS constructs were germinated on agar and inoculated with cysts of G. t. solaneaceous. Seedlings were incubated for 24 h, harvested at different time intervals and stained for GUS activity. It was observed that Hmg2:GUS activity was localized at the nematode feeding site (Cramer, unpublished data). These preliminary experiments provided significant results confirming Hmg2 as a nematode responsive plant promoter.

In a similar study, Westwood et al. (1998) allowed a parasitic plant, Egyptian broomrape (O. aegyptiaca) to parasitize the roots of transgenic tobacco plants expressing Hmg2:GUS constructs. GUS activity was observed 24 h following penetration of the host root by the parasite’s radicle. The blue precipitate was localized in the tissues immediately surrounding the site of ingress. As the parasite further developed, the activity of GUS increased as well. GUS activity was also detected in areas where the parasite “secondary roots” contacted the host. This expression was not observed in control plants that contained a construct of the constitutive 35S
cauliflower mosaic virus promoter fused to GUS. These results confirm that \textit{Hmg2} is responsive to the parasitic angiosperm Egyptian broomrape.

\textbf{I.4 ENGINEERING RESISTANCE TO GLOBODERA}

\textbf{I.4.1 General}

It is evident that there is a need to develop more environmentally favorable strategies for the control of plant parasitic nematodes such as \textit{Globodera} spp. Over the past decade, the investigations of molecular events that mediate parasite interactions have been made possible through the availability of modern molecular tools. Several breakthroughs have been made in understanding the changes involved in interactions between the cyst nematode and its hosts (Cramer et al., 1993; Gheysen et al., 1996; Opperman and Bird, 1998; Urwin et al., 1998, 1997; and Yang et al., 1991). This knowledge supports the ability to employ new genetic engineering resistance strategies against this parasite. Engineering disease resistance to the cyst nematode requires two criteria: (1) a root parasite-responsive gene promoter such as \textit{Hmg2}, and (2) nematode-inhibitory or toxic gene product. There are several possible approaches for developing transgenic plants with enhanced nematode resistance, including anti-invasion and migration strategies, feeding cell attenuation, and anti-nematode feeding strategies (Urwin et al., 2001). The latter approach is based on utilizing proteinase inhibitors that are important elements of natural plant defense mechanisms.

\textbf{I.4.2 Plant proteinases}

The metabolism of all living cells depends on proteolysis. Proteolysis is essential in the regulation of protein synthesis and catabolism. Proteases (or peptidases) can be defined as enzymes capable of hydrolyzing proteins. Depending on the position of the peptide bond cleavage within the protein, proteases are categorized as exopeptidases or endopeptidases (proteinases). These proteolytic enzymes mediate two types of hydrolytic processes involved in important cellular functions, referred to as limited or extensive proteolysis. Limited proteolysis can be described as cleavage of one or more peptide bonds, for example, to release a biologically active protein. This process is important in the regulation of intercellular proteins and/or the recycling of amino acids for protein synthesis. In contrast, extensive proteolysis is the complete degradation of a protein through the hydrolysis of its peptide bonds (Michaud, 2000). Proteinases are classified according to their active site catalytic mechanisms, resulting in four mechanistic classes including serine-, cysteine-, aspartic-, and metallo-proteases (Laskowski and Kato, 1980).
I.4.3 Plant proteinase inhibitors (PI’s)

Proteinase inhibitors (PI’s) are polypeptides that form complexes with proteinases and inhibit their proteolytic activity (Ryan, 1990). These proteinaceous protein inhibitors appear to be widespread in nature, present in bacteria, fungi, and animal species. Plants produce an array of proteinase inhibitors that are involved in developmental regulation of endogenous proteinases and defense. There are at least 10 protease inhibitor families that have been identified in plants. The inhibitor families that have been recognized correspond to each of the four mechanistic classes of proteinases (See Ryan, 1990 and Laskowski & Kato, 1980 for detailed reviews). The majority of PI families identified are active toward serine proteases.

Proteinase inhibitors are relatively small proteins ranging from 3000 to 25,000 kD in size and are generally present in high concentrations in storage tissues (up to 10% of total protein content) (De Leo et al., 2002). The most abundant source of PI’s can be found in the seed of monocotyledonous and dicotyledonous species, although they are not limited to this location. Proteinase inhibitors are also present in fruits, tubers, roots, bulbs, leaves, and stems (See Leiner and Kakade, 1980 for detailed review). Proteinase inhibitors can also be found in multiple organs of a single plant. In some legumes (mung and field bean), high levels of PI’s are present in the seeds as well as leaves; while in tuberous plants such as white and sweet potato, PI’s are primarily in tubers and leaves; and among cereals (corn, barley, wheat, and rye), they are located mainly in the endosperm and to a lesser extent in the germ (Leiner and Kakade, 1980).

I.4.4 Physiological role of proteinase inhibitors in plants

The two physiological roles of PIs in plants are (1) to target endogenous proteases during plant growth, development, and senescence; and (2) to inhibit exogenous or foreign proteases produced by invading herbivores. The role of inhibitors of endogenous proteases has not been fully resolved. There are conflicting reports regarding PI activity during germination and maturation. In barley (Kirsi and Mikola, 1971), lettuce (Shain and Mayer, 1965), cowpea (Xavier-Filho, 1973), and mung bean (Baumgarten and Chrispeels, 1976), it was shown that inhibitors that are active against endogenous proteinases disappear during germination (Leiner and Kakade, 1980). This may reflect a role of PIs as a seed storage protein rather than regulation of inhibitory activity.

However, other scientists reported that PI’s isolated from plants are incapable of inhibiting proteases of the same plant. This phenomenon has been reported for soybean (Birk and Applebaum,
Xavier-Filho (1973) reported that there was no change in inhibitory activity in soybean seeds during germination, while Weder (1981) observed an increase in kidney bean. PIs have been reported to prevent the degradation of storage proteins in various plant species (Kirsi and Mikola, 1971) and to increase in activity during seed maturation (Kirsi, 1974).

### I.4.5 Role of proteinase inhibitors in defense mechanisms

Proteinase inhibitors are also an important aspect of natural plant defense strategies that are developmentally regulated or activated in various plants in response to wounding, insect and/or pathogen attack (Ryan, 1990). The role of proteinase inhibitors as defensive proteins against herbivorous pests such as insects and nematodes has been clearly established (see Ryan, 1973, 1981, 1989, and 1990 for detailed reviews).

The effect of PIs on insect development has been of interest since 1947; however, it was not until 1972 that Ryan and Green demonstrated a role in defense. Plants that have not been parasitized or mechanically wounded normally exhibit low levels of PIs in the leaves, but Green and Ryan (1972) observed a high level of PI induction in the leaves of potato and tomato plants following predation by the Colorado potato beetle (*Leptinotarsa decemlineata*) or mechanical damage. They found that PIs accumulated not only at the site of damage, but also in adjacent plant tissues. This inhibitor response was believed to be mediated by a wound hormone, or protease inhibitor-inducing factor (PIIF), which triggers the synthesis of PI’s systemically via the phloem following leaf injury (Leiner and Kakade, 1980). In young solanaceous plants, the accumulation of serine PIs begins 4 to 5 hr after a single wound and can be significantly magnified by a second wound 15 and 72 hr after the first (Jongsma and Bolter, 1997).

The activity of PIs is due to their ability to form stable complexes with target proteases thereby blocking, altering, or preventing access to the enzyme catalytic site. The inhibitory activity of PIs against extracellular proteases such as those present in the midguts of herbivores, constitutes the utilization of PIs as potential tools in broad-based resistance strategies against various pests and pathogens. The findings by Ryan and his colleagues (Green and Ryan, 1972) afforded the opportunity for subsequent exploitation of PI compounds in crop protection by genetic manipulation. Inhibitors can be produced by the activation of a single gene and are primary gene products, as a result several transgenic plants expressing PIs have been generated in the last 15 yr and tested for enhanced
defensive capacities with particular efficacy against pest insects (De Leo et al., 2002). The major digestive proteolytic enzymes utilized by herbivores correspond to the four classes of proteases (serine-, cysteine-, aspartic-, and metallo-proteases). Trypsin and chymotrypsin are serine proteinases that are major enzymes responsible for the initial digestion of protein in the gut of nematodes, chewing and sucking insects, animals, and humans (Ryan, 1990). Several PIs are produced by plants that inhibit trypsin and chymotrypsin activity. Serine PIs are the most widely studied protease inhibitors with respect to plant defense. The activities of these enzymes have been reviewed in detail by Ryan (1990).

I.4.6 Effects of proteinase inhibitors on insect feeding

Proteinase inhibitor proteins affect the growth and development of pests by attenuating enzyme function necessary for metabolic processes such as protein turnover, or proteolytic digestion required for nutrient assimilation (Koiwa et al., 2000). These proteins have been shown in various feeding studies to cause detrimental effects on the digestive systems of insect herbivores and mammals by reducing the digestibility and nutritional quality of leaves. (Ryan, 1990; Johnson et al., 1989b). Studies have been performed that firmly support that the expression of various trypsin inhibitor genes in transgenic plants confers resistance to insect pests (Ryan, 1990).

Transgenic strategies have been used to demonstrate the ability of PIs to confer enhanced insect resistance. The first gene of plant origin to be transferred into another plant species to produce enhanced resistance to the tobacco budworm (*Heliothis virescens*) was isolated from cowpea by Hilder et al. (1987). This gene encoded a Bowman-Birk type serine PI that contained two inhibitory reactive sites against bovine trypsin (CpTI). Constructs containing a full-length CpTI cDNA clone under the expression of the 35S Cauliflower Mosaic Virus (CaMV) promoter were transformed into tobacco. Several CpTI transgenic plants were generated and they expressed the PI at various levels ranging from as little as no detectable protein to the highest levels of 1% of total soluble protein. Utilizing the latter in bioassays with first instar larvae, they found that CpTI-expressing plants reduced damage up to 50% compared to untransformed plants and reduced instar survival and biomass again by as much as 50%. Larvae that fed on CpTI-expressing plants caused limited damage and usually died or failed to develop compared to those on control plants (Gatehouse and Gatehouse, 1998).

Feeding studies have subsequently been used to demonstrate the effectiveness of recombinant CpTI against many other Lepidopteran pests including the corn earworm (*Heliothis zea*), Mediterranean climbing cutworm (*Spodoptera littoralis*), and tobacco hornworm (*Manduca sexta*)
(Gatehouse and Gatehouse, 1998). In addition to tobacco, the gene encoding CpTI has been transferred into a number of important crops such as potato, oilseed rape, rice, and strawberry (Gatehouse and Gatehouse, 1998).

Not only have the genes encoding PIs from cowpea been shown to confer resistance expressed in transgenic crop plants, but the tomato inhibitor I and II genes, when expressed in solanaceous plant species were also shown to confer insect resistance (Gatehouse and Gatehouse, 1998). Tomato has small gene families which encode for two powerful classes of serine proteinases, proteinase inhibitor I and II. Proteinase inhibitor I is an oligomeric protein containing subunits of Mr 8,100; the major form is a tetramer with a Mr of 39,000 (Michaud, 2000). PI-I has a low cysteine content and is a strong inhibitor of chymotrypsin and a weak inhibitor of trypsin. PI-II is a dimer with a Mr of 12,300 that contains two reactive sites and inhibits both trypsin and chymotrypsin (Johnson et al., 1989). Walker-Simmons and Ryan (1977) showed that PI-I and II were synthesized as preproteins of Mr 2000-3000 larger than the mature proteins in cytoplasm, and mature proteins were accumulated in the central vacuoles of leaf cells. Genes and cDNA’s that encode for tomato PI-I and II have been cloned and characterized.

Johnson et al. (1989) used the genes encoding tomato PI-I and II to assess their potential in increasing the natural defenses of crop plants against tobacco hornworm predation. Gene constructs containing 35S:tomato PI-I and 35S: tomato PI-II were stably introduced into tobacco. Tobacco hornworm larvae were allowed to feed on transgenic and control plants for several d and larval weights were recorded during the experiments. Leaves expressing PI-II proteins at 50 µg/g of tissue moderately affected the larval growth rate, whereas levels above 100 µg/g or more of tissue caused severe inhibition of growth to tobacco hornworm larvae when compared to larvae feeding on untransformed plants (Ryan, 1990). Leaves expressing PI-I did not significantly affect the larval growth, thus indicating that trypsin inhibitor activity plays a more significant role in digestion by tobacco hornworms than chymotrypsin. Similarly, the level of resistance of transgenic tobacco and potato plants expressing three soybean trypsin inhibitors (Kti3, C-II, and PI-IV) were tested against the Mediterranean climbing cutworm in an insect feeding study (Marchetti et al., 2000). The level of insect resistance was higher in Kti3-expressing tobacco transformants than in C-II and PI-IV transgenic plants and led to larval death in as little as 16 hr and in no more than 48 hr of feeding. In transgenic potatoes, the larval mortality was significantly less than those that fed on tobacco when compared to
the larvae feeding on control plants. Larval weights of instars that fed on control plants for five consecutive days were four times greater than on transgenic potatoes. In plants expressing the highest levels of Kti3, C-II, or PI-IV inhibitors, a 50% reduction in larval weight was observed as well as a reduction in leaf damage. The number of insect resistance studies based on inhibitor gene expression has grown significantly over the years and has utilized additional PIs and crops (Table 5).

Based on the interactions of PI-I and II and the enhanced resistance demonstrated by their activities in these studies, PI-I and II are excellent candidates for testing the effectiveness of this strategy against nematode parasitism.

### 1.4.6 Effects of proteinase inhibitors on plant parasitic nematode feeding

Several possible approaches exist for developing transgenic plants with improved nematode resistance. When considering these approaches, one can target the general life cycle of an endoparasitic plant nematode where there are many stages to implement transgenic intervention. The nematode resistance strategy can either directly affect the nematode or attenuate the plant cells with which they interact. These include anti-invasion and migration, feeding cell attenuation, or anti-nematode feeding strategies (Atkinson, 1996). Overexpression of PIs in transgenic plants to suppress digestive proteases represents a broad-spectrum anti-feeding resistance strategy against chewing and sucking insects, and plant parasitic nematodes. This strategy is based on a common utilization of digestive proteolytic enzymes for nutrient assimilation among these parasites.

There are several studies that demonstrate the effectiveness of recombinant PIs towards growth, development, sexual fate, and fecundity of both cyst and root knot nematodes (Urwin et al., 1997; Lilley et al., 1996; Urwin et al., 1995; Atkinson, 1996; Hepher and Atkinson, 1992). The serine proteinase inhibitor CpTI, cysteine proteinase inhibitor isolated from rice seed, oryzacystatin (Oc-I), and an improved engineered oryzacystatin (Oc-IΔ86) are the primary PIs used in these studies. For example, Urwin et al. (1997) constitutively expressed Oc-IΔ86 in Arabidopsis to engineer resistance to both the cyst (H. schachtii) and root knot (M. incognita) nematodes. Plants were allowed to grow for four weeks and were inoculated with approximately 100 second-stage juveniles of either nematode species. Samples were harvested at 3, 4, 5, 6, 7, and 8 wk post-inoculation. At these time points, the growth and developmental stages of nematodes were observed. The growth and development of cyst nematodes on Arabidopsis expressing Oc-IΔ86 differed from the pattern described for the controls. Nematode size was significantly reduced
compared with the controls at all time points except weeks 5 and 8. The reduction in size of root knot nematodes was similar to the cyst nematodes. The fecundity of both nematode species was profoundly affected as well. Depending on the nematode species, approximately 300 to 600 eggs are usually produced by females. In this study, the females did not achieve the minimum size necessary for egg production. Furthermore, the smallest cyst (outline area of 0.075mm$^2$) recovered from the soil had an egg content of 32 eggs, which correlates to the lower size limit of cysts with eggs that naturally occur (Urwin et al., 1997). They concluded that the growth and normal development of females of both species was similarly inhibited by expression of Oc-I$\Delta$86.

In recent preliminary experiments performed by Zhang, Eisenback, Cramer, Radin, and Ryan (unpublished data), the effect of PI expression on formation of root galls, nematode development, and egg mass production of the root knot nematode were tested in vitro and in vivo. Untransformed tomato seedlings and those expressing 35S:PI-I and 35S:PI-II gene constructs were germinated on agar medium and inoculated with 1000 eggs of $M. \text{hapla}$. There was a significant reduction in the number of galls present on the transgenic plants compared to the untransformed control plants when these plants were harvested 15 d after inoculation. The effect of the PI’s on nematode development were also determined by inoculating three week old seedlings (grown on agar medium) with 1000 eggs per seedling. The roots of these plants were harvested 20 and 35 d after inoculation. When nematode developmental stages were assessed 20 d following inoculation, only 35% of the nematodes present on PI-I transgenic plants and 51% of nematodes on PI-II transgenic plants were able to successfully develop into 3$^{rd}$ and 4$^{th}$ stage juveniles when compared to 77% of the nematodes on control plants. Thirty-five days after inoculation, approximately 50% of nematodes on non-transgenic plants developed into adult females. In comparison, 9.5% developed to adult females on PI-I expressing plants and 11.6% progressed adult females on PI-II-expressing plants. This study showed that both PI-I and PI-II functioned to inhibit growth and development of root knot and cyst nematodes.

The impact of the PI-I and PI-II transgenes on production of egg masses was also analyzed in soil-grown plants inoculated in the greenhouse (Zhang, 1994). Untransformed tomato seedlings and those expressing PI-I and PI-II were grown in the greenhouse then inoculated with 1000 eggs. The roots of these plants were harvested and the egg masses were measured at 8 and 10 wk intervals following inoculation. After 8 wk, egg masses were significantly lower on transgenic
plants as compared to egg masses on untransformed tomato plants. Compared to the untransformed plants, there was a decrease of approximately 60% to 80% in the number of eggs found on 35S:PI-I and 35S:PI-II transgenic plants, respectively. However, 10 wk after inoculation, the egg masses on all transgenic plants increased and there were no significant differences between these plants and the untransformed controls. These early results indicated that PIs are effective nematocidal gene products, with PI-I more effective than PI-II. The fact that PIs provided strong but transient resistance against *M. hapla* indicates that the loss of resistance could be attributed to the 35S constitutive promoter used in this study. Consistent with the 35S being a key factor in the short-term nature of this PI-based delay in nematode disease development, Goddjin et al. (1993) showed that nematodes suppressed the expression of 35S:GUS in nematode feeding structures within days after nematode infection. Thus, the use of PI-I and II for effective nematode control will require use of different promoters.

Urwin et al. (1998) explored a novel approach to enhance nematode resistance and gene durability by stacking plant defense genes. They reported the co-delivery of two distinct PI’s in *Arabidiopsis* to examine resistance against cyst and root knot nematodes. In this study, the principal approach was to use peptide linkers to translate the coding regions of the PIs Oc-IΔ86 and CpTI, as a single fusion protein. Previous studies have shown that cystatin suppresses growth of developing females (Urwin et al., 1997), and CpTI influences sexual fate (Atkinson, 1996). Unfavorable environmental conditions and poor nutrient availability can stimulate plant parasitic nematodes, which are parthenogenic in nature, to revert from female to male until conditions are conducive and can support reproduction and growth. In addition to the cysteine and serine PIs, two distinct peptide linkers were used to determine the PI delivery mode. The first linker is part of a plant metallothionin-like protein (PsMTa) and is susceptible to *in planta* cleavage, while the other linker was derived from the fungal enzyme galactose oxidase (GO) and is refractory to *in planta* cleavage. The genes encoding the PIs were joined as translational fusions by one of two peptide linkers producing four individual gene constructs (cystatin, CpTI, cystatin:GO:CpTI, and cystatin:PsMta:CpTI).

To determine if the PIs were ingested by the nematodes, proteins were extracted from females that fed on the transgenic *Arabidiopsis* roots, fractionated on SDS-PAGE gel, then cross-reacted with either a cystatin or CpTI antibodies. Western analysis with both antibodies revealed
that root knot nematode females had ingested Oc-I\(\Delta86\) or CpTI when recovered from plants expressing single PI constructs. The intact products of Oc-I\(\Delta86\):GO:CpTI were detected by both antibodies, while products of Oc-I\(\Delta86\):PsMTa:CpTI did not show detectable levels. They concluded that both products of dual constructs should have been detected given that both inhibitors were present in the host plant, however, fractionated plant material demonstrated that products of Oc-I\(\Delta86\):PsMTa:CpTI were membrane associated but were not integral membrane proteins (Urwin et al., 1998).

The effect of PI constructs on sexual fate and ratio was determined by the percentage of nematode stages (including adult females) was observed at 20 and 45 d following inoculation with either nematode species on control and transgenic plants. At 20 d post-inoculation, CpTI expressing single and dual constructs showed a lower proportion (40% reduction) of saccate females of both cyst and root knot nematodes than found on control plants. However, this was not observed on transgenic plants expressing Oc-I\(\Delta86\) alone. This phenomenon was also observed at 45 d following inoculation as well. The ratio of individuals within the two groups was such that plants expressing Oc-I\(\Delta86\) alone or Oc-I\(\Delta86\):GO:CpTI possessed smaller saccate females than those on control plants. Plants expressing CpTI alone had some affect on the developing females, whereas plants expressing Oc-I\(\Delta86\):PsMTa:CpTI had no effect on nematode size. These results suggest that PsMTa-linked CpTI affected sexual development of cyst nematodes. These results were consistent with previous studies that demonstrated enhanced resistance of plants expressing CpTI to cyst nematodes (Atkinson et al., 1996). These studies also demonstrate that the cystatin component from the PsMTa construct had no effect, indicating that ingestion is necessary for effectiveness. The delivery of dual inhibitors linked by GO linker showed a clear additive effect compared to delivery of a single inhibitor. This strategy could have obvious advantages for stacking distinct gene products active against various pests (Vrain, 2000).

I.5 SIGNIFICANCE AND OBJECTIVES OF RESEARCH

The overall goal of this project is to enhance resistance to the tobacco cyst nematode with the use of the tomato proteinase inhibitor I gene via genetic engineering. Previous studies clearly demonstrated the effectiveness of PIs against sedentary endoparasitic nematodes. In addition to the use of inhibitor genes to increase nematode resistance, the choice of promoter utilized to drive the expression of such genes influences the effectiveness of this strategy as well. Studies
performed by Zhang et al. (unpublished) directly suggests that the strategy of inserting a single nematode inhibitory protein such as PI-I, fused to the inducible nematode responsive promoter, Hmg2, could effectively confer, improve, and extend resistance to the sedentary endoparasitic nematodes of the genera *Heterodera*, *Globodera*, and *Meloidogyne* than resistance achieved through the use of a constitutive promoter. The specific objectives of this research were to (1) amplify tomato PI-I gene via PCR amplification; (2) generate gene constructs containing the Hmg2 promoter fused to full length and truncated PI-I cDNA genes; (3) stably integrate these constructs into tobacco plants via *Agrobacterium*-mediated transformation; (4) characterize transgenic plants by determining genomic insertion and copy number, transcript and protein production; and to (4) test host-parasite interactions via greenhouse inoculation studies.

### I.6 OROBANCHE PLANT DISEASE

#### 1.6.1 Economic Importance, Distribution, and Host Range

Egyptian broomrape (*Orobanche aegyptiaca* Pers.) is a parasitic angiosperm that attacks the roots of many economically important dicotyledoneous plants including *Solanaceae*, *Fabaceae*, *Cruciferae*, *Compositae*, and *Umbelliferae* (Parker and Riches, 1993). This chlorophyll-lacking holoparasite draws nutrients, photosynthates, and water from the host plants, creating such a strong osmotic gradient that the host may have difficulty maintaining water in the shoots. Partitioning nutrients in such a manner causes severe damage to the host significantly reducing crop yield quantity and quality (Goldwasser et al., 2001). Plants parasitized by *Orobanche* spp. not only appear stunted but also experience potassium and nitrogen depletion. *Orobanche* spp. are considered among the most damaging parasites, causing significant crop losses particularly in southern Europe, Central Asia, and the Mediterranean (Joel, 2000; Parker and Riches, 1993; Musselman, 1980). It is recorded among the most serious weed species in not less than 14 countries, which include Afghanistan, Cuba, Nepal, Pakistan, India, Saudia Arabia, Turkey, and Uganda (Acharya et al., 2002). For example, Parker and Riches (1993) state that areas of crops affected by *O. aegyptiaca* include over 50,000 ha of cucurbits in China, 35,000 hectares (ha) of tomatoes and tobacco in Greece, 4,000 ha of these two crops in Cuba, 1,500 ha of tobacco in Bulgaria, and 1,000 ha of tomatoes in Ethiopia.
I.6.2 Orobanche Life Cycle

Like plant parasitic nematodes, Egyptian broomrape also requires the establishment of a close relationship with its host plant, and has evolved its own complex mechanisms of communication and physiological interactions with the host. Egyptian broomrape has two primary life phases: (a) the independent life phase, and (b) the parasitic life phase (Joel, 2000). The independent life phase begins with seed preconditioning and germination. Specific chemical germination stimulants are exuded from the roots of a compatible host and only seeds within the host root rhizosphere will germinate (Goldwasser and Yoder, 2001; Parker and Riches, 1993). Germination is one of the most critical and vulnerable stages of the parasitic life cycle because the seeds are extremely small (0.3 mm in length) possessing limited food reserves and must attach to a host root to ensure its survival. Once the seed germinates, the radicle (generally less than 1mm in length) emerges and contacts the host. From the tip of the radicle a multicellular organ develops called the haustorium. Once the haustorium develops, the independent life phase is terminated and the parasitic phase is initiated. The haustorium is an extremely important organ because it acts as a physiological bridge conducting the transfer of nutrients between host and parasite (Musselman, 1980). This organ adheres to the roots and penetrates the epidermis and cortex of host root tissues by both mechanical force and enzymatic digestion of the middle lamella utilizing two enzymes, pectin methylesterase (PME) and polygacturonase (PGA) (Losner-Goshen et al., 1998). In response to Orobanche parasitism, it is believed that PME initiates the degradation of the middle lamella, thereby loosening host cell wall adhesions and PGA completes pectin digestion allowing the intrusive parasitic cells to penetrate host cells intercellularly without rupturing them (Griffits, 2001; Losner-Goshen et al., 1998; Joel and Losner-Goshen, 1994). Vascular connections are formed between the two angiosperms and the parasite extracts water and nutrients from the host xylem and phloem. Broomrape sieve elements share plasmodesmatal connections with sieve elements of the host (Dörr, 1996), and the parasite essentially becomes a metabolic sink for carbohydrates produced by the host (Musselman, 1980). As a result, the broomrape radicle, which remains outside the host root, increases in size as it develops into a bulbous mass of tissue referred to as a tubercle. The final event in the parasitic life cycle is the development of a floral meristem into a floral spike, which emerges from the soil beside of the parasitized host. This spike may produce as many as 250,000 new seeds that can re-infest the field.
1.7. *OROBANCHE* CONTROL METHODS

Overall, broomrape management is difficult due to the close association that the parasite has with its host. The implementation of any post emergent control methods is negatively affected by the timing of parasitism. For example, it is difficult to detect the parasite early in the season because the majority of the parasitic lifecycle occurs in the rhizosphere, and damage to the host has already occurred prior to the parasite’s floral meristem emerging from the soil. The current control methods of Egyptian broomrape include mechanical, cultural, biological, or chemical means (Musselmann, 1980). However, no control measure, that is both effective and economically feasible, has been found for *Orobanche* spp. (Muller-Stover et al., 2002).

1.7.1 Mechanical Methods

Mechanical methods such as hand-pulling are the least effective of the current control measures because not only is it very time-consuming, but also can be detrimental to host that is being parasitized. *Orobanche* forms strong vascular connections with its host plant, which makes it difficult to eradicate the parasite from the soil without disturbing the host root system or completely uprooting the crop (Parker and Riches, 1993). The parasitic lifecycle occurs primarily subterranean, thus damage has occurred to the crop plant prior to the emergence of the parasite’s floral shoot. Hand-pulling is only beneficial to the small farmer with light infestations and/or when sufficient labor is available to employ this practice in reducing the spread of seed.

1.7.2 Cultural Methods

There are a number of cultural practices implemented to decrease *Orobanche* seed bank in infested fields. The methods utilized most frequently include crop rotation using catch or trap crops, and solarization. Complete control of broomrape cannot be achieved through a single cultural method, so integrated management is gaining importance and the use of catch crops is one of the major components (Acharya et al., 2002). Catch crops are normal hosts that stimulate the parasite to germinate and develop, but are destroyed parasite prior to seed production. Traps crops are hosts that also stimulate parasite germination, but do not permit attachment or parasite development (Parker and Riches, 1993).

Soil solarization is a non-conventional method used to reduce *Orobanche* soil seed bank. Infested fields are covered with plastic sheets during warm summer months thereby increasing the soil temperatures to levels lethal to *Orobanche* seeds or other soil-borne pathogens. Requirements
for successful solarization include moist soil, high air temperatures and solar radiation, and adequate length of exposure (Parker and Riches, 1993). It has been well established that long periods of solarization, 6 to 7 wk, are needed to control *Orobanche*, however, this is not feasible in many agricultural systems. Recent studies show that amending the soil with chicken manure can help reduce solarization time. In Lebanon, chicken manure is widely used as an organic fertilizer and significantly reduced *O. ramose* in infested potato fields (Haidar and Sidahmed, 2000). Haidar and Sidahmed (2000) investigated the effects of solarization with and without chicken manure on cabbage fields infested with *O. crenata* at soil depths ranging from 0 to 10 cm. Studies were conducted for a period of 0 to 6 wk. They found that solarization treatments alone killed *Orobanche* seeds at 0 cm soil depth only. The combination of solarization and chicken manure killed *Orobanche* at all soil depths, effectively from 2 to 6 wk of treatment and cabbage yields were increased.

1.7.3 Biological Control Methods

The use of microbial herbicides or mycoherbicides to control *Orobanche* has greater opportunity for application than non-selective herbicides in intensive agriculture and has gained much attention in recent years (Muller-Scharer et al., 2000). The fungus, *Fusarium oxysporum* Schlecht f. sp. *orthoceras* (Appel. and Wollenw.), is a natural pathogen of *Orobanche* species, that attacks underground plant parts such as seeds, tubercules, and shoots. Fungal spores incorporated in the soil before planting resulted in significant control of *O. cumama* under field conditions. Despite the level of *Orobanche* control that is achieved by inoculating with *F. oxysporum*, more information is needed to understand the interaction between *Orobanche* and *F. oxysporum*. The availability of storable formulations has been limited in the past. However, new formulations techniques have resulted in the development of an effective formulation that can be stored under cold conditions for a year without unacceptable loss of viability (Muller-Scharer et al., 2000).

1.7.4 Chemical Methods

With respect to chemical control, the application of herbicides to the subterranean parasite is difficult and there is a general lack of selectivity between the host and parasite. However, the most effective means of broomrape control has been achieved through the use of chemical fumigants such as methyl bromide, metham sodium, and dazomet (Foy et al., 1989). Soil fumigants have the ability to permeate and kill the seeds prior to germination, but they are highly
volatile, toxic compounds that are dangerous to the environment and yield inconsistent results (Goldwasser et al., 2001). There are only few reports of post-emergence and soil-residual herbicides effectively controlling broomrape. It has been reported that glyphosate applied postemergence at 60 g/ha controlled broomrape in crops such as carrot (*Daucus carota*), common vetch (*Vicia sativa*), celery (*Apium graveolens*), and faba bean (*Vicia faba*) (Aly et al., 2001). Soil-applied sulfonylurea and imidazolinone herbicides have been reported to show activity in controlling *Orobanche*, but their selectivity vary among crops (Aly et al., 2001; Hershenhorn et al., 1998).

In a greenhouse study, Hershenhorn et al. (1998) investigated the effectiveness of several sulfonylurea herbicides for controlling the parasite on tomato grown in pots. They reported that herbicides were effective when applied through chemigation (irrigation water) to the tomato root but not to the crop foliage. Chemigation has limited use due to the risk of groundwater contamination; consequently, this method is only registered in Israel for specific pesticides applied to cotton and orchard fields that are not in the vicinity of water supplies for human or animal consumption (Hershenhorn et al., 1998).

Field experiments were performed by Hershenhorn et al. (1998) to study the effectiveness of chlorosulfuron and triasulfuron applied via chemigation for the control of *O. aegyptiaca* on tomato. Three split applications of chlorosulfuron (2.5 g a.i./ha) and triasulfuron (7.5 g a.i/ ha) applied 10 to 14 d apart, consistently decreased *O. aegyptiaca* shoot emergence by 90% and 80%, respectively. Crop yields were increased from 25% to 47% in chlorosulfuron treated crops and by 30% in triasulfuron treated crops as compared with the infested untreated control. Slightly higher control was observed when the repeated applications of the same herbicides were applied at half rates than a single application at a double rate. Slight phytotoxicity was observed on tomato treated with chlorosulfuron, however, it was no longer observed two weeks following herbicide treatment.

Suicidal germination using a chemical germination stimulant has become an attractive control strategy to reduce disease pressure in infested fields. It is critical for the parasite to receive germination signals exuded from the host roots to stimulate germination. Suicidal germination entails introducing a germination stimulant agent (strigol analogues) into the soil prior to sowing to induce the parasite seeds to germinate in the absence of a host (Nefkens et al., 1997).
However, this has not been demonstrated to be a cost-effective strategy for the control of *Orobanche* in the field.

1.7.5 Herbicide Resistant Crops

With the loss of methyl bromide, which is being phased out due to international accords, there are few effective alternatives to combat *Orobanche* species other than suffering severe yield reductions or the discontinuation of cultivating high value crops. The use of herbicide resistant cultivars would provide a useful alternative method to control broomrape because it would allow the herbicide to be translocated through the host to the parasite (Joel et al., 1995). Two types of resistance to herbicides can be genetically engineered into plants. Either the target site of the herbicide can be modified and to the systemic translocation of unmetabolized herbicide from the host directly to the parasite, or can be engineered to degrade the herbicide to non-toxic products.

Joel et al. (1995) initiated this research by testing transgenic crops expressing genes for resistance to the four herbicides glufosinate, chlorosulfuron, glyphosate, and asulam. The application of chlorosulfuron, glyphosate, and asulam to their respective herbicide resistant transgenic plants effectively controlled *O. aegyptiaca*. A single application of chlorosulfuron on chlorosulfuron-resistant tobacco plants resulted in 95% normal growth and flowering of the host. Glyphosate-resistant transgenic plants also developed normally after glyphosate was applied and were free of parasites. Foliar application of asulam reduced parasitization of a single asulam-resistant host plant by 70% (Joel et al., 1995). Unlike chlorosulfuron-, glyphosate-, and asulam-herbicide resistant plants, the application of glufosinate to glufosinate-resistant tomato plants failed to control *Orobanche* due to degradation of the herbicide. Effectiveness of the herbicide-resistant crops was attributed to the movement of the herbicides through the host plant conductive tissue without being degraded prior to exposure to the parasite. In a similar study, Surov et al. (1998) found that that the foliar application of asulam on asulam-resistant transgenic potatoes completely eradicated the parasite. These results indicate that controlling young parasites on the roots of host plants is an effective strategy for limiting damage due to parasitic weeds.

1.7.6 *Orobanche*-Resistant Cultivars

Genetic resistance is considered a major component of integrated pest management, however, little is known about genetic resistance against parasitic weeds (Goldwasser and Yoder, 2001). Genetic resistance to *Orobanche* can be attained by different mechanisms including low
germination stimulant production by the host roots (Alonso, 1998; Aalders and Pieters, 1986), physical barriers such as lignification of the host root blocking further penetration by the parasite (Antonova, 1978), or reduced host root systems (Nassib et al., 1984). By histological observations of the host-parasite interface, Labrousse et al. (2001) demonstrated several resistance mechanisms in resistant sunflower species (*Helianthus* spp.) to *O. cumana* that include cell wall deposition, vessel occlusion, and broomrape cellular disorganization. In addition, a rapid cell necrosis was observed during early stages of parasite development. Goldwasser et al. (1997) also characterized resistance in vetch (*Vicia sativa*) to *O. aegyptiaca* by the presence of necrotic lesions in the region of parasite attachment. Chemical or mechanical barriers present at the endodermis have been attributed for preventing the haustorium from completely penetrating the root (Labrousse et al., 2001). This impassable encapsidation layer is accompanied by the secretion of an unidentified material produced at the host-parasite interface (Goldwasser et al., 2000).

Although these mechanisms of resistance have been observed in several host species (sunflower, faba bean, vetch, tomato, tobacco, eggplant, curcurbits, rapeseed, hemp, and carrot) (Parker and Riches, 1993), further investigations are needed to understand the genetics and biochemical events involved in *Orobanche* resistance. There are very few *O. aegyptiaca* resistant cultivars currently available, and these have not been well characterized.

1.8. ENGINEERING RESISTANCE TO *OROBANCHE*

1.8.1. General

There is a clear need for the development of new control strategies for *Orobanche* spp. Plants resist diseases caused by plant pathogens by producing effective local resistance responses that would inhibit disease progression. Recruiting this mechanism to create hosts resistant to *Orobanche* would be a valuable contribution.

The local resistance response, or hypersensitive response, is induced upon interactions between the products of plant resistance (R) genes and the corresponding pathogen avirulence (avr) gene products. Several breakthroughs have been made in understanding R gene structure and function and the interactions that are involved in pathogen recognition (Dangl and Jones, 2001)(See Hamond-Kosak and Jones, 1997 for detailed review). In recent years, gene-for-gene resistance strategies have gained attention for their their potential to controlling diseases caused by plant pathogens (Jones et al., 1994). Research efforts are underway in an effort to discover
Orobanche-specific toxins, however, there continues to be a need for new resistance strategies. The use of genetic engineering may facilitate these efforts. Engineering disease resistance to Orobanche requires two criteria: (1) two Orobanche-responsive gene promoters, such as Hmg2, and (2) a novel R-avr gene pair that produces a HR upon co-expression.

1.8.2. Orobanche-responsive Promoters

The utilization of R gene-mediated resistance requires two promoters to drive the expression of the R and avr gene pair products. The success of this strategy relies on two important caveats: (1) the promoters utilized must be induced by Orobanche parasitism, and (2) their expression patterns cannot otherwise occur within the same cells during normal plant development. The impact of overlapping expression patterns would cause cell death in the areas of promoter:R-gene-avr gene co-expression. Westwood et al. (1998) demonstrated that Hmg2 is activated in response to Orobanche development and parasitism. Hmg2 expression was observed by histochemical staining of GUS activity in transgenic tobacco plants expressing Hmg2:GUS constructs. GUS activity was localized in the areas surrounding sites of ingress and secondary root attachment. If the Hmg2 promoter is utilized to drive expression of an R-gene, a second Orobanche-responsive promoter must be identified whose developmental expression pattern is distinct from Hmg2. We have explored the potential of using alderthe bean CHS8 promoter.

Chalcone synthase (CHS), catalyzes the stepwise condensation of three acetyl units from malonyl-CoA with 4-coumaroyl-CoA to yield naringenin chalcone, which is the first committed step in the branch pathway of phenylpropanoid metabolism specific for flavanoid biosynthesis (Schmid et al., 1990). The phenylpropanoid pathway gives rise to secondary metabolites such as flavanoid pigments (anthocyanins) and UV-protectants, which are ubiquitous in higher plants. In leguminous plants, CHS8 is involved in the synthesis of isoflavanoid-derived phytoalexins that are induced in response to wounding, infection, and exposure of cells to biotic or abiotic elicitors (Lindsay et al., 2002; Ryder et al., 1987).

It is known that CHS is encoded by a small gene family in many plant species. Of particular interest is the gene family in French bean (Phaseolus vulgaris). It has been demonstrated that the haploid genome of bean contains as many as eight CHS genes that are tightly clustered (Durbin et al., 2000; Ryder et al., 1987)
CHS mRNA and enzyme levels are highly regulated during plant development associated with the tissue- and cell type-specific accumulation of flavanoid pigments and in response to environmental stimuli (Schmid et al., 1990). Schmid et al. (1990) examined the developmental and environmental regulation of a 1.4 kb promoter fragment of the bean CHS gene. By histochemical staining, GUS activity was observed in transgenic tobacco expressing CHS8:GUS constructs. The CHS promoter was highly expressed in the root apical meristems and petals, while low expression was observed in other floral organs, mature leaves, and stems. The promoter was also active in lateral root initiation sites, greening cotyledons, and primary leaves, but not in shoot apical meristems (Schmid et al., 1990).

CHS8 promoter is strongly induced in response to pathogen attack and chemical treatment. Doerner et al. (1990) inoculated leaves of CHS8:GUS transgenic tobacco with an isolate of the phytopathogenic bacterium, *Pseudomonas syringae*, that causes HR on tobacco. Intense GUS activity was observed at the periphery of the HR lesion 72 h after inoculation. Tobacco leaves were infiltrated with a heavy metal salt, HgCl$_2$, incubated for 8 h, and assayed for GUS activity. Chemical treatment resulted in strong induction of CHS8:GUS transgene in the area of application.

### 1.9. GENE FOR GENE RESISTANCE

#### 1.9.1. General

Currently, there are no broomrape-specific inhibitory gene products available. Considering the close association between host and parasite, and apparent reliance of the parasite on living host cells, we believe that necrosis in the area of haustorial penetration will effectively limit further parasite development. Therefore, our approach to genetically engineer resistance to broomrape will be based on creating a novel gene-for-gene resistance in response to parasitism.

Plant defenses are often activated by specific interactions between the product of a disease resistance (*R*) gene in the plant and the product of a corresponding avirulence (*Avr*) gene of the pathogen (Jones et al., 1994). Several *R* genes have been cloned and characterized at the molecular level and are specific for viral, bacterial, nematodal, and fungal pathogens and aphids (Dangl and Jones, 2001; Ellis et al., 2000; Martin, 1999; Hammond-Kosak and Jones, 1997; Bent, 1996; Ganal et al., 1995; Staskawicz et al., 1995; Martin et al., 1993; Mindrinos et al., 1994; Jones et al., 1994; Whitham et al., 1994). There are five classes of *R* genes that are grouped according to their structural characteristics and predicted protein products (Fig. 1.1). It is thought that *R* genes
encode proteins that activate signaling cascades that coordinate the initial plant defense response to impair pathogen ingress (Hammond-Kosak and Jones, 1997). In a compatible host-pathogen interaction, a strong resistance response is induced, known as the hypersensitive response or HR. Resistance attained through the tobacco N gene and TMV-interaction will be discussed. Hammond-Kosak and Jones (1997, 1996) and Bent (1996) have reviewed molecular interactions involving plant disease resistance genes in detail.

1.9.2. Plant Disease Resistance Genes

There are four well characterized systems used to study \( R-Avr \) gene-dependent events which include: N gene-mediated resistance to TMV in tobacco, \( Cf \) gene-mediated resistance to \( Cladosporium fulvum \) in tomato, R gene-mediated resistance to \( Pseudomonas syringae \) in \( Arabidopsis thaliana \), and \( Mla \), \( Mlg \), and \( Mlo \) gene-mediated resistance to \( Erysiphe graminis f sp hordei \) in barley. In these plant systems, the co-expression of \( R-Avr \) gene components results in an enhanced level of resistance to their respective pathogens. For example, tomato resistance genes, \( Cf\)-2, \( Cf\)-4, \( Cf\)-5, and \( Cf\)-9 confer resistance to specific races of the leaf mold fungus \( C. fulvum \) that express the corresponding genes \( Avr2 \), \( Avr4 \), \( Avr5 \), and \( Avr9 \), respectively, which have been cloned and characterized. The co-expression of each \( Cf-Avr \) gene combination results in the arrest of hyphal growth during a distinct stage of colonization (Hammond-Kosak and Jones, 1997). \( Cf-Avr \) gene-mediated resistance was produced when 14 day-old tomato seedlings, which individually carried \( Cf\)-2 and \( Cf\)-9 resistance genes, were infiltrated with an elicitor preparation that contained both \( Avr2 \) and \( Avr9 \) gene products. More specifically, \( Avr9 \) specifies a 28 amino acid peptide that elicits a necrotic response when injected into tomato plants harboring the \( Cf\)-9 resistance gene (Jones et al., 1994). These \( Cf-Avr \) gene products may provide an excellent opportunity to enhance plant resistance to broomrape through genetic manipulations, however, these strategies are limited to the availability of these particular gene products.

1.9.3. Tobacco N Gene

The tobacco N gene has proved to be a durable source of resistance against tobamoviruses. The TMV-induced HR is the classical model system for studying disease resistance responses to pathogens and is characterized by the formation of necrotic lesions in tobacco bearing the N gene (NN tobacco) (Whitham et al., 1994).
**Fig. I. 2.** Schematic representation of the location and structure of the five main classes of plant resistance (R) gene products. LRR= leucine rich repeat, NBS= nucleotide binding site, CC= coiled-coil domains, TIR= *Toll* interleukin 1 receptor, S/T Kinase = serine/threonine kinase
In 1929, Holmes identified resistance in *N. glutinosa* by inoculating various *Nicotiana* species with TMV. *Nicotiana glutinosa* inoculated plants very rapidly formed local lesions that were much smaller and more uniform than the other species. TMV infection of NN tobacco induces HR within 48 hrs post infection and restricts TMV to the region surrounding the necrotic lesions, while cultivars lacking the N gene (nn tobacco) allow the virus to spread systemically producing mosaic symptoms (Spence, 1997). The experiments performed by Holmes facilitated the use of *N. glutinosa* as the standard in quantifying TMV resistance by local lesion assays (Bagley, 2000). Holmes (1938) concluded that *N. glutinosa* resistance was attributed to a single locus dominant gene known as the N gene. Interspecific hybridization was performed to introduce the N gene from the TMV-resistant species, *N. glutinosa*, to the TMV-sensitive cultivar, *N. tabacum* var. Samson (Holmes, 1938; Clausen and Godspeed, 1925). The genetic history of the N locus has been described in detail (Dunigan et al., 1987).

### 1.9.4. N-gene Mediated Resistance to Tobacco Mosaic Virus (TMV)

Whitham et al. (1994) isolated the first plant resistance gene utilizing the maize Ac transposon for insertional mutagenesis. By employing a positive selection scheme, TMV-susceptible mutants were isolated by using genomic DNA sequences flanking the Ac transposon. Genomic DNA sequences were used to identify cDNA and genomic clones containing the N gene. Complementation tests were performed using TMV-susceptible plants with a genomic N gene fragment and transferring resistance to the TMV-sensitive plant confirming that the N gene had been cloned. Sequence analysis revealed that N gene encodes a protein of 131.4 kDa and the amino terminal domain has similarity to the cytoplasmic domains of the *Drosophila melanogaster* Toll protein and the interleukin-1 receptor in mammals (Sims et al., 1989). The protein contains a putative nucleotide binding site (NBS), and 14 imperfect leucine-rich repeats (LRR) making it a member of the TIR-NBS-LRR plant resistance genes (Fig. 1.1). The presence of these functional domains in the predicted N gene product is consistent with the hypothesis that the N gene functions in a signal transduction pathway (Spence, 1997). Similarities of N to Toll and the interleukin-1 receptor suggest similar signaling mechanisms leading to the rapid gene induction and TMV resistance (Dinesh-Kumar et al., 1995).

The plant resistance genes belonging to the TIR-NBS-LRR class have been predicted to encode multiple transcripts. The N gene is complex and includes 5 exons and 4 introns. Dinesh-
Kumar et al. (2000) performed quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) on wild type and transgenic tobacco plants to demonstrate that N gene exons can be alternatively spliced from a single gene and result in two transcripts, N_S and N_L. Transcript N_S is produced by default splicing of the 5 exons and encodes the full length 131.4 kDa N protein, while N_L transcript is generated from alternative splicing of an alternative exon (AE) in intron III. The inclusion of AE in N_L transcript results in a shift in the reading frame and subsequently causes premature translation termination after the first LRR in exon 4 (Dinesh-Kumar et al., 2000). N_L transcripts encode a truncated protein, Ntr, that is 75.3 kDa.

Several N gene intron deletion constructs were generated to determine the functional significance of AE. Deletion constructs were transformed into TMV-sensitive tobacco plants and tested for TMV response at various times points. Plants expressing N gene lacking introns I, II, and IV showed an HR following TMV inoculation. However, deletion of intron III containing AE led to a partial TMV resistance that ultimately resulted in systemic spread of the virus. These results suggest that the AE sequence within intron III is required to confer complete resistance to TMV (Dinesh-Kumar et al., 2000). Additionally, Dinesh-Kumar et al. (2000) investigated the minimum N sequences necessary to confer complete TMV resistance. Several reconstructed cDNAs were generated using N gene cDNA and genomic clones, transformed into TMV-sensitive tobacco plants, and tested for TMV response. They found that complete resistance to TMV required not only the coding region and AE of intron III, but also an additional genomic sequence located 1.4 kb downstream of the N gene stop codon. These results confirm that the AE and 3’ GS are the N gene minimum sequences required to confer complete resistance to TMV.

The TMV genome has been explored to identify potential avr components that interact with the N gene to trigger HR. TMV is a single-stranded, positive-sense RNA virus. Its genome comprises 6395 nt and encodes four proteins: two for viral replication synthesized from genomic RNA (126 kDa and 183 kDa), one for cell-to-cell movement (30 kDa), and one for viral RNA encapsidation (17.5 kDa) both translated from sub-genomic RNAs. TMV replicase-mediated resistance was first demonstrated by Padgett and Beachy (1993). Padgett et al. (1997) identified the 126/183 kDa TMV replicase protein as the corresponding Avr ligand involved in N gene-mediated HR. To demonstrate that a portion of the replicase gene is required for induction of an N gene-mediated HR, chimeric virus genomes were constructed from genes encoding the 126/183
kDa protein from a strain of TMV that elicits a HR sensitive response and the resistance-breaking Ob tobamovirus. Each hybrid virus genome contained the 5' untranslated region (UTR) and replicase gene of TMV joined to the sequences encoding the MP, CP, and 3' UTR of the Ob viral genome. Tobacco lines that harbor the N gene react hypersensitively to infection by tobacco mosaic virus (TMV) except the tobamovirus Ob (Padgett et al., 1997). After inoculating the leaves of a susceptible tobacco host, *N. tabacum* cv. Xanthi nn, with transcripts of the viral hybrids, systemic infection was observed. These results suggest that viral functions required for replication, local and systemic invasion were maintained. In addition, when viral hybrids transcripts were inoculated onto Xanthi NN plants, necrotic local lesions were observed on plants expressing the TMV replicase with the Ob MP, CP, and 3'UTR. On the other hand, plants inoculated with the Ob viral replicase, MP, CP, and 3' UTR transcript spread systemically without causing a HR. They concluded that the TMV replicase protein sequence is required to elicit a N gene-mediated HR.

The tobacco N-gene-mediated resistance system is unique because is provides a second level of complexity to study the activation of this defense response. For nearly two decades, it has been known that N-mediated resistance is sensitive to high temperatures. More specifically, at 20 to 27°C, resistance is maintained, while at temperatures above 28°C, TMV overcomes the effects of the N gene (Ito et al., 2002). Padgett et al. (1997) also wanted to determine if thermosensitivity of N-gene-mediated HR would be induced by various Ob mutants carrying components of the TMV replicase. Xanthi NN plants were inoculated with these viral mutants then maintained in a growth chamber at constant temperatures ranging from 18 to 28°C (at 2°C increments). Plants were observed for the presence of necrosis and systemic symptoms for a two wk period. It was found that the TMV virus was localized at 24°C and below and that the Ob-viral mutants were localized by HR at 18 °C and below. From these results they concluded that the thermosensitivity of the N gene response is induced at the level of interaction between the virus and the defense response mechanism (Padgett et al., 1997).

### 1.10. SIGNIFICANCE AND OBJECTIVES OF RESEARCH

The development of new resistance strategies are urgently needed to control Egyptian broomrape, especially in areas where the parasite causes serious economical losses. The specific objectives of this research were to (1) generate gene constructs containing the *Hmg2* promoter...
fused to N gene sequences required to confer complete TMV resistance; and the CHS8 promoter fused to the TMV replicase gene; (2) stably integrate the CHS8:TMV replicase construct into TMV-resistant tobacco plants via Agrobacterium-mediated transformation; (3) characterize transgenic plants by determining genomic insertion and copy number, and transcript production; and to test host-parasite interactions via polyethylene bag inoculation studies.
I.11. REFERENCES


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Chapter II

ENGINEERING NOVEL GENE-FOR-GENE RESISTANCE TO THE PARASITIC ANGIOSPERM *OROBANCHE*
II.1 INTRODUCTION

Orobanche aegyptiaca Pers. (Egyptian broomrape) is an obligate root parasite that affects many economically important dicotyledoneous plants including Solanaceae, Fabaceae, Cruciferae, Compositae, and Umbelliferae (Parker and Riches, 1993). These achlorophylous holoparasites obtain all their nutrients and water from their host plants, causing severe damage and reducing crop yield and quality (Goldwasser et al., 2001). O. aegyptiaca causes significant crop loss in semiarid regions of the world, particularly in southern Europe, central Asia, the Middle East, and the Mediterranean (Joel, 2000; Parker and Riches, 1993; Musselman, 1980). O. aegyptiaca is intimately associated with its host roots and causes the most damage prior to its emergence, thereby making control difficult. The parasite completes its lifecycle by producing a floral spike that may contain many flowers and as many as 250,000 seeds that readily disperse and can remain viable in the soil for years (Goldwasser et al., 2001). Conventional control methods, including mechanical, cultural, biological, and chemical means, have had limited success in controlling O. aegyptiaca. The most effective current means of broomrape control is achieved through the use of chemical fumigants such as methyl bromide, metham sodium, and dazomet. However, these are highly volatile compounds that are expensive and dangerous to the users and the environment (Foy et al., 1989). The development of O. aegyptiaca-resistant crop cultivars would provide the best long-term strategy to control the parasite, but, little is known about genetic resistance against parasitic weeds and there has been limited success with the development of O. aegyptiaca-resistant cultivars (Goldwasser and Yoder, 2001; Cubero, 1991). No control measures that are both effective and economically feasible have been found for O. aegyptiaca spp. (Muller-Stover et al., 2002). There is clearly a need for the development of new strategies to effectively control O. aegyptiaca spp. Genetic engineering techniques offer the possibility of creating plants that can defend against parasites by appropriately inducing their natural “gene-for-gene” pathogen recognition and defense response systems. Plant disease resistance, or R-genes, specific for mediating resistance to specific viral, bacterial, nematodal, and fungal pathogens and aphids have been cloned and characterized (Dangl and Jones, 2001; Ellis et al., 2000; Martin, 1999; Hammond-Kosak and Jones, 1997; Bent, 1996; Staskawicz et al., 1995; Jones et al., 1994; Whitham et al., 1994; Martin et al., 1993). Resistance genes are hypothesized to encode proteins that activate signaling cascades that coordinate the initial plant defense response to impair pathogen ingress by producing a hypersensitive response or HR
Like plant pathogenic agents, *O. aegyptiaca* has close associations and has evolved complex interactions with its host. It initiates parasitism by penetrating host cells to form direct vascular connections. Although putative R-genes for *O. aegyptiaca* resistance have been identified genetically in sunflower (Lu et al., 2000; Antonova, 1994), these genes have not been cloned. We are interested in determining if an *O. aegyptiaca*-specific gene-for-gene system can be engineered by using a well characterized R-gene/avr-gene pair, the tobacco N gene and the Tobacco Mosaic Virus (TMV) replicase gene, and placing it under the control of *O. aegyptiaca*-inducible promoters.

N gene-mediated resistance to TMV in tobacco is a model system used to study hypersensitive disease resistance (HR) responses in plants. The N gene was initially cloned based on transposon tagging (Whitham et al., 1994) and encodes a protein of the TIR-NBS-LRR class of plant resistance genes (see chapter I for discussion of plant R-genes). The N gene comprises about 12.0 kb of coding region interrupted by four introns (Dinesh-Kumar et al., 2000). Dinesh-Kumar et al. (2000) demonstrated that N gene exons can be alternatively spliced, resulting in two transcripts, Ns and Nl, and that regulatory regions within intron III and 3’ genomic sequences are needed to confer complete resistance to TMV.

The N gene product interacts with the replicase of TMV to trigger a hypersensitive response (Padgett et al., 1997). TMV is a single-stranded, positive-sense RNA that encodes four proteins: the 126 kDa and 183 kDa proteins are required for viral replication, a 30 kDa protein needed for cell-to-cell movement, and a 17.5 kDa protein required for viral RNA encapsidation. Padgett et al. (1997) identified the amino acids 692 to 1116 as the portion of the replicase gene that is responsible for N gene-mediated HR.

N gene-mediated resistance was selected to test the feasibility of creating a novel gene-for-gene response because it offers an additional mechanism to regulate TMV-induced HR. N gene-mediated HR is temperature sensitive and elevated temperatures (above 28 °C) weaken the interaction between the viral elicitor (viral replicase) and the N gene product, blocking HR and allowing TMV replication and spread. N gene thermosensitivity can be used to regulate TMV-induced HR while testing systems for proof of principle.

In addition to selecting the tobacco N gene and the TMV replicase as the R-avr gene pair for triggering HR, engineering disease resistance to *O. aegyptiaca* based on this strategy requires
the identification of two *O. aegyptiaca*-responsive gene promoters. The success of this strategy relies on two important caveats: (1) the promoters utilized must be induced by *O. aegyptiaca* parasitism, and (2) their expression patterns cannot overlap within the same tissues during normal plant development because cell death would result. The tomato *Hmg2* promoter has been well characterized as an *O. aegyptiaca*-responsive promoter (Westwood et al., 1998) and is also induced by wounding and pathogen invasion (see chapter 1) (Weissenborn et al., 1995; Park et al., 1992). The bean (*Phaseolus vulgaris*) chalcone synthase 8 promoter (CHS8) has been identified as an inducible defense-related gene associated with phytoalexin synthesis in response to pathogenic fungi and bacteria (Harrison et al., 1991; Doerner et al., 1990). The CHS8 promoter, introduced into tobacco as CHS8:GUS construct, has also been shown to be responsive to *O. aegyptiaca* parasitization (Westwood et al., 1999). According to Griffits (2001) and Westwood et al. (1999), the pea (*Pisum sativum*) β-subunit farnesyltransferase (PsFtb), tobacco (*Nicotiana tabacum*) Prla, and bean PAL genes appear to be *O. aegyptiaca*-responsive and could be additional candidate promoters for our studies.

The development of new resistance strategies are urgently needed to control Egyptian broomrape. Pathogen-induced HR resulting from *R*-gene/*avr*-gene interactions have been successful in limiting the development and spread of phytopathogens. The *O. aegyptiaca* lifecycle is highly specialized for parasitism (Goldwasser et al., 2001). *O. aegyptiaca* behaves analogously to sedentary endoparasitic nematodes in that it establishes close associations with its host plant during nutrient acquisition. Based on these similarities, is it possible to redirect a well characterized *R*-gene/*avr*-gene pair to create resistance to *O. aegyptiaca*? Our goal is to test this hypothesis. Transgenic plants were generated expressing the TMV replicase gene with an endogenous N gene under the control of an *O. aegyptiaca*-responsive promoter, bean CHS8 and monitored for *O. aegyptiaca* parasitism. This study characterizes the impact of an *O. aegyptiaca*-inducible *avr* gene, CHS8:TMV replicase, on *O. aegyptiaca* parasitism and provides an initial proof of concept that N-gene mediated HR can enhance resistance against parasitic weeds.

II.2 MATERIALS AND METHODS

II.2.1 Assay of tobacco seeds for GUS expression

Tobacco seeds of lines 330.1-002 (Yu, 1995), pDZ.1 no.7 (Zhou, 1997), and CHS8-GUS (β-glucuronidase) (Schmid et al., 1990) expressing tomato *Hmg2* promoter:GUS, the pea β-
subunit farnesyltransferase promoter PsFTb:GUS, and the bean chalcone synthase promoter, CHS8:GUS gene fusions (provided by C. J. Lamb, Salk Institute for Biological Studies, La Jolla CA) were surface sterilized with ethanol, 20% sodium hypochlorite, and sterile distilled water. Seeds were germinated on MS media (Murashige and Skoog, 1969) amended with kanamycin (200 mg/ml) and incubated at 23 °C for 3 wk. To visualize developmental expression patterns of each promoter, seedlings were harvested and histochemically stained for GUS activity (Jefferson, 1987). Seedlings were vacuum-infiltrated with 1mM of 5-bromo-4-chloro-3-indolyglucuronide (X-gluc, Sigma, St. Louis, MO) substrate and incubated at 37 °C for 24 hr. Following incubation, seedlings were cleared of chlorophyll and stored in 70% ethanol.

II.2.2 Plant growth

Tobacco seeds expressing CHS8:GUS reporter gene fusions were surface sterilized (see above), germinated on MS media amended with kanamycin (200 mg/ml), and kanamycin-resistant seedlings were transferred into 17.5 cm pots containing Promix BX/PGX soil mix (2:1 ratio; Wetsel, Harrisonburg,VA). Leaf tissue was harvested from fully expanded leaves 3 wk after transplanting.

II.2.3 Generation of CHS8:TMV replicase construct

For the production of CHS8:TMV replicase gene constructs, the bean CHS8 promoter was amplified from transgenic tobacco carrying the CHS8:GUS gene. Tobacco genomic DNA was extracted from leaf tissue using the DNeasy genomic extraction kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s recommendations. Sequence data for the entire CHS8 promoter was not available. Therefore, the promoter was amplified using the polymerase chain reaction from a transgenic tobacco line carrying the CHS8:GUS transgene utilizing primers designed based on the multiple cloning site region of pBI101.1 (CHS85’: 5’GCAGCC AAGCTTGCATGCCTGCAG3’) and the 5’ end of the GUS coding region CHS8:GUS3’Mar01 (5’TGGGTTTCTACAGGACGTAACATGATATC3’). The oligonucleotide primer CHS85’ provided a flanking HindIII restriction site (bold, underlined). PCR amplification from 100 ng genomic template resulted in the amplification of a 1.4 kb fragment. The PCR amplification protocol included: 1 cycle at 95 °C for 1 min; 39 cycles at 94 °C for 1 min, 59 °C for 2 min, and 72 °C for 3 min, and 1 cycle 94 °C for 1 min, 59 °C for 2 min, and 72 °C for 6 min. Fidelity of the PCR product was confirmed by sequencing (Virginia Bioinformatics Institute DNA
Sequencing Facility, Blacksburg, VA). Based on these results an additional primer, CHS8’apr01 (5’GCAGTCCCCGGAGTTTTCTTGAAATAGAAGTAATGAGTG3’) was designed to anneal to the 3’ end of CHS8 promoter and provided a flanking SmaI restriction site. The previous PCR protocol was used to amplify a 1.4 kb fragment. The PCR product was digested with HindIII and SmaI and ligated into the respective sites of a pBluescript cloning vector (Stratagene, La Jolla, CA) to yield a construct designated as pCHS8Blue the construct was sequenced for confirmation.

The plasmid pTMV004 (wild type TMV cDNA clone) containing the coding region of the tobacco mosaic virus (TMV) was kindly provided by Dr. Dennis Lewandowski (Univ. of Florida, Gainsville, FL). Oligonucleotide primers TMV rep5’ (5’GCGGATATCGT GGA CAT GCCTGCGCTTGAC3’) and TMV RepHIS3’ (5’GGTACCTCA ATGGTGATGGT GA TGG TGTGTTGCCTTGCAATCGACCTT3’) were used to amplify a region of the TMV replicase gene (1689 nt to 3419 nt) with flanking EcoRV and KpnI restriction sites (bold, underlined). A histidine tag (underlined) was also incorporated into the primer TMV RepHIS3’. The PCR protocol used to amplify the replicase gene was as follows: 1 cycle at 96 °C for 1 min; 30 cycles at 94 °C for 1 min, 59 °C for 2 min, and 72 °C for 3 min, and 1 cycle 94 °C for 1 min, 56 °C for 2 min, and 72 °C for 6 min. The 1.6 kb PCR product was digested with EcoRV and KpnI and ligated into EcoRV and KpnI digested pBluescript. The resulting plasmid, pTMVBlue, was sequenced to confirm fidelity of the PCR product.

To construct the CHS8:TMV replicase gene, the plasmid pCHSBlue was digested with HindIII and SmaI. The plasmid pTMVBlue was digested with EcoRV and KpnI. The promoter and replicase fragments were gel purified by performing the crush and soak technique (Sambrook et al., 1989). pBluescript and the Agrobacterium tumefaciens transformation vector, pBIB-kan (Becker, 1990) were digested with HindIII and KpnI restriction enzymes and gel purified. A tri-molecular ligation was performed with the CHS8 promoter, TMV replicase gene, and pBluescript according to standard protocols (Sambrook et al., 1989) and junctions were confirmed via sequencing. This plasmid was designated as pCHS8:TMVBlue. pCHS8:TMVBlue was digested with HindIII and KpnI restriction enzymes, and the CHS8:TMV replicase fragment was gel purified and ligated into HindIII and KpnI sites of pBIB-kan.

II.2.4 Generation of Hmg2::N gene construct
The Hmg2 MeGA promoter was provided by CropTech Corporation (Blacksburg, VA) in a plasmid vector designated as pCT151. The tobacco N gene was provided as a 12.0 kb XhoI-XhoI fragment in the genomic clone pGEMN (Dinesh-Kumar et al., 2000) by Dr. Paul Rangel (Univ. of California, Berkeley, CA). The tobacco N gene is a complex gene containing five exons and four introns. Due to the complexity of the tobacco N gene and lack of useful restriction endonuclease sites, several cloning steps were performed to create the Hmg2:N gene construct. Briefly, the plasmid pCT151 was mutagenized to create a SmaI restriction site upstream of the HindIII site using HindIII-Smal adaptors according to manufacturer’s recommendations (New England BioSystems, NEB, Beverly, MA). The clone was sequenced to confirm restriction site insertion. The resulting mutagenized plasmid, ∆pCT151, and the pGEMN were digested with NcoI and KpnI restriction enzymes. Digestion of pGEMN rendered two N gene fragments of 2.6 kb (NcoI-NcoI) and 4.0 kb (NcoI-KpnI) in size. The vector and N gene fragments were gel purified using the crush and soak standard techniques (Sambrook et al., 1989) and ligated into the vector in two steps. All junctions were confirmed via sequencing. To obtain the 3’ genomic sequence downstream of the stop codon, an oligonucleotide primer was designed to anneal to the 10406 nt (primer NG 10406), of the N gene and used with the M13 reverse universal primer which annealed to vector sequences of pGEMN (12346 nt). The PCR protocol used included: 1 cycle at 95 °C for 3 min; 30 cycles at 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 2 min, and a final extension cycle at 72 °C for 10 min. A 1.4 kb PCR product was obtained, digested with the restriction enzyme SacI, and ligated downstream of the 4.0 kb N gene fragment into the respective restriction site in the vector ∆pCT151. Gene insertion and orientation was confirmed via restriction digestion and PCR amplification with NG 10406 and M13 reverse primers as previously described. The sequence fidelity of the N gene was confirmed via sequencing. In the final cloning step, the entire construct containing the Hmg2:N gene was digested with SmaI, resulting in an 8.4 kb fragment. This fragment was gel purified and ligated into the SmaI restriction site of the Agrobacterium tumefaciens transformation vector, pBIB-hyg (Becker, 1990). Vectors containing the appropriate orientation of Hmg2:N-gene with respect to the terminator were identified by PCR and designated pNGFL (N gene full length).

Plasmids containing the CHS8 promoter (pCHS8Blue), TMV replicase gene (pTMVBlue), and the constructs CHS8:TMV replicase (pCHS8TMV) and Hmg2:N gene
(pNGFL) were transformed into electrocompeent *Escherichia coli* strain DH5α using the One Shot transformation kit according to the manufacturers recommendations (Invitrogen Life Technologies, Carlsbad, CA) and *Agrobacterium tumefaciens* strain LBA4404 using the freeze-thaw method (Holsters et al., 1978). The CHS8:TMV genes were introduced into tobacco plants (*N. tabacum* var. Xanthine) via *Agrobacterium*-mediated transformation using the petiole method (Medina-Bolivar et al., 2003). Transgenic plants were generated and the integration of the genes was confirmed via PCR amplification of genomic DNA (100 ng) with oligonucleotide primer CHS302F (5’GACAGGTCAGTGGGTAACCTAG3’) and CHS83’apr01’ (described above).

**II.2.5 CHS8:TMV induction by wounding**

Leaves from axenically-grown CHS8:TMV transgenic plants (3 cm in diameter) were excised and wounded with a glass capillary tube (0.2 cm in diameter). Wounding was performed by positioning the capillary tube upright on the leaf surface and pressing firmly. Leaves were stored in a Petri dish on water-moistened filter paper and monitored for the development of HR or necrosis. In order to confirm the wound induction of the CHS8 promoter, CHS8:GUS transgenic leaves were excised, wounded, and stained for GUS activity.

**II.2.6 RNA isolation and northern hybridization**

Fully expanded leaves of greenhouse-grown transgenic plants containing CHS8:TMV replicase gene constructs were wounded by passage through a pasta maker (producing uniform leaf strips approximately 2 mm in diameter), and incubated in a petri dish at room temperature for 0 and 24 hrs. Total RNA was extracted from the wounded leaves with TRIsreagent (MRC, Cincinatti, OH) according to manufacturers recommendations with an additional phenol/chloroform purification step. Total RNA (15 µg) was size separated by electrophoresis in a 1.2% agarose denaturing formaldehyde gel and capillary transferred onto Hybond N+ nylon membrane (Amersham Pharmacia, Piscataway, NJ). The membrane was hybridized overnight at 60°C with the 1.6 kb 32P-labeled TMV replicase fragment labeled as previously described. Filters were washed under high stringency conditions with a final wash at 0.2X SSC and 0.1% SDS.

**II.2.7 O. aegyptiaca inoculation**
Transgenic tobacco lines containing CHS8:TMV gene constructs were vegetatively propagated from parent plants to obtain five replicates from each line. In addition, non-transformed Xanthi and transgenic tobacco plants containing CHS8:GUS gene constructs were used as controls and propagated in the same manner. Meristem cuttings were transferred to 17.5 cm pots containing Profile ceramic mix (Landscape LLC, Roanoke, VA), fertilized with Peters fertilizer (Spectrum Group, St. Louis, MO), and allowed to root for 7 d. Plants were transferred into polyethylene (PE) bags containing glass fiber filter paper and watered with 0.5X Hoaglands solution as described by Westwood et al. (1998). Plants were allowed to grow for 7 d under fluorescent lighting (100 μE . s⁻¹ . m⁻²) at 25 ºC.

Seeds of *O. aegyptiaca* (provided by Dr. Jim Westwood, Virginia Tech, Blacksburg, VA) were surface sterilized using 70% ethanol and 1% sodium hypochlorite, rinsed with sterile distilled water, placed (800 to 1000 seeds) “in excess” around and below the roots of tobacco plants using a small paintbrush, and allowed to precondition for 7 d. A chemical germination stimulant, GR-24 (1mg/L), was injected with a 10mL syringe into the back of the PE bag and allowed to diffuse into the membrane. Plants were grown under light and temperature conditions as described above. Parasitized plants were harvested approximately 6 wk after inoculation to observe the number of attachments and size of the parasite. These analyses were performed under quarantine in collaboration with Dr. James Westwood. Images were captured using the Synscroscopy digital imaging system (Synoptics, Ltd., Fredrick, MD).

**II.2.8 Statistics**

Two experiments were performed and results of experiments were not significantly different, therefore, data were pooled and averaged. Means were separated by Duncan’s multiple range test. Lines were significantly different at the $P< 0.001$ level. Data were analyzed by ANOVA.

**II.3 RESULTS**

**II.3.1 Promoter expression studies**

Our long-term goal is to engineer plants that elicit an HR response upon *O. aegyptiaca* ingress by using *O. aegyptiaca*-responsive promoters to direct the expression of an *R-avr* gene pair. Success of this strategy will depend on tight regulation of *R-avr* gene pair since co-expression within a cell leads to cell death (Jones et al., 1994). Local cell necrosis, or HR, is
Figure II.1. Assay of tobacco seeds for promoter GUS expression. Transgenic tobacco seedlings expressing (A) Hmg2:GUS construct, (B) PsFTb:GUS (farnesyltransferase) construct, and (C) CHS8:GUS construct. Transgenic tobacco (*Nicotiana tabacum*) seeds expressing a *Hmg2* promoter:GUS, FT promoter:GUS, or CHS8 promoter:GUS (chalcone synthase 8) constructs were germinated on MS media amended with kanamycin for and incubated at 23 °C for 3 wk. Promoter activity was visualized using GUS assay (blue stain). Seedlings were vacuum-infiltrated with 1mM of 5-bromo-4-chloro-3-indolyglucuronide (X-gluc, Sigma, St. Louis, MO) substrate and incubated at 37 °C for 24 hr. Following incubation, seedlings were cleared of chlorophyll and stored in 70% ethanol.
required to limit the development and spread of *O. aegyptiaca*, however, its expression is not desired under any other conditions. We previously demonstrated that the tomato *Hmg2* promoter is strongly induced by *O. aegyptiaca* parasitism during development. In order to identify *O. aegyptiaca*-inducible promoters with non-overlapping developmental expression patterns, we compared GUS activities in transgenic tobacco expressing GUS driven by the following *O. aegyptiaca*-responsive promoters: 0.4 kb of *Hmg2*, 3.2 kb of *PsFTb*, or 1.4 kb of *CHS8* promoter (Westwood and Cramer, 1999; Weissenborn et al., 1995). As previously shown (Weissenborn et al., 1995), *Hmg2* is expressed throughout cotyledons of young seedlings and at the site of lateral root initiation in older roots (Fig II.1). The *psFTb* transgenic seedlings showed strong GUS activity in the cotyledons, hypocotyls, and root tips. In *CHS8* seedlings, GUS activity was seen primarily in root tips and restricted areas within lateral root initiation sites. Based on these studies, *CHS8* has the most restricted expression during early seedling development and its promoter was selected to drive the expression of TMV replicase as the *avr* component for engineering *O. aegyptiaca* resistance.

II.3.2 Production of tobacco expressing CHS8: TMV replicase

The portion of the TMV replicase from amino acid 692 to 1116 effectively elicits N gene-mediated HR (Padgett et al., 1997). A vector was constructed that fused this region of the TMV replicase gene to the 1.4 kb *O. aegyptiaca*-responsive CHS8 promoter (Fig. II.2A). CHS8 and TMV replicase sequences were generated by PCR amplification to provide flanking restriction sites and a HIS tag. The PCR resulted in CHS8 and TMV replicase products that were 1.4 kb and 1.6 kb, respectively (Fig. II.2B and II.2C). CHS8 and TMV replicase fragments were ligated into pBluescript and subsequently introduced into the binary transformation vector pBIB-kan as a 3.1 kb *HindIII–KpnI* fragment (Fig. II.3). This construct was introduced into TMV resistant tobacco cultivar Xanthi ne using *Agrobacterium tumefaciens*-mediated transformation. More than 35 transgenic lines were regenerated with the pCHS8:TMV construct. Transgenic tobacco plants were initially screened for T-DNA insertion via PCR amplification of CHS8 products with CHS8 gene specific primers (CHS302F and CHSapr013’) and yielded the expected 800 bp product in 23 out of 35 plants (see Appendix A for summary of experiments).
Figure II.2. pCHS8:TMV with a histidine tag. Construction of the CHS8:TMV replicase construct. (A) Diagram showing CHS8 promoter:TMV gene construct and CHS83’apr01 100ng of tobacco genomic DNA from plants carrying CHS8:GUS as template. (B) PCR amplification of CHS8 promoter. PCR results in all lanes show a 1.4 kb product amplified using CHS8 specific primers CHS85’. (C) PCR amplification of TMV replicase gene. PCR results show a 1.6 kb product amplified using TMV replicase gene specific primers TMVRep5’ and TMVRep 3’ and 25 ng of pTMV004 (TMV cDNA clone) plasmid DNA. M represents the 1 kb molecular weight markers (Invitrogen Life Technologies, Carlsbad, CA).
Figure II.3. Map of T-DNA from Agrobacterium tumefaciens plant transformation vector pBIB-kan displaying CHS8:TMV transgene insertion. The vector contained a kanamycin resistance gene (NPTII), plant promoter (pAg7), plant terminators (pAnos, Pnos), and the T-DNA border sequences (LB, RB) that define the region to be transferred into the plant genome.
II.3.3 Construction of \( Hmg2: \) N gene vectors

To create transgenic plants that express the tobacco N gene under the control of an \( O. \ aegyptiaca \)-inducible promoter, a second construct was generated that fused the coding region of the N gene to the 0.4 kb \( O. \ aegyptiaca \)-responsive \( Hmg2 \) promoter (Fig. II.4). It has been demonstrated that there are non-coding sequences in the N gene that are important for conferring resistance to TMV (Dinesh-Kumar et al., 2000). These include sequences within intron III and 1.4 kb region downstream of the stop codon. Due to the large size and lack of useful restriction sites, the cloning of the N gene was performed in several steps (see Methods). Restriction endonuclease digestion of pGEMN with \( NcoI \) and \( KpnI \) yielded two N gene fragments 2.6 kb (\( NcoI-NcoI \)) and 4.0 kb (\( NcoI-KpnI \)) in size (Fig. II.5A) which were sequentially ligated into \( \Delta pCT151 \) which contained the \( Hmg2 \) promoter. The 1.4 kb of the N gene 3’ untranslated region was generated by PCR amplification, digested with \( SacI \), and ligated into \( \Delta pCT151 \) downstream of the 4.0 kb N gene fragment of (Fig. II.5B). The resulting construct contained the entire N gene from the ATG start codon to 1.4 kb downstream of the stop codon. The \( Hmg2: \) N gene was ligated into the binary transformation vector pBIB-hyg as a 8.4 kb \( SmaI-SmaI \) fragment yielding pNGFL (Fig. II.4). Because construction of the \( Hmg2: \) N gene vector was so challenging and time-consuming, tobacco transformation using this construct was not undertaken during this initial feasibility project. Instead, CHS8:TMV replicase constructs were introduced directly into tobacco lines containing an endogenous copy of the N gene (Xanthi NN). This strategy is not optimal because the limited developmental expression of the CHS8 promoter in tobacco could be problematic during regeneration and normal growth (\( i.e., \) lead to local cell death). However, the ability to utilize elevated temperatures to minimize replicase-N interactions should permit generation of plants for testing of \( O. \ aegyptiaca \) interactions.
Figure II.4. Construction of the *Hmg2:*N gene and introduction into the *Agrobacterium tumefaciens* plant transformation vector, pBIB-hyg to yield pNGFL. *Hmg2:*N gene was inserted into the T-DNA region of pBIB-hyg as a 8.4 kb *Sma*I-*Sma*I fragment. The vector contained a hygromycin resistance gene (HPT), plant promoter (Pnos), plant terminators (Pnos, pAg7), and the T-DNA border sequences (LB, RB) that define the region to be transferred into the plant genome.
Figure II.5. Construction of Hmg2:N. (A) Restriction digestion of pGEMN with NcoI and KpnI yielded two N gene fragments 5.4 kb (NcoI-KpnI) and 2.6 kb (NcoI-NcoI). (B) Restriction digestion of 3’ genomic sequences (GS) with SacI yielding a 1.4 kb fragment.
II.3.4 CHS8:TMV replicase expression

The CHS8 promoter is induced by wounding in bean hypocotyls (Ryder et al., 1987). As a first assessment that the transgenic plants could express effective levels of the TMV replicase, leaves of axenically grown CHS8:TMV and CHS8:GUS (control) transgenic tobacco were wounded and monitored for HR. Necrosis was observed in approximately 4 h after wounding in the cells encompassing the wound site in two of five CHS8:TMV transgenic lines tested (Fig. II.6A). No necrosis was observed on wounded leaves of non-transformed tobacco or the CHS8:GUS control. In CHS8:GUS transgenic leaves, GUS activity was localized to the wound site 6 h after wounding. These initial results suggest an HR induction caused by the interaction of the TMV replicase with an endogenous N gene upon wounding. Based on these results, CHS8:TMV transgenic tobacco lines displaying a necrotic phenotype were tested for transcript expression of the pCHS8:TMV construct.

For northern blots, total RNA was extracted from fully expanded tobacco leaves that were wounded by passage through a pasta maker and incubated at room temperature for 0 and 24 hr (Fig. II.6B). RNA from wounded wild-type tobacco plants were used a negative control. As shown in Fig. II.6B, the TMV replicase probe cross-hybridized to RNA of approximately 1.6 kb from transgenic lines. This transcript size is consistent with the size of the CHS8:TMV transgene. No cross-hybridization was detected with RNA from the non-transgenic control (Figure II.6B, lane 2). Transgenic plants displayed only low levels of expression. (Figure II.6B, lanes 4 and 6). It should be noted that 24 hr may not represent the optimal time for RNA accumulation and that since the replicase avr activity functions as a first step in defense signal transduction, so high levels of product should not be required to elicit an HR.
Figure II.6. Expression of transgenic CHS8:TMV. (A) Wounded leaves from untransformed and CHS8:TMV transgenic tobacco *N. tabacum* var. Xanthi. NTX (Non-transformed Xanthi) (left panel) displays no necrosis at wound site, CHS8:TMV plant #8 transgenic leaf (middle panel) and CHS8:TMV plant #9 transgenic leaf (right panel) display necrosis at wound site. (B) Northern hybridization analyses of RNA from transgenic tobacco leaves. Northern blot of total RNA (15 µg) was hybridized with with 32P-labeled TMV replicase fragment (1.6 kb). Lane 1: Nontransformed *N. tabacum* var. Xanthi at 0 hr, Lane 2: Nontransformed *N. tabacum* var. Xanthi at 24 hrs; Lane 3: CHS8:TMV plant #8 at 0 hr; Lane 4: CHS8:TMV plant #8 at 24 hrs; Lane 5: CHS8:TMV plant #9 at 0 hr; Lane 6: CHS8:TMV plant #9 at 24 hrs (top panel). Ethidium bromide-stained gel displaying total RNA (15 µg) isolated at 0 and 24 hr from fully expanded leaves of CHS8:TMV transgenic plants was used for northern blotting shown (bottom panel).
II.3.5 Impact of R-gene mediated resistance on *O. aegyptiaca* parasitism

The effect of CHS8:TMV replicase transgene on *O. aegyptiaca* parasitism was evaluated. Five CHS8:TMV replicase transgenic tobacco lines were inoculated with seeds of *O. aegyptiaca* and monitored for parasite attachment and development. Nontransformed tobacco cv. Xanthi (NTX) plants and those expressing CHS8:GUS transgenes were used as negative controls. Plants were harvested six weeks after inoculation and enumerated for parasite attachment. Three developmental stages (radicle, tubercle, “spider stage”) of *O. aegyptiaca* were observed micro- and macroscopically on inoculated tobacco plants. Phenotypic differences in the interaction were observed among three of five transgenic lines. During the radicle and tubercle developmental stages a distinct “darkening” phenotype was observed at the site of attachment in three transgenic lines but not in control roots (Fig. II.7). The host cells directly underneath haustorium penetration were brown in color or necrosis suggesting the induction of an HR. This phenotype was observed in multiple transgenic lines indicating it was due to the transgene and does not represent a gene insertion event. At later stages in parasite development, the darkening was observed throughout the region of vascular attachment (Fig. II.7I and II.7L). In some cases, this phenotype was associated with darkening of the tubercle as well, suggesting reduced viability of the parasite. Host cell necrosis was not observed on all attachments in which *O. aegyptiaca* progressed to advanced developmental stages (Fig. II.7F).

For all inoculated lines, some parasite attachments that successfully progressed beyond the radicle stage and “spider stages” were subsequently observed (as shown in Fig. II.7). The number of tubercles and spider stage attachments were significantly greater on NTX plants than those observed on CHS8:TMV transgenic lines 1, 5, 8, and 9. Line CHS8:TMV-9 showed a 75% reduction in successful attachment and progression of *O. aegyptiaca* suggesting partial or enhanced resistance to the parasite was attained (Figure II.8).

To confirm localization of CHS8 induction during *O. aegyptiaca* interactions, tubercles were excised from CHS8:GUS roots and stained in X-gluc for GUS activity. CHS8 activity was localized at tubercle attachment (Figure II.7 M-O). During early tubercle development, low GUS activity was observed in cells surrounding attachment sites. As the parasite developed into the spider stage, strong GUS activity was observed throughout the host cells in the area of parasite attachment and those surrounding cells.
Figure II.7. Early interactions of *O. aegyptiaca* grown on non-transformed *Nicotiana tabacum* cv. Xanthi (A-C) and CHS8:TMV transgenic lines (D-L). During early parasite development, CHS8:TMV lines (1, 5, and 9) displayed a darkening of host cells beneath *Orobanche* attachment sites indicating HR. CHS8:GUS transgenic plants (M-O) were used as a control to confirm promoter induction. GUS activity was localized to the site of parasite attachment.
Figure II.8  Observations of Orobanche aegyptiaca growth on CHS8: TMV in Xanthi. Non-transformed N. tabacum var. Xanthi, (NTX), CHS8:TMV transgenic lines (1, 5, 8, 9, 18), and CHS8:GUS transgenic plants were inoculated with seeds of O. aegyptiaca. Plants were harvested 6 weeks after inoculation and enumerated for macroscopic parasitic attachments. Transgenic lines 8 and 9 were shown to express the TMV replicase. As controls, non-transgenic NTX, CHS8:GUS tobacco and CHS8:TMV-18, which did not show replicase expression, were used. Data represent means of 10 observations (5 replicates per line, combined from two experiments) with bars representing standard error. Bars with the same letters are not significantly different at $\alpha=0.05$. Means were separated by Duncan’s multiple range test.
II.4 DISCUSSION

We have successfully generated transgenic plants expressing CHS8:TMV replicase gene in tobacco lines carrying an endogenous N gene. The plants appeared normal in phenotype and the number of transgenic lines generated were typical of routine Xanthi transformation, suggesting that basal CHS8:TMV replicase activity was not detrimental to regeneration or growth. CHS8:TMV replicase appears to confer enhanced host resistance to *O. aegyptiaca*. During early host-parasite interactions, a transgene-dependent darkening at the site of *O. aegyptiaca* attachment was observed which extended to the vascular system. This phenotype was seen in three of five transgenic lines expressing the TMV replicase; CHS8:TMV replicase lines 1, 5, and 9. It suggests that the co-expression of endogenous N gene and transgenic TMV replicase genes resulted in a successful N gene-mediated HR. This phenotype was observed in multiple lines, which indicates that it is due to transgene expression and is not a consequence of site of insertion. The transgene-associated darkening was observed in CHS8:TMV replicase lines 5 and 9 during the later stages of *O. aegyptiaca* development. The most dramatic response to *O. aegyptiaca* development and parasitism was observed on CHS8:TMV replicase line 9, resulting in a 75% reduction in successful attachments. Some attachments in all transgenic lines were successful in progressing to the “spider” stage suggesting that resistance was incomplete.

The current strategy utilizes an endogenous N gene under the control of its own promoter. The expression patterns of the N gene in roots are not known and it is possible that *O. aegyptiaca* interactions may suppress N gene expression. The current results show promise for future experiments utilizing a R/avr gene pair to engineer resistance to *O. aegyptiaca*. Expression of the TMV replicase leads to a significant reduction in *O. aegyptiaca* parasitism. It is possible that the resistance could be further enhanced by introducing the N gene under the control of Hmg2, an *O. aegyptiaca*–responsive promoter. In the future we will develop transgenic plants that co-express the Hmg2:N transgene and CHS8:TMV replicase transgenes in tobacco that lacks the endogenous N gene. Transgenic plants can be generated by two approaches: (1) introduce the Hmg2:N gene construct and CHS8:TMV replicase construct into two separate tobacco lines and cross plants to obtain both genes in a single tobacco line, or (2) co-cultivate the Agrobacterium tumefaciens strains which harbor the Hmg2:N gene and CHS8:TMV replicase constructs during plant transformation and select for kanamycin and hygromycin resistant plants.
Further demonstration of CHS8:TMV-mediated *O. aegyptiaca* resistance will require interaction in soil-grown plants to assess the entire *O. aegyptiaca* lifecycle. However, these results, demonstrating the co-expression of CHS8:TMV replicase transgene and an endogenous N gene contained within *N. tabacum* cv. Xanthi, is among the first examples of engineering enhanced resistance to a parasitic weed based on a novel gene-for-gene strategy. The co-expression of a *R-avr* gene pair to create novel phytopathogen resistance has been established (Jones et al., 1994). Several studies have utilized distinctly different molecular approaches to try to enhance resistance of plants to *O. aegyptiaca*. Hammamouch et al. (unpublished) has used the sacrotoxin gene from the flesh-fly, *Sarcophaga peregrina*, that encodes a potent antibacterial toxin which appears to be preferentially toxic to the parasite. Sarcotoxin was expressed in transgenic tobacco and initial assessments suggest it is effective in reducing *O. aegyptiaca* development and parasitism. Joel et al. (1995) generated herbicide-resistant crops that expressed a particular herbicide (see Chapter 1 for details). The herbicide resistant transgenic crops survived herbicide application that successfully reduced the number of parasite attachments, resulting in normal growth and flowering of the host. This research represents a different strategy for engineering *O. aegyptiaca* resistance that allows the herbicide to be translocated through the host plant directly into the vascular system of the parasite. This strategy has the potential to provide long-term control of *O. aegyptiaca* disease in the field. However, new races of the parasite may evolve from the continued use of herbicide-resistant transgenic crops, and extensive herbicide use may not be financially feasible in many regions of high *O. aegyptiaca* infestation.
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Chapter III

THE EFFECT OF INDUCIBLE PROTEINASE INHIBITOR EXPRESSION
ON GLOBODERA TABACUM DEVELOPMENT AND DISEASE
II.1 INTRODUCTION

Sedentary endoparasitic nematodes are destructive obligate root parasites that occur worldwide. These nematodes cause over $100 billion per year in annual crop losses and are most severe in temperate and tropical regions (Sasser and Freckman, 1987). Damage caused by the cyst (Globodera and Heterodera spp.) and root knot nematodes (Meloidogyne spp.) accounts for 80% of these losses, thus making these the species major nematode pests of agriculture. Of particular interest is the impact of cyst nematode disease on economically important crops. Unlike the root knot nematode, cyst nematodes have narrow host ranges and are particularly severe on Solanaceae, Leguminosae, Graminaceae, and Cruciferaceae crops. Infections caused by the cyst nematode can reduce crop yields by as much 30% to 75% (Agrios, 1988).

The control of cyst nematodes relies heavily on the use of nematicides. Nematicides have been the most unacceptable class of pesticides and tend to be toxic, expensive to use, and dangerous to the environment (Urwin et al., 1998). Several of the most effective and widely used nematicides are being now banned, due to their potential hazard to human health (Giannakou et al., 2002). Nematode-resistant cultivars could provide the best long-term control and are safer for the environment than using nematicides. Although traditional breeding has been successful in some crops, resistance genes have not been identified for many important crops (Whitehead, 1998; Gheysen et al., 1996). It is evident that there is a need to develop more environmentally favorable strategies for the control of plant parasitic nematodes such as Globodera spp.

The lifecycle of both the cyst and root knot nematodes require the establishment of complex and intimate relationships with their hosts. Infectious second-stage juveniles penetrate the host root and migrate intercellularly by using enzymatic secretions and mechanical force. During migration, the nematode secretes cell wall degrading enzymes such as β-1,4-endoglucanases, into the plant cell to aid in the cell wall degradation and intercellular movement (Wang et al., 1999; Smant et al., 1998). To establish a feeding site, the nematodes use their stylet to puncture five to six procambial cells surrounding their head and begin to feed on the cytosolic nutrients. Nematode feeding induces these cells to undergo a series of metabolic changes that modify the host cells to support nematode development and survival. During feeding, contents of their salivary glands include digestive proteases chymotrypsin and trypsin.
There are critical steps in the lifecycle of sedentary endoparasitic nematodes that can serve as potential targets for the control of nematodes. Possible approaches can include the development of transgenic plants with enhanced nematode resistance, including anti-invasion and migration strategies, feeding cell attenuation, and anti-nutrient/anti-feeding strategies (Urwin et al., 2001). Plants produce inhibitors specific for the nematode proteinases, chymotrypsin and trypsin. Proteinase inhibitor (PI) proteins have been identified as defense proteins that can protect plants against predation by insects, plant parasitic nematodes, and microbial pathogens (see Ryan, 1973, 1981, 1989, and 1990 for detailed reviews). Many studies have utilized PIs specific for serine and cysteine classes of proteases in anti-feeding strategies to enhance crop resistance to plant parasitic nematodes (Urwin et al., 1997; Lilley et al., 1996; Atkinson et al., 1996; Urwin et al., 1995; Hepher and Atkinson, 1992).

Zhang (1994) showed that transgenic expression of potato PI-I (chymotrypsin inhibitor) significantly reduced nematode development, and root galling on tomato (*Lycopersicon esculentum*) after *M. hapla* inoculation. For this experiment, transgenic plants were generated that constitutively expressed the coding regions of either PI-I or PI-II under the control of the 35S Cauliflower Mosaic Virus (35S) promoter. Transgenic seedlings were inoculated with *M. hapla* eggs. PI-I and PI-II transgenic plants reduced the number of adult females by 40% and 90%, respectively when compared to adult females present in nontransformed plants. Transgenic tomato (*Lycopersicon esculentum*) plants grown in the greenhouse and inoculated with nematodes were significantly reduced in the number of galls and eggs at 5 and 8 wk after inoculation compared to the nontransformed control plants. However, 10 wk after nematode inoculation, resistance disappeared and there were no significant differences in galling or egg mass production on control or PI transgenic lines. These results suggest that PI proteins are effective toxins (anti-feedants) against sedentary endoparasitic nematodes, however, the 35S promoter may not be capable of driving expression throughout the period of parasite interaction. In fact, Goddijn et al. (1993), using the 35S:GUS transgenic plants, demonstrated that the cyst and root knot nematodes specifically inhibited the 35S promoter within the syncytia and giant cells, respectively. Based on these results, our goal is to test the hypothesis that the PI-I gene linked to a nematode-inducible promoter will provide more effective and long-term protection. The tomato *Hmg2* promoter has been characterized as a nematode-inducible promoter (Cramer et
al., 1993) and was selected in this study to locally express the tomato PI-I gene in response to parasitism by the cyst nematode.

Additionally, the impact of cellular location of PI proteins (secreted versus cytosolic) on the effectiveness of this anti-feeding strategy was tested. Proteinase inhibitors are normally targeted to vacuoles or secreted to the extracellular space (Narvaez-Vasquez et al., 1993). However, if the majority of nutrient acquisition for the nematode is via the stylet, which is inserted into the host cell, cytosol localization of PI-I may be more effective in controlling nematode development. This study addresses two questions: (1) can a nematode-inducible promoter drive production of effective levels of PI-I to enhance cyst nematode resistance and (2) will PI-I retained intracellularly be more effective than the extracellular deposition of inhibitor proteins? To address these research questions, transgenic tobacco (*Nicotiana tabacum*) plants were generated that expressed an intracellular PI product (lacking its signal peptide) and an extracellular PI product (signal peptide included) under the control of the nematode-inducible promoter, *Hmg2*, and tested for their interactions with the tobacco cyst nematode, *Globodera tabacum tabacum*.

II.2 MATERIALS AND METHODS

III. 2.1 *HMG2* induction by cyst nematode

Tobacco seeds of line 330.1-002 (Yu, 1995) expressing *Hmg2* promoter:GUS reporter gene fusions (Cramer et al., 1993) were surface sterilized with ethanol, 30% sodium hypochlorite, and sterile distilled water. Seeds were germinated on 1X MS agar plates that were positioned vertically to allow tobacco roots to grow parallel to the agar surface. *Globodera tabacum tabacum* cysts were surface sterilized with 10% sodium hypochlorite, rinsed with sterile distilled water, and placed on plates containing germinated transgenic tobacco seedlings. Infectious second-stage juveniles hatched 4 to 5 d following inoculation. Induction of the *Hmg2*:GUS transgene was visualized by using the GUS reporter gene assay (Jefferson, 1987). Nematodes were stained in pink with acid fuschin.

III. 2.2 Construction of *Hmg2* promoter: PI-I vectors

The *Hmg2* MeGA promoter was provided by CropTech Corporation (Blacksburg, VA) in a plasmid vector designated as pCT151. The tomato proteinase inhibitor gene was provided by Dr. Clarence Ryan (Washington State University, Pullman, WA.) in a plasmid vector. The following oligonucleotide primers were designed to amplify the entire PI-I sequence and provide
flanking NcoI and SstI restriction sites (underlined): primer 1-5' GCACCATGG ACTCAA AGTATTGCTC 3', primer 2-5' TGAACCTCGA GTAATTAAG TCACCACAGGC 3'. To determine if proteinase inhibitors are more effective against nematode feeding if retained intercellularly, a PI-I sequence lacking the 5' end of the coding region encoding the PI-I signal peptide was generated using a third primer, primer 3-5' CTACCATGG CACGAAAAG AGAATTG 3' and primer 2. For the amplification of the full-length PI-I (secreted form), the PCR parameters (temperature, time, number of cycles) were as follows: 6 cycles at 98 °C, 1:30 min; 58 °C, 2 min; 72 °C, 2 min; 6 cycles at 98 °C, 1:30 min; 44 °C, 2 min; 72 °C, 2 min; 35 cycles at 98 °C, 1 min; 58 °C, 1 min; 44 °C, 1 min; 72 °C, 2 min; and a final extension of 72 °C, 5 min. The PCR parameters for the retained PI-I gene product included: 1 cycle at 95 °C, 1 min; 44 °C, 5 min; 72 °C, 2 min; 5 cycles at 95 °C, 1 min; 44 °C, 2 min; 72 °C, 2 min; 30 cycles at 95 °C, 1 min; 58 °C, 2 min; 72 °C, 1:50 min; and final extension at 95 °C, 1 min; 58 °C, 2 min; 72 °C, 5 min. PCR products of 350 bp (for vacuolar secreted PI) and 300 bp (for retained PI) were generated, digested with NcoI and SstI (Invitrogen Life Technologies, Carlsbad, CA), gel purified, and inserted into pCT151, a pUC18 vector containing the 0.4 kb Hmg2 promoter with an NcoI site engineered at the ATG start codon. Ligations were performed according to the protocols described by Sambrook et al. (1989). Plasmids were transformed into electrocompetent Escherichia coli strain DH5α using the One Shot transformation kit according to the manufacturers recommendations (Invitrogen Life Technologies, Carlsbad, CA). Sequence fidelity of PCR products and ligations were confirmed by sequencing (Virginia Bioinformatics Institute DNA Sequencing Facility, Blacksburg, VA).

III. 2.3 Genomic extractions

Leaf tissue (3 g) from PI 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 (Murray and Thompson, 1980). Presence of the transgene was initially established by PCR amplification using 100 ng of genomic DNA and conditions described above, and yielded products of the expected 300 bp.

III. 2.4 RNA isolation and RT-PCR

Seeds of transgenic plants were germinated on MS (Murashige and Skoog, 1969) media containing kanamycin (200mg/L), harvested after three weeks, and frozen in liquid nitrogen.
Total RNA was purified from transgenic cotyledons with TRIreagent (MRC, Inc., Cincinnati, OH.) according to manufacturers recommendations with an additional phenol/chloroform purification step. First strand cDNAs were synthesized using the Prostar cDNA kit (Stratagene, La Jolla, CA) and commercial oligo d(T) primer. PCR was subsequently performed with gene specific primers: primer 4, MeGA-PI forward (5’AATCCCAAAACAACACTT3’)) and primer 5, MeGA-PI reverse (5’AGAGGAACTCGATCACATAGA3’). Primers were designed to anneal to the 3’UTR of the MeGA promoter and the coding region of the PI gene, respectively. Using pURETaq Ready-To-Go beads (Amersham Pharmacia, Piscataway, NJ), a 285 bp fragment was amplified with the following PCR protocol: 1 cycle at 95 °C for 3 min; 25 cycles at 94 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min; and 1 cycle at 72 °C for 10 min.

III. 2.5 Protein extraction and western analysis

To accumulate PI protein, fully expanded leaves (propagated under greenhouse conditions) were harvested from 46 cm plants, wounded by passage through a pasta maker (producing uniform leaf strips approximately 2 cm in width), and incubated on the bench at room temperature for 48 hrs. Samples were homogenized in 1X PBS (Phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$) at a 1:2 ratio of leaf tissue to buffer. Protein samples were processed and concentrated by a series of ammonium sulfate precipitations at 30% and 50% w/v according to standard techniques (Sambrook et al., 1989). Proteins were size-separated on 16% SDS-PAGE gel (Invitrogen, La Jolla, CA) under reducing conditions used for western immunoblots. Gels were electrophoresed at room temperature with constant voltage (110V) and capillary transferred onto Hybond N+ nitrocellulose membrane (Amersham Pharmacia, Piscataway, NJ). Gel electrophoresis and blotting was carried out as described by Sambrook et al. (1989).

Immunological detection was performed with rabbit anti-potato PI-I antibodies. Purified potato proteinase inhibitor I (8 kDa) was used as a standard. PI-I protein standard and antibodies, were kindly provided by Dr. Gregory Pearce (Washington State University, Pullman, WA). Primary antibody concentration was used at a 1:10,000 to detect the presence of PI proteins approximately 8 kDa in size. Commercial alkaline-phosphate–conjugated goat-anti rabbit IgG-AP antibodies were used as secondary antibody at a dilution of 1:5000 (Biorad Laboratories, Hercules, CA).
III. 2.6 Nematode interactions

Cysts of tobacco cyst nematode (*Globodera tabacum tabacum*) were obtained from Dr. Jonathan Eisenback (Virginia Tech, Blacksburg, VA). The protocols developed by Zhang (1994) were used to test the transgenic tobacco plant-nematode interactions. In these procedures, both control and transgenic \textit{Hmg2}:PI-I plants (primary transformants) were clonally propagated to obtain three replicates from each plant line and grown in 36 cm pots containing Promix BX/PGX soil mix (2:1 ratio; Wetsel, Harrisonburg, VA). After growing for 2 weeks, the plants were inoculated with 50 cysts. Primary roots were harvested 7 weeks after inoculation. To harvest, plants were ratooned and using a metal cylinder the primary root soil cores (13 cm diameter by 23 cm deep) were collected for each sample. Three 250 cc soil samples were obtained from soil that was mechanically removed from the primary root cores. Soil samples were processed using a semi-automatic elutriator to remove cysts present in the soil. (Byrd et al., 1976). To recover additional cysts, each root core was manually rinsed above a stack mesh sieves; numbers 25, 60, and 400 $\mu$m, respectively. Recovered cysts from elutriated soil samples and manually rinsed root samples were combined and enumerated. The effects of the proteinase inhibitor gene expression on root biomass and cyst development were determined. Statistical analyses were performed using a students t-test in a SPSS statistical package. Images were captured using a Sony Cybershot digital camera (Model no. DSC-S70).

III.2.7 Statistics

One experiment was performed and numbers of cysts present on different lines were significantly different. Data represent means of three observations (3 replicates per plant line) and means were separated by a students T-test. Lines were different at the 0.00001 level. Analysis was performed by ANOVA.

III. 3 RESULTS

III. 3. 1 \textit{HMG2} induction by cyst nematode infection

The tomato \textit{Hmg2} promoter was previously shown to be induced by the root knot nematodes, \textit{M. incognita} and \textit{M. hapla} (Cramer et al., 1993). To determine if \textit{Hmg2} is also induced in response to parasitism by the cyst nematode (*G. t. tabacum*), axenically grown transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) seedlings expressing \textit{Hmg2} promoter:GUS reporter gene constructs were inoculated with \textit{G. t. tabacum} cysts. At various times after
addition of cysts, seedlings were harvested and histochemically stained for GUS activity (X-gluc) and the presence of nematodes (acid fuschin). As shown in Fig. III.1, GUS activity was localized to the nematode feeding site. *Hmg2*:GUS induction was observed in the majority of sites of ingress (Umayam and Cramer, unpublished results) indicating that the *Hmg2* promoter is activated by both cyst and root knot nematodes.
Fig. III.1. Induction of tomato Hmg2 in response to parasitism by the cyst nematode, *Globodera tabacum*. Seedlings of transgenic tobacco expressing a Hmg2 promoter:GUS construct were inoculated with cysts and incubated to allow nematode ingress and establishment of feeding sites. Hmg2 promoter activity was visualized using GUS assay (blue stain). Nematodes were stained with acid fuchsin (pink stain). Experiments were performed by Lowell Umayam as a part of an undergraduate research project under the supervision of Cramer and McMeans.
III. 3. 2 Production of tobacco expressing *Hmg2* promoter: PI-I gene constructs

Two constructs were generated that fused sequences encoding the tomato proteinase inhibitor I gene to 0.4 kb of the nematode-inducible *Hmg2* promoter. The first construct (EM1) included a truncated tomato PI-I coding region, which lacked the PI-I signal peptide, fused to the *Hmg2* promoter. The EM1 product should be retained in the cytosol. The second construct (EM2) consisted of the full length tomato PI-I coding region fused to the *Hmg2* promoter and should be secreted to the extracellular space or to the apoplast. PI sequences were generated by PCR amplification to provide flanking restriction sites and an ATG start site. The PCR resulted in PI products that were 290 bp and 354 bp, respectively, (Fig. III.2) that were fused to the tomato *Hmg2* promoter (Fig. III.3). The constructs, EM1 and EM2 were ligated into the binary transformation vector pBIB-kan as 698 bp or 754 bp *SalI-SstI* fragments, respectively, and introduced into tobacco using *Agrobacterium tumefaciens*-mediated transformation by the petiole transformation method (Medina-Bolivar et al., 2003).

More than 30 transgenic tobacco lines were regenerated for each construct. Because tobacco contains closely related PI-I homologs to tomato, plants were initially screened for T-DNA insertion by PCR amplification of PI-I and NPTII. A total of 20 plants (10 EM1 plants and 10 EM2 plants) were screened via PCR. PCR amplification of PI-I products with primers 2 and 3 (Fig. III.3) yielded the expected 290 bp products in 14 out 20 plants (9 EM1 plants and 5 EM2 plants). Using NPTII (neomycin phosphotransferase) gene-specific primers 6 and 7 (Fig. III.4a), amplification resulted in 291 bp products in plants (Fig. III.4b).
**Fig. III.2.** Electrophoresis of PCR amplification products of tomato proteinase inhibitor I (PI-I) genes. PCR results show a (A) 354 bp product amplified with PI-I gene-specific primers 1 and 2 and a (B) 290 bp product with PI-I gene specific primers 2 and 3 which excludes the region encoding the signal peptide. PI-I plasmid DNA from tomato (25 ng) was used as template in both reactions.
Fig. III.3. *Hmg2* promoter:PI-I gene constructs EM1 (A) and EM2 (B) displaying gene specific primers as described. SP: signal peptide; tss: transcription start site; ATG: translational start site.
Fig. III.4. Construction of Hmg2:PI-I Agrobacterium tumefaciens plant transformation vector, by insertion into pBIBkan. (A) T-DNA region of engineered vector. NPTII gene specific primers shown as P6 and P7. The vector contained a neomycin phosphotransferase resistance gene (NPTII), plant promoter (Pnos), plant terminators (Pnos, pAg7), and the T-DNA border sequences (LB, RB) that define the region to be transferred into the plant genome. (B) PCR amplification of neomycinphosphotransferase gene (NPTII) fragments 291 bp in size to confirm stable integration of T-DNA into tobacco. Genomic DNA (100 ng) from transgenic (1-8) and Xanthi nontransformed plants (NTX) (Lane 9) were used as template with NPTII gene specific primers. Positive control (lane C) consists of 25 ng of pBIBkan plasmid DNA used as template.
### III. 3. 3 Expression analysis of PI in transgenic plants

Analysis of the expression of the PI-I transgenes in plants at the transcript and protein level was challenging due to the presence of endogenous defense-inducible PI homologs. Tobacco PI-I shows 72% identity at the nucleic acid level compared to the tomato PI-I transgene. Because this similarity exists among tobacco and tomato homologs, it is difficult to accurately analyze the expression of PI transgenes. Cross-reactivity presents a challenge as well in regards to immunodetection with PI-I specific antibodies.

The *Hmg2* promoter is developmentally regulated and induced in response to wounding and/or pathogen attack (Cramer et al., 1993). From previous studies it has been established that the genes under the control of this promoter are highly expressed throughout the cotyledons during the seedling development (Cramer unpublished). Therefore, transcription of the tomato PI-I gene was analyzed by reverse transcription polymerase chain reaction (RT-PCR) with total RNA from kanamycin-resistant 1st generation transgenic tobacco seedlings of 11 lines expressing EM1 or EM2 (Fig. III.5). To ensure that RT-PCR of fragments represented products from the transgene and not endogenous PIs, primers p4 (from 5’-UTR from *Hmg2*) and P5 or P2 (from PI-I) were used (primer positions shown in Fig. III.3). Fragments of approximately 280 bp were amplified from EM1 samples using primers 4 and 5. Among the EM1 lines, plant PI-300#5 was identified as the highest expressor based on RT-PCR results (Fig. III.5). Amplification products of 370 bp were obtained from EM2 samples using primers P2 and P4.

Based on detection of PI-I transcript, four transgenic tobacco lines containing EM1 (PI-300 #3, 5, 8, and 13) and three transgenic tobacco lines containing EM2 (PI-350 #1, 3, and 5) were tested at the protein level. To induce PI-I proteins, fully expanded leaves were harvested, wounded by passage through a pasta maker, and incubating at room temperature for 48 hr. Proteins were extracted as previously described and analyzed by western immunoblotting using rabbit anti-potato PI-I antibodies. Protein from nontransformed wild type plants were used as a negative control. As shown in Fig. III.6, the anti-PI-I antibodies cross-reacted with PI-I proteins and sizes were consistent with the purified potato PI-I used as a standard. Proteins were detected in PI-I transgenic tobacco plants containing EM1 and EM2 that were 8 kDA in molecular weight (Fig. III.6). Consistent with transcript expression results, protein detected from lines containing EM1 were greater than EM2 expressing plant lines. No proteins were detected in Xanthi nontransformed control plants.
Fig. III.5. Reverse transcriptase-polymerase chain reaction (RT-PCR) with promoter and PI-I gene-specific primers. PCR amplification of EM1 resulted in 285 bp products using primers p4 and p5 (Lanes 1-9). PCR amplification of EM2 resulted in 370 bp products using primers P2 and P5 (Lanes 1-9). PI-I expression was determined by RT-PCR with total RNA from the cotyledons of 2 wk old transgenic tobacco seedlings. Positive control (C) consisted of 25 ng EM2 plasmid DNA used as template with primers P4 and P5 resulting in the amplification of a 280 bp product.
**Fig. III.6.** Western analyses of EM1 and EM2 transgenic plants. Total soluble protein extracted from leaves 48 hrs after wound induction was denatured and separated on 16% SDS-PAGE gels. Twenty micrograms of total proteins were loaded for each sample. PI-I protein was detected using rabbit anti-potato PI-I antibodies at 1:10,000 dilution to detect of tomato PI-I proteins in transgenic tobacco plants. Nontransformed Xanthi (NTX) protein used as a negative control, samples 3, 5, 8, 13 contain EM1 proteins; samples 1, 3, 5 contain EM2 proteins. Commercial goat anti-rabbit secondary antibodies IgG were used at 1:5000 dilution (Biorad Laboratories Inc, Hercules, CA). Purified potato PI-I was 8 kDa in molecular weight and was used as a standard (std.).
III. 3. 4 Impact of transgenic on cyst nematode interaction

The effects of the *Hmg2:PI-I* transgenes on cyst nematode disease development was evaluated. A total of seven lines (four of EM1 encoding PI-I without a signal peptide and three of EM2 encoding native PI-I) were inoculated with *Globodera tabacum* cysts under greenhouse conditions and evaluated for cyst reproduction and disease development. Nontransformed tobacco plants were inoculated in the same manner and used as negative controls. Plants were harvested 7 wk after inoculation and the primary root soil cores were removed (see Methods for description). Soil samples were collected from the root cores and elutriated to extract cysts present in the soil. In addition, root cores from each plant line were manually rinsed for cyst removal and cysts were pooled with respective elutriated soil samples. There was a significant reduction in the number of cysts extracted from plants expressing EM1 compared to the number of cysts on control and EM2 expressing plants (Fig. III.7). Differences in root masses were also observed (Fig. III.8). The most severe disease symptoms were observed on the roots of nontransformed and transgenic tobacco line EM2-3 (Fig. III.8A and III.8G). These plants displayed stunted roots that were brown in color or necrotic-like in appearance (Fig. III.8). EM1 transgenic plant lines 3, 5, 8, and 13 appeared to have been protected by the expression of PI-I retained product, having fewer cysts present and larger root masses than nontransgenic plants (Fig. III.8 and Fig. III.8B-D). EM2 transgenic plant lines 1, 3, and 5 had significantly more cysts present than EM1 expressing plants, however, there was no significant difference between the root masses of EM2 transgenic lines 1 and 5 (Fig. III.8F and III.8H) according to the statistical analysis. These results suggest that the intercellular PI-I form, EM1, is more effective on cyst nematode development and disease than the vacuolar/extracellular PI-I form, EM2.
Fig. III.7. Cyst nematode production on PI-I tobacco roots. Nontransformed *Nicotiana tabacum* var. Xanthi, *Hmg2*: PI-I transgenic lines, EM1 (PI300-encodes the intercellular PI; -SP3, -SP5, -SP8, -SP13) and EM2 (PI350-encodes native PI that is secreted; +SP1, +SP3, +SP5) were inoculated with 50 *Globodera tabacum* cysts per pot. Plants were harvested 7 wk after inoculation and enumerated for cysts. Data represent means of three observations (3 replicates per plant line) with bars representing standard error. Means were separated by T-test. One experiment was performed and lines were different at the 0.00001 level. Analysis was performed by ANOVA.
Fig. III.8. Cyst nematode disease on nontransformed *Nicotiana tabacum* cv. Xanthi (A) and *Hmg2*:PI-I transgenic lines (B-H) 7 wk after inoculation. Transgenic PI plants expressing EM1 (B-E) reduced the number of cysts on plants, while transgenic PI-I plants expressing EM2 has similar amounts of cysts when compared to the nontransformed plants. Nontransformed Xanthi (A) and transgenic PI-I line expressing EM2-PI350#3 (G) appeared to have a significant reduction in the root mass and severity of nematode disease.
III. 4 DISCUSSION

Previous studies have established the effectiveness of proteinase inhibitor genes against herbivore predation. Urwin et al. (1997) shown that transgenic *Arabidopsis thaliana* expressing a serine PI-I from rice, oryzacystatin, under the control of the cauliflower mosaic virus 35S promoters effectively reduced the growth, development, and fecundity of both the cyst and root knot nematodes. These results were confirmed by Urwin et al. (1998). Mechanisms for the co-delivery of multiple effectors were explored via a single transgene under the control of the 35S CaMV. A cysteine and serine PI, that showed distinct effects against the cyst and root knot nematodes, were joined as translational fusions by one of two peptide linkers and expressed in *A. thaliana*. The cowpea trypsin inhibitor, CpTI, was selected due to its influence on sexual fate, while the modified cystatin suppresses nematode growth. The properties of the peptide linkers established the mode of delivery either separately or as a fusion product with the ability to be cleaved *in planta* (see chapter 1 for details). Plants expressing cystatin alone or with CpTI+ refractory peptide linker, showed a significant decrease in the number of both the cyst and root knot saccate females as compared to control plants. In addition, plants expressing cystatin and/or CpTi in the presence of the peptide linker that is cleaved within the plant had no effect on nematode development or disease. In a similar study Zhang, Eisenback, Cramer, Radin and Ryan (unpublished data), evaluated the effect of constitutively expressed the tomato PI-I or PI-II on root knot nematode disease and development. PI-I reduced the formation of root galls, nematode development, and egg mass production of the root knot nematode during the earlier nematode infection stages but did not during later stages indicating that resistance was lost. Results to date suggest that strategies using recombinant PI-I’s can effectively enhance plant resistance to nematodes. However, the majority of these studies utilize a constitutive promoter to over-express PI genes in nematode resistant transgenic plants. It has been demonstrated that nematode parasitism can down-regulate some promoters including 35S CaMV (Goddijn et al., 1993).

The current study represents the next generation of promoters used in PI-based anti-feeding defense strategies against plant parasitic nematodes. We generated transgenic plants expressing the coding regions of the tomato PI-I gene under the control of the *Hmg2* nematode-inducible promoter. In an effort to delineate the most effective mechanism of resistance, this strategy included a second level of complexity. It has been established that sedentary
endoparasitic nematodes ingest plant cell components while both migrating through the host root in search of a feeding site and during feeding cell establishment. Based on this knowledge, two types of PI transgenic plants were generated whose products should either be retained in the cytosol or secreted out into the apoplast or extracellular space (EM2). Sedentary endoparasitic nematodes (particularly females carrying eggs) feed on host cells for a longer period of time during feeding site establishment than migrating nematodes. Transgenic plants that produced the retained PI-I product (EM1) significantly reduced the number of cysts as compared to the cysts found on nontransformed plants and transgenic plants expressing the secreted PI-I product (EM2). The results from this study suggest that the intercellular PI-I product enhanced plant resistance to the cyst nematode and significantly reduced cyst development. Reducing the amount of female nematodes could greatly impact the severity of diseases caused by nematodes in the field by significantly lowering disease pressure. Utilizing a cytosolic PI-I product under the control of an inducible promoter shows promise and could potentially provide effective, long-term control of cyst nematode disease in the field. In addition, an anti-feedant expressed locally has significant implications from the point of view of food crops. PI products that result from constitutive expression, may be available for consumption in foods crops. The advantage of using an inducible locally expressed PI versus constitutively expressed PI is that the localized expression of these products would alleviate the production of proteinase inhibitors in food crops by only synthesizing PI’s when needed upon pathogen attack.

The study demonstrates that PI-I retained intracellularly inhibited the development of the cyst nematode, however, further experiments are needed to characterize the effect of PI-I transgenes on cyst nematode disease. This feasibility study represented primary infections (one lifecycle) caused by cyst nematode. To understand the long-term impact of transgenic inducible PI expression on cyst nematode disease and to mirror field infestations where cyst nematodes go through multiple lifecycles, future experiments will be performed for a longer duration to increase disease pressure and to assess plant biomass and yield. Additionally, the site of PI transgene accumulation must be determined to efficiently evaluate the effect of PI expression on cyst production. The use of biochemical methods to assay PI activity or location in syncytia would offer more definitive results, however, nematode infection sites involve relatively few cells and are unlikely to provide further delineation of PI site accumulation in EM1 versus EM2 plants. Immunolocalization studies represent an efficient and reliable method to establish the
subcellular location of our transgenic PI’s and to better characterize PI transgenic plants. The development of more definitive tools (e.g., more sensitive and specific antibodies or epitope tagged PI) will also facilitate screening of transgenic plants to identify lines that express PI’s at higher levels. Confocal microscopy of immunofluorescent labeled PI-I would allow transgenic PI-I accumulation to evaluated more effectively.
III. 5 REFERENCES


Chapter IV

CONCLUSIONS AND FUTURE DIRECTIONS
IV.1 CONCLUSIONS AND FUTURE DIRECTIONS

Over billions of dollars are lost each year due to infections caused by plant pathogens which compromise the world’s food supply. *Orobanche aegyptiaca* (Egyptian broomrape) and *Globodera tabacum tabacum* (cyst nematode) are obligate root parasites that cause severe yield and quality losses in several important dicotyledoneous crops (Parker and Riches, 1993; Urwin et al., 1997). Although these parasites represent two diverse classes, they share significant similarities. Both parasites establish complex and close relationships with their hosts during initial infection and nutrient acquisition. Conventional methods have had limited success in controlling these recalcitrant pests resulting in the search for effective alternative methods. Understanding the molecular mechanisms involved in these host-parasite interactions has facilitated the development of resistance strategies that utilize genetic engineering.

Plants have evolved elaborate defense mechanisms against invading pathogens and herbivores. These defense responses are either constitutive, providing pre-existing biological or physical barriers, or induced by the invading organism. Upon recognition of a pathogen, the plant initiates a cascade of signal transduction events activating defense-related genes and subsequent synthesis of proteins encoded by these genes. The activation of these genes directs the synthesis of phytoalexins, proteinase inhibitors (PIs), reactive oxygen species, cell wall fortification materials, pathogenesis-related (PR) proteins, as well as genetically programmed cell death of infected cells or hypersensitive response (HR). Additionally, plants can mediate apoptosis by the induction of disease resistance (R) gene products. For centuries, agricultural crops have bred for increased resistance against pathogens, identifying a number of genetic traits primarily due to single genes (Ryan and Jagendorf, 1995). Although the molecular mechanisms of these interactions are not fully understood, several R genes have been cloned from a variety of hosts and characterized in recent years. It is thought that R genes encode putative receptors that interact with a pathogen avirulence (*avr*) gene products or ligands (Bent, 1996). In a compatible host-pathogen interaction, gene products of matched specificity result in a local cell necrosis (or HR) limiting the development and spread of the invading organism. Based this resistance response, R genes have been transferred to susceptible hosts of the same species or to other plant species through genetic transformation to enhance resistance to a variety of pathogens.

Plant gene products such as R genes and proteinase inhibitors, have been used in genetic engineering strategies to enhance resistance to a broad array of organisms. In particular, gene-
for-gene resistance strategies have been used to successfully create resistance to fungi, bacteria, viruses, and nematodes (Bent, 1996; Hammond-Kosak and Jones, 1997; McDowell and Woffenden, 2003). Although resistance genes against Egyptian broomrape have been identified, they are not well characterized and no gene-for-gene resistance system is available. We utilized a well-known $R/avr$ gene pair, the tobacco N gene and the Tobacco Mosaic Virus replicase gene (TMV), to engineer resistance to Egyptian broomrape (Whitham et al., 1994; Dinesh-Kumar et al., 1995). In an effort to test this strategy, we successfully generated transgenic plants expressing TMV replicase gene under the control of a broomrape-responsive promoter the bean chalcone synthase 8 (CHS8) promoter. Constructs were introduced into tobacco lines carrying an endogenous N gene. Because CHS8 is wound-inducible, wounding experiments were performed on transgenic leaves to determine if TMV replicase expression resulted in the production of local cell necrosis, defined as the HR. Two replicase-expressing tobacco lines (CHS8:TMV-8 and CHS8:TMV-9) showed necrosis around the wound site periphery. Northern analysis confirmed that these lines expressed the TMV replicase gene at low levels. *O. aegyptiaca* inoculation studies, performed in polyethylene bag (PE) to permit observation, showed local necrosis or darkening directly underneath the site of parasite attachment during the earlier stages. This darkening was observed in multiple transgenic lines and not in nontransformed Xanthi control plants. These results suggest that this phenotype resulted from the expression of transgene products and not the site of transgene insertion. The number of parasite attachments were enumerated on transgenic and nontransformed control plants. There was 75% reduction in tubercle on a single TMV-expressing line, CHS8:TMV-9 when compared to those on nontransformed control plants. Additionally, advanced developmental stages were observed on both transgenic and nontransformed control plants. These results suggest that N gene-mediated resistance can enhance resistance to *O. aegyptiaca*, however, partial resistance was obtained. N gene expression has been well characterized in leaf tissues (Dinesh-Kumar et al., 2000), but these expression patterns are not known in roots. The fact that partial resistance was obtained may be attributed to low endogenous N gene expression under the control of its own promoter or possibly parasite mediated suppression of activity. In future experiments this problem may be alleviated by the introduction of a recombinant N gene under the control of the additional *O. aegyptiaca*-inducible *Hmg2* promoter. To determine N gene expression levels in
roots, Northern analysis or real-time RT-PCR can be performed on RNA extracted from uninoculated and \textit{O. aegyptiaca} parasitized root tissues.

The HR is characterized by a rapid collapse of host cells in the vicinity of pathogen ingress that appears with the inhibition of pathogen growth (Innes, 1995). The production of HR is primarily associated with R-gene mediated resistance. We believe that the darkening observed throughout \textit{O. aegyptiaca} parasitism is a true HR resulting from interaction of the N gene and TMV replicase gene products. Confirmation of local cell death can be facilitated by the use of vital dyes. Vital stains such as Evans blue are routinely used to evaluate cell death \textit{in situ} (Baker and Mock, 1994; Costet et al., 1999). In future experiments, the \textit{O. aegyptiaca} inoculation studies will be repeated and following enumeration of parasite attachments, host root tissues with \textit{O. aegyptiaca} tubercles attached will be excised and evaluated for HR by the use of Evans blue. In addition, data will include the further delineation of dead tubercles versus living tubercles to determine the effectiveness of our engineered gene-for-gene resistance on parasitism. These experiments will include inoculation studies performed in soil to assess the transgene impact on the full lifecycle of \textit{O. aegyptiaca}. Plants will be harvested when wildtype control plants show emergence of multiple parasitic flowering spikes (but prior to seed production due to quarantine restrictions) and the overall parasite biomass will be measured. This should provide a more representative test of potential impacts of \textit{O. aegyptiaca} parasitism over the long-term in the field. This evaluation will determine the field utility of N gene-mediated resistance system in response to \textit{O. aegyptiaca} development and parasitism.

We believe that these results show promise in potentially providing complete and durable resistance to \textit{O. aegyptiaca}. This is the first demonstration of gene-for-gene resistance against a parasitic weed. When using gene-for-gene resistance strategies, it has been a concern that resistance is short-term and can be overcome by evolving pathogenic strains. This phenomenon occurs when the pathogen is continuously exposed to hosts with resistance based on a particular \textit{avr} gene. This provides selective pressure for pathogens that have mutated the \textit{avr}, therefore changing the conformation of the \textit{avr} ligand. In this event, the host R gene can no longer “recognize” the \textit{avr} gene resulting in loss of resistance. In this strategy, the N gene only recognizes counterparts from TMV (replicase or coat protein which is encoded in the host genome). Therefore \textit{O. aegyptiaca} does not have ability to overcome resistance through mutation of the TMV replicase. The trigger for replicase induction is presumably elicitors.
released by wounding and/or plant cell degradation, critical components for successful initiation of *O. aegyptiaca* parasitism. Thus parasite mutations that would block CHS8:TMV induction would likely be incapable of effective attachment. Thus our strategy may have significant advantages over traditional gene-for-gene resistance for durability in the field. In fact, utilizing *N* gene-mediated resistance may provide long-term and durable resistance to other serious weed species such as *Striga* spp. and a broad array of fungal, bacterial, or nematodal pathogens. To test this hypothesis, additional pathogenicity studies can be performed with tobacco pathogens such as *Rhizoctonia solani* (causal agent of target spot), *Pseudomonas syringae* pv. *tabacina* (causal agent of angular leaf spot), or *Globodera tabacum* (cyst nematode disease).

Proteinase inhibitors can have been used as effective anti-feedants to enhance resistance to insects and nematodes (Urwin et al., 1997; Thomas et al., 1995; Johnson et al., 1989). Tomato PI-I and PI-II, constitutively expressed in transgenic tomato (*Lycopersicon esculentum*) plants have significantly reduced *Meloidogyne* (root-knot nematode) development, egg mass production, and disease symptoms such as root galling (Zhang, 1994). In this study, enhanced resistance was observed during the earlier stages of nematode infection but not at later stages. These results suggest that recombinant PIs can increase resistance to nematodes, however, constitutive expression driven by the CaMV 35S promoter does not provide a long-term durable source of resistance. Goddijn et al., (1993) showed that the 35S promoter was down-regulated by nematode parasitism.

For cyst nematode resistance, our strategy utilized the nematode-inducible promoter, *Hmg2*, to locally express the tomato PI-I in transgenic tobacco (*Nicotiana tabacum* cv. Xanthi). It has been previously demonstrated that the *Hmg2* promoter is activated in response to attack by sedentary endoparasitic nematodes and promoter activity was localized to the feeding site using the GUS reporter gene system (Cramer et al., 1993; Umayam and Cramer, unpublished). Based on this expression pattern, we believed that constructs containing *Hmg2* promoter fused to genes encoding toxins would be expressed in the same tissues upon nematode parasitism. The coding sequence of the tomato PI-I gene, including its signal sequence was fused to the *Hmg2* promoter (EM2) and introduced into tobacco. This gene product is normally targeted to vacuoles or secreted out into the apoplast or extracellular space, thus targeting the cyst nematode during intercellular migration through the host cell. Because the cyst nematode expends a substantial amount of their lifecycle at feeding sites (or syncyntia), we believe that the intracellular retention
of a PI product would be more effective in enhancing cyst nematode resistance. To generate this gene product, the tomato PI-I coding sequence excluding the signal sequence, was fused to the *Hmg2* promoter (EM1). Reverse-transcriptase polymerase chain reaction (PCR) of transgenic plants confirmed that RNA expression in transgenic tobacco. Western analysis revealed that EM1 plants produced PI-I protein, while either low or no protein was detected in EM2 plants. By performing greenhouse experiments, we demonstrated that intracellular PI products reduced cyst production when compared to the number of cysts from nontransformed control plants or those expressing extracellular PI products. The lack of resistance observed in EM2 expressing plants could directly be attributed to the low PI protein production demonstrated via western analysis. Future experiments will test additional EM2-expressing lines to identify those containing comparable amounts of PI-I as shown to be effective in reducing nematode damage in EM1 lines. These lines can then be compared to the assess effectiveness of intracellular PI localization versus extracellular localization. Additionally, the next level of confirmation will delineate the subcellular localization of PI products. Immunolocalization studies are excellent tools that will allow the visualization of transgene accumulation to support our primary data suggesting that PI products retained in the cytosol enhances resistance to the cyst nematode. Greenhouse experiments will be repeated and extended to 12-14 wks to determine the long-term effectiveness of locally expressed PI products. This will permit the production of multiple infection cycles similar to cyst nematode disease in the field.

Inducible expression of PI proteins shows promise in enhancing plant resistance in many ways. Locally expressed PIs represent effective anti-feedants that can potentially protect plants in the field from both plant parasitic nematodes as well as herbivore predation. This dual resistance would allow the farmer to rely on a reliable single source of resistance without the use of toxic pesticides. The use of transgenic PI plants would not only be user and environmentally friendly, but would be a less expensive control method. Previous studies have constitutively expressed PIs in transgenic plants, which has led to concerns of PI human consumption. Our protection strategy has the advantage that PIs are induced locally at the site of ingress, leaving the majority of the plant, including parts for human consumption, free of the anti-feedant proteins. Once this construct has been moved into food crops, assessment of PI levels in consumed organs and any associated toxicity will need to be directly tested.
IV.2 REFERENCES


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APPENDIX A

Transgenic lines expressing CHS8:TMV replicase and the experiments performed on each line.

<table>
<thead>
<tr>
<th>CHS8:TMV PLANT LINES</th>
<th>WOUND RESPONSE</th>
<th>RNA</th>
<th>LOCALIZED DARKENING DURING PARASITISM</th>
<th>DECREASE IN PARASITE ATTACHMENT</th>
<th>SEED PRODUCTION</th>
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VITA

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