THE ROLES OF PROTEINASES AND PROTEINASE INHIBITORS IN PLANT-NEMATODE INTERACTIONS

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ABSTRACT

The primary objective of this study was to investigate the roles of plant proteinase inhibitors in plant-nematode (*Meloidogyne* spp.) interactions. Transgenic tomato and tobacco plants were employed to examine the effects of proteinase inhibitor I or II transgene on nematode disease development. In the first part of this study, tomato and tobacco root cultures and seedlings aseptically grown in agar medium were developed to test the roles of proteinase inhibitor transgenes in enhancing plant resistance against nematodes. Root galling in cultured root and seedlings expressing inhibitor I or II gene was reduced as compared with controls. Nematode development was also retarded in proteinase inhibitor-expressing root cultures.

In the second part of this study, the effects of high expression of proteinase inhibitor I or II transgene on nematode disease development were examined in whole plants grown under greenhouse conditions. It was found that both root galling, nematode egg and egg mass production were inhibited in transgenic tomato plants during the early infection stage. However, this inhibition ceased during the late infection stage.

The suitability of cauliflower mosaic virus (CaMV) 35S promoter used for transgene constructs was evaluated in this study. It was found that the expression of proteinase inhibitors, driven by the CaMV 35S promoter, decreased in root tissues of transgenic plants during late nematode infection stage. The developmental expression pattern of proteinase inhibitors in root tissues was clearly correlated with nematode disease development. In addition, the GUS gene, driven by CaMV 35S promoter, was not expressed in gall tissues containing feeding nematodes during the late infection stage. The results of this study suggested that CaMV 35S promoter might not be suitable for engineering nematode resistant crop plants.

Additional experiments were performed to identify the proteolytic activity present in root-knot nematodes at different developmental stages. Both trypsin and chymotrypsin activities were detected in second-stage juvenile extracts. Only trypsin activity was found in female extracts. Both tomato proteinase inhibitor I and II were induced in root tissues in response to nematode infection. The preliminary results of this study further confirmed the involvement of proteinases and proteinase inhibitors in plant-nematode interactions.

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Chapter I

INTRODUCTION

The Problem and Its Setting

Statement of Problem

Root-knot nematodes (*Meloidogyne* spp.) are among the most widespread pathogens limiting world agricultural productivity. Almost all crops are susceptible to infection by root-knot nematodes (Sasser and Carter, 1985). Conventional breeding methods have been successfully used to generate new commercial root-knot nematode resistant cultivars in some crops. However, in others, traditional methods have not succeeded due to the lack of nematode resistance genes (Fassuliotis, 1985).

Advances in genetic engineering technology provide potential new tools to develop root-knot nematode resistant cultivars that have been difficult to produce with conventional breeding procedures. However, before genetic engineering can be used for practical crop improvement schemes, new nematode resistance genes must be identified, characterized, and their roles in plant nematode defense must be understood.

Plant proteinase inhibitors have been found to play roles in defending plants against insects and microbial pathogens (Ryan, 1990). Proteinase inhibitors are thought to protect plants from insects or microbial pathogens by interfering with protein digestion needed for pathogen growth (Ryan, 1990). Recent studies have shown that genetically engineered tobacco (*Nicotiana tabacum*) plants expressing high levels of proteinase inhibitor transgenes were more resistant to feeding by insect larvae than unaltered plants (Hilder *et al.*, 1987; Johnson *et al.*, 1989).

To our knowledge, the potential roles of proteinase inhibitors against nematode attack have not been evaluated. Transgenic plants with high levels of expression of

proteinase inhibitors have not yet been tested for possible defense against nematodes. To consider proteinase inhibitors as potential candidate genes for improving plant nematode resistance by genetic engineering technology, we must first evaluate their role in the plant-nematode interaction. The effects of proteinase inhibitor transgenes on plant growth and nematode disease development must be understood.

Hypothesis

If proteinases play critical roles in digesting host proteins during the feeding phase of the plant-nematode parasitic relationship, then constitutive overexpression of appropriate proteinase inhibitors in host plants could increase their resistance by interfering with nematode digestion of host proteins.

Goal and Objectives

The overall goal of this research is to investigate the role of proteinase inhibitors in plant-root-knot nematode interactions, and to test the potential for employing proteinase inhibitor genes for improving nematode resistance. The specific objectives are:

1. To examine the effects of proteinase inhibitor I or II transgene expression on the susceptibility of host plants to nematode attack by employing genetically engineered tomato (*Lycopersicon esculentum*) and tobacco plants expressing high levels of proteinase inhibitor I or II driven by cauliflower mosaic virus (CaMV) 35S promoter.

2. To determine the different temporal and tissue-specific expression of proteinase inhibitor transgenes in root tissues of transformed plants inoculated with root-knot nematodes. In addition, the suitability of CaMV 35S promoter used for gene constructs will be evaluated by correlating the level of expression of proteinase inhibitors in root tissue with nematode disease development.

3. To determine the induced expression of proteinase inhibitors in root tissues of wild type tomato plants in response to root-knot nematode infection.

The Rationale and Significance of the Study

Tomato and tobacco plants contain two types of inhibitors of serine proteinases, inhibitor I and inhibitor II. Proteinase inhibitor I shows strong chymotrypsin inhibitory activity, whereas proteinase inhibitor II inhibits both trypsin and chymotrypsin (Plunkett *et al.*, 1982). Since chymotrypsin and trypsin are primary enzymes present in digestive systems of insects (Applebaum *et al.*, 1985; Broadway, 1989; Houseman, *et al.*, 1989), proteinase inhibitors were thought to protect plants from insects by interfering with the digestive processes of insects (Ryan, 1990).

Information is available that supports defensive roles of proteinase inhibitors against insects. However, the possible involvement of proteinase inhibitors in protecting plants against microbial pathogens and nematodes is not fully understood. If the insect model holds for microbes, proteinase inhibitors could play roles in defending plants against microbial pathogens by inhibiting their trypsin or chymotrypsin proteinases, and thereby limiting availability of amino acids for growth, development, and reproduction of the pathogenic organisms (Ryan, 1990). Thus, the possible involvement of proteinase inhibitors in plant-root-knot nematode interactions will be investigated in this study.

Transgenic tomato and tobacco plants with high levels of the proteinase inhibitor I or II will be employed to test directly for the possible roles of the proteinase inhibitors in defending plants against root-knot nematodes. Such genetically modified plants with single gene changes provide a new system for direct analysis of the roles of proteinase inhibitors in enhancing nematode resistance. This proposed research also allows testing the effectiveness of this new genetic engineering approach for improving nematode resistance in crop plants. Furthermore, the nematode *Meloidogyne* species we will

study in this research have very broad host ranges and induce root-knot diseases in many of the major crops in the world. The knowledge gained from this research should, therefore, be potentially useful for improving nematode resistance in other economically important crops.

Literature Review

PLANT ROOT-KNOT NEMATODE DISEASE

Economic Importance

The plant root-knot diseases caused by *Meloidogyne* species of pathogenic nematodes are among the most important crop diseases limiting world agricultural production. Root-knot nematodes occur throughout the world, infect all major crop plants, and cause substantial reduction in crop yield and quality (Sasser and Carter, 1985). Average crop yield losses caused by *Meloidogyne* spp. have been estimated to be about 5% (Taylor and Sasser, 1978). These losses are not evenly distributed and can exceed 90% in highly infested soils.

Life Cycle of *Meloidogyne*

Root-knot nematode *Meloidogyne* species exhibit a complex life cycle (Fig. 1). Following embryogenesis, the first-stage juvenile molts once within the egg producing a second-stage juvenile. This motile, vermiform and infective juvenile migrates through the soil and penetrates the root of a susceptible host at a site just posterior to the root tip. Then it migrates to a feeding site within the root and establishes a specialized parasitic relationship with the host (Eisenback and Triantaphyllou, 1991). The consequence of this interaction for the nematode is development of the egg-producing female and completion of its reproductive cycle within the host plant. As it feeds on host cells at a feeding site, the second-stage juvenile undergoes profound morphological changes and becomes flask-shaped. Without further feeding it molts three times into the third- and fourth-stage juveniles, finally reaching the adult female stage. Shortly after the last molt the saccate female resumes feeding and develops into a mature female

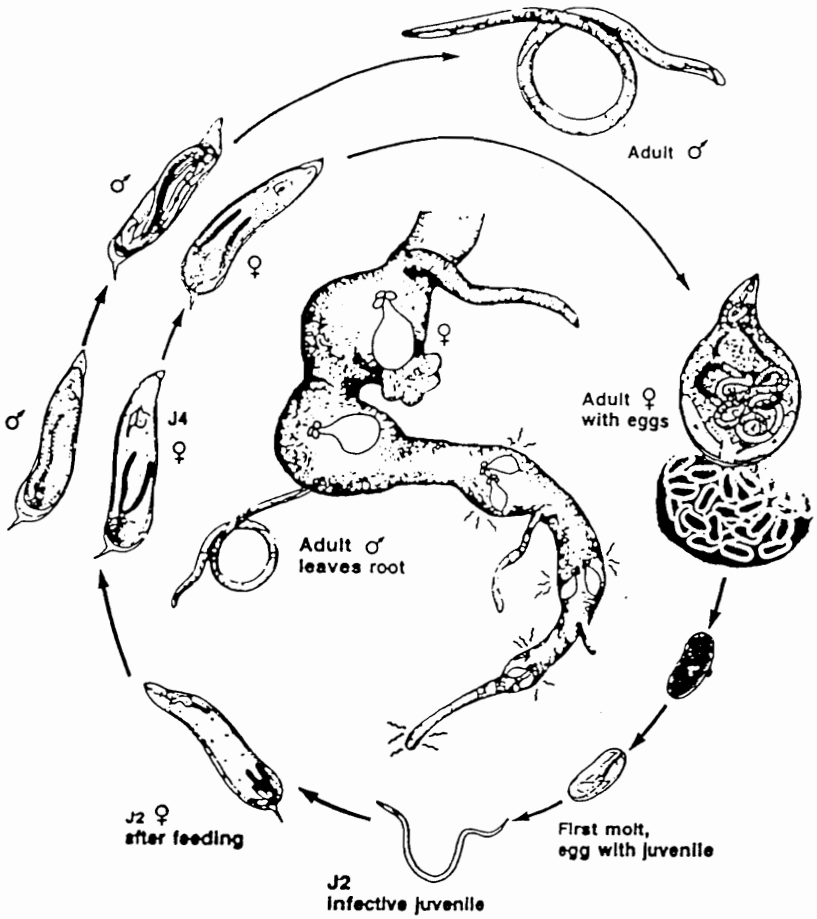

Fig. 1. Life cycle of the root-knot nematode, *Meloidogyne*. The third-stage juvenile is not shown in diagram. J2 and J4 represent second-stage and fourth-stage juvenile respectively (after H.D. Shew)

(Eisenback and Triantaphyllou, 1991). It then deposits eggs, which are enclosed in gelatinous egg masses (sacs), on the surface of galled roots. The length of the *Meloidogyne* spp. life cycle is greatly influenced by temperature. Although its life cycle is completed faster at higher temperature, it normally completes its life cycle in about 19-21 days at 29°C (Eisenback and Triantaphyllou, 1991).

Host-nematode Parasitic Relationships

Root-knot nematodes show specialized and complex relationships with their hosts. Establishment of the parasitic association involves a sequence of interactions between nematode and plant root tissue. Infection of host root tissue by a second-stage juvenile results in the development of a feeding site consisting of a few nutritive giant plant cells, from which the juvenile obtains food. Giant cells are formed following repeated nuclear division in the absence of cytokinesis. Giant cells act as transfer cells passing nutrients from the plant to the nematodes (Huang and Maggenti, 1969; Jones and Payne, 1978). The contents of the host giant cells are partially digested extracorporeally prior to ingestion by the nematode (Doncaster, 1971). Nematode stylet secretions are probably involved in this extracorporeal digestion (Hussey, 1989). Most nematodes are able to digest host carbohydrates and proteins (Lee and Atkinson, 1978). However, the origins of hydrolytic enzymes functioning in carbohydrate and protein digestion are not known. Enzymes, such as amylase (Zinovier, 1957) and proteinase (Dasgupta and Ganguly, 1975; Zinovier, 1957), have been detected in *Meloidogyne* species. Dasgupta and Ganguly (1975) isolated, purified, and characterized a trypsin-like proteinase from second-stage larvae of the root-knot nematode, *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949. Studies on this enzyme revealed many similarities with vertebrate and invertebrate trypsins including those of insects. It was suggested that root-knot nematodes excrete proteolytic enzymes to assist in extracorporeal protein digestion.

Concurrent with giant cell formation, root tissues surrounding the nematode and its feeding site undergo hypertrophy giving rise to characteristic root galls (Hussey, 1985). Such galled roots are less efficient for absorption and translocation of water and minerals, thereby limiting the plant's growth and development.

Effect of Temperature On Nematode Pathogenicity

Temperature is one of the most important environmental factors affecting nematode development. Embryogenesis, hatching, root penetration, migration, growth, and reproduction of nematodes within the roots are altered by changes in temperature (Van Gundy, 1985). Generally, nematode embryogenesis, root penetration, and migration are favored by high temperatures (Van Gundy, 1985). The entire nematode life cycle is completed faster at higher temperature. Moreover, high temperature may reverse some resistance responses to susceptible responses (Dropkin, 1969b; Zacheo *et al.*, 1988; Omwega *et al.*, 1990). It is possible that high temperature makes plants more vulnerable to nematode infection both by favoring nematode growth and by causing stress responses in plants (Van Gundy, 1985).

Host Resistance Response To Nematode Infection

Resistance to nematode infection may occur by interruption of any of the steps required for establishment of the plant-nematode parasitic relationship. Often, resistance to root-knot nematodes results from the inhibition of giant cell formation (Huang, 1985). Resistance is also associated with a hypersensitive reaction (HR) characterized by rapid cell death around the nematode infection site (Huang, 1985).

Although the mechanisms of plant resistance to nematode infection are little understood, some biochemical information is now available. Accumulation of several defense-related compounds has been reported. Phenolics are involved in the formation of necrotic lesions and a positive correlation between phenolics present in plant roots

and degree of plant nematode resistance has been found (Singh and Choudhury, 1973; Hung and Rohde, 1973; Bajaj and Mahajan, 1977). Cell wall lignification (Giebel *et al.*, 1971) and increased cell wall hydroxyproline (Giebel and Stobiecka, 1974) have been reported to be correlated with disease resistance. It was suggested that increased lignification and hydroxyproline leads to reduced plasticity of cell walls, thus preventing nematode invasion.

Phytoalexins have also been examined in plant-nematode interactions. Kaplan *et al.* (1980) reported that the phytoalexin glyceollin increased in roots of resistant soybean (*Glycine max*) and remained low in susceptible cultivars after *M. incognita* inoculation. Terpenoid phytoalexin levels were also shown to increase in resistant cotton (*Gossypium* spp.) roots inoculated with *M. incognita* (Veech and McClure, 1977). However, no correlation between high tomatine (a sequiterpenoid phytoalexin) levels and resistance toward *M. incognita* was apparent (Elliger *et al.*, 1988). The involvement of defense-related enzymes has also been reported. Phenylalanine ammonia lyase (PAL), a regulatory enzyme in the biosynthesis of lignin and phenolic compounds was induced significantly 12 hours after *M. incognita* inoculation in tomato roots (Brueske, 1980).

Genetics of Root-knot Nematode Resistance

Although inheritance of resistance to *Meloidogyne* spp. has been studied in various host species, the tomato-*M. incognita* system is the most explored. Resistance to root-knot *M. incognita* in tomato is genetically dominant and conditioned by one major genetic locus called *Mi* (Gibert and McGuire, 1956; Sidhu and Webster, 1981). This gene is also effective against several other *Meloidogyne* spp. that attack tomato, but not to *M. hapla* Chitwood, 1949 (Sidhu and Webster, 1981). The resistant phenotype is characterized by rapid cell death of infected root tissue, a typical hypersensitive reaction (HR). Additional resistance genes against *Meloidogyne* spp. have been

identified in other agricultural hosts such as alfalfa (*Medicago sativa*), beans (*Phaseolus* spp.), cotton, peach (*Prunus persica*), pepper (*Piper* spp.), soybean, tobacco (*Nicotiana tabacum*) and *Vitis* spp. Most of these resistance genes are monogenic and dominant in action (Sidhu and Webster, 1981).

The gene product encoded by *Mi* is unknown. It was found that the *Mi* locus is located at position 35 of chromosome VI of the tomato genetic linkage map and tightly linked to *Aps-1* gene encoding an isozyme of acid phosphatase (Medina-Filho and Tanksley, 1983). The *Aps-1* gene has been purified and characterized from a nematode resistant tomato cultivar (Paul and Williamson, 1987). Molecular biology approaches are being utilized to clone the *Aps-1* gene and identify the *Mi* gene by "chromosome walking" techniques (Aarts *et al.*, 1991).

Recently, virulent lines of *M. incognita* from avirulent populations have been developed by repeated propagation on resistant tomatoes (Dalmasso *et al.*, 1991; Jarquin-Barberena *et al.*, 1991; Castagnone-Sereno *et al.*, 1993). Those virulent populations of *M. incognita* could overcome the *Mi* resistant gene. One protein was found to be different between the virulent and avirulent *M. incognita* isogenic lines from a single female (Dalmasso *et al.*, 1991). Therefore, such isogenic virulent and avirulent lineages could be used for identifying one or more nematode virulence genes and studying gene-for-gene interactions between plants and root-knot nematodes.

Classical Methods For Control of Plant Nematode Disease

Non-chemical management strategies for minimizing the loss caused by root-knot nematodes include a combination of good cultural practices, crop rotation, biological control agents, and physical treatments of nematode-infested soils. Limiting crop losses due to *Meloidogyne* through the use of nematicides has been effective in the past (Fassuliotis, 1985). However, the banning of several nematicides, such as DBCP (1,2-dibromo-3-chloropropane) and EDB (ethylene dibromide), because of their potential

hazards to human health, has reduced the number of available chemicals to less effective nonfumigant and systemic nematicides (Fassuliotis, 1985).

The lack of safe and effective nematicides has made breeding for root-knot nematode resistance an attractive alternative. In several crops where nematode resistance genes are available, conventional breeding methods have been successfully used to generate new commercial root-knot nematode resistant cultivars (Dukes *et al.*, 1979; Fery and Dukes, 1984; Wyatt *et al.*, 1983). However, the limiting factor in other crops with serious root-knot nematode disease problems has been the lack of nematode resistance genes (Fassuliotis, 1985). Since breeders can successfully work only with plants that are cross-fertile, it is usually impossible to introduce nematode resistance from other species by traditional methods.

Application of Genetic Engineering to Nematode Resistance

Advances in genetic engineering technology provide new tools to develop root-knot nematode resistant cultivars. Genetic engineering offers the promise of transferring root-knot nematode resistance gene(s) from one species to another. By introducing a single nematode resistance gene, improved nematode resistant crops may be generated. Therefore, it may be feasible to utilize this technology to develop host-nematode-resistant cultivars in crops where conventional breeding methods have not succeeded. However, before genetic engineering can be used for practical crop improvement schemes, new nematode resistance genes must be identified, characterized and their roles in plant nematode defense must be understood.

Recently, a novel gene was identified and characterized from root tissues of potato plants infected with potato cyst nematodes (*Globodera rostochiensis* Wollenweber, 1923) (Gurr *et al.*, 1991). It was suggested that this gene encodes a protein responsible for establishment and maintenance of nematode feeding sites. The isolation of this susceptible gene offers possibilities for engineering nematode resistant plants.

Specifically, the antisense transcripts of this gene could be introduced to plants to block expression of this gene product, therefore inhibiting the establishment or maintenance of nematode feeding sites. The overall strategy is to construct an "antisense gene" in which the orientation of transcribed regions is reversed with respect to the promoter resulting in synthesis of RNA (antisense RNA) complimentary to the normal gene transcript (sense RNA). The antisense RNA hybridizes to sense RNA and blocks subsequent translation (Ecker and Davis, 1986). Alternatively, the promoter of this gene could be used to regulate nematode resistant genes in transgenic plants. Most recently, an HMGR gene (coding for enzyme 3-hydroxy 3-methylglutaryl CoA reductase) promoter was found to be specifically expressed in gall tissues induced by root-knot nematodes (Dr. Deb Weissenborne and Dr. Carole Cramer, unpublished results). Thus, this promoter may also be a potential candidate promoter for engineering nematode resistant plants.

PROTEINASE INHIBITORS IN PLANTS

Plant Proteinase Inhibitors

Proteinase inhibitors are polypeptides that form complexes with proteinases and inhibit their proteolytic activity (Ryan, 1990). One role of proteinase inhibitors may be to regulate proteolytic activity, thereby protecting tissues from degradation by foreign proteinases (Neurath, 1984). Inhibitor families specific for each of the four mechanistic classes of proteolytic enzymes, i.e. serine, cysteine, aspartic, and metallo-proteinase have been identified. By far the majority of known plant proteinase inhibitors are specific for serine proteinases (Ryan, 1990).

Tomato and potato plants contain two types of proteinase inhibitors, inhibitor I and II. Proteinase inhibitor I contains chymotrypsin inhibitory activity, whereas proteinase inhibitor II inhibits both trypsin and chymotrypsin activity. Little information is

available regarding the regulation of synthesis of the two inhibitors in tomato leaf cells. Previous study (Walker-Simmons and Ryan, 1977) showed that proteinase inhibitors were synthesized as preproteins of Mr 2000-3000 larger than the mature proteins in cytoplasm and mature proteinase inhibitors were accumulated in the central vacuoles of leaf cells. A number of plant-derived chemicals have been found to regulate proteinase inhibitor I and II synthesis. These include oligouronides (Bishop *et al.*, 1981), the plant growth regulators abscisic acid and auxin (Peña-Cortes *et al.*, 1989; Kernan and Thornburg, 1989), jasmonic acid and methyl jasmonate (Farmer and Ryan, 1990), and an 18-amino acid polypeptide, systemin (Pearce, *et al.*, 1991). It was suggested that these compounds may be involved in complex signaling networks that regulate proteinase inhibitor I or II synthesis (Farmer and Ryan, 1992).

Regulation of Plant Endogenous Proteolytic Enzymes

Proteolytic enzymes have been discovered in plant tissues such as seeds, leaves, flowers, and fruits (Ryan, 1973). They are important enzymes in regulating plant growth, development, and senescence. The possible regulation of these proteolytic enzymes by endogenous inhibitors is not well understood. Earlier studies have shown that proteinase inhibitors in soybean seeds did not function as inhibitors of endogenous proteolytic enzymes (Ofelt *et al.*, 1955). Baumgarner and Chrispeel (1976) also demonstrated that a proteinase inhibitor in the cotyledons of mung bean (*Phaseolus aureus*) seeds was not involved in the regulation of endogenous proteolytic enzymes. Another study (Santarius and Belitz, 1978) reported that a serine proteinase inhibitor extracted from leaves of *Solanaceae* did not inhibit endogenous proteolytic enzyme activity. Instead, they affected serine proteinases from microorganisms and animals.

However, a number of studies have suggested possible interactions between inhibitors and endogenous proteolytic enzymes. Shain and Mayer (1965) showed that a proteinase inhibitor found in lettuce (*Lactuca* spp.) seeds inhibited an endogenous

trypsin-like proteinase. The possible regulation of endogenous proteolytic enzymes by inhibitors was also found in cowpea (*Vigna* spp.) seeds (Gennis and Cantor, 1976).

Defensive Role of Plant Proteinase Inhibitors Against Insects

Animals are usually able to digest the proteins they consume. Generally, proteins are digested in animals, such as insects, extracorporeally by hydrolytic enzymes in the stomach, intestine, rumen, or midguts (Ryan, 1990). Serine proteinases have been identified in extracts from the digestive tracts of insects and most of them were inhibited by serine proteinase inhibitors (Applebaum, 1985; Broadway, 1989; Houseman *et al.*, 1989). Because of the essential role of serine proteinases in the digestive processes of insects, serine proteinase inhibitors are thought to protect plants from insect attack by interfering with their digestion of proteins.

Earlier studies have shown that soybean serine proteinase inhibitors inhibited growth of *Tribolium confusum* (meal worm) (Lipke *et al.*, 1954) and *Tribolium casteneum* (Birk and Applebaum, 1960). Similarly, the Bowman-Birk inhibitor isolated from soybeans was also found to inhibit the growth of *Tribolium* species at 5% of the diet (Birk, 1985). Steffens *et al.* (1978) demonstrated that larval growth in European corn borer (*Ostrinia nubilalis*) was severely retarded and pupation was delayed when diets included 2-5% bovine trypsin inhibitor. A similar study found that trypsin inhibitor at 10% of the diet was toxic to larvae of *Callosobruchus maculatus* (Brucid beetle) (Gatehouse and Boulter, 1983). A third study also reported that added trypsin inhibitor from soybeans in the diet slowed the growth of *Manduca sexta* (Shukle and Murdock, 1983).

The wound-induced expression of serine proteinase inhibitors has been found in tomato leaves (Green and Ryan, 1972). Levels of serine proteinase inhibitors were also shown to increase dramatically in response to insect attack in plants such as alfalfa (Brown and Ryan, 1984) and soybean (Kraemer *et al.*, 1987), which suggested a

possible defensive function of serine proteinase inhibitors against insects in plants. In support of this idea, growth of larvae of *Spodoptera littoralis* (Edwards *et al.*, 1985) was severely retarded when fed with wounded tomato leaves expressing high levels of serine proteinase inhibitors.

Two recent research reports have confirmed the defensive role of serine proteinase inhibitors against insects by employing transgenic plants with high levels of proteinase inhibitors. Hilder *et al.* (1987) demonstrated that transformed tobacco plants expressing a foreign cowpea trypsin inhibitor, driven by cauliflower mosaic virus (CaMV) 35S promoter, were more resistant to feeding by larvae of *Heliothis virescens* than untransformed plants. Johnson *et al.* (1989) showed that tobacco leaves expressing high levels of tomato proteinase inhibitor II (with trypsin and chymotrypsin inhibitory activity), also driven by the CaMV 35S promoter, were toxic to larvae of *Manduca sexta*. The growth of larvae was greatly inhibited as compared to larvae fed on untransformed leaves. These studies demonstrated the effectiveness of using genetic engineering approaches to develop insect resistant plants.

A Potential Role of Proteinase Inhibitors For Defense Against Pathogen Attack

Although evidence is increasing that supports defensive roles of proteinase inhibitors against insects, indirect evidence for a possible role of proteinase inhibitors in defending against other plant pathogens is only just emerging (Ryan, 1990). Proteinase inhibitors are suspected to arrest pathogen invasion by inhibiting proteolysis and limiting availability of amino acids required for growth, development and reproduction of the pathogen (Ryan, 1990).

Proteinase inhibitors that specifically inhibit proteolytic enzymes in microorganisms, but not in plants or animals, have been found in plants. Inhibitors of a subtilisin-enzyme secreted by *Bacillus subtilis*, have been purified and characterized from many cereal and legume species (Garcia-Olmedo *et al.*, 1987). Potato proteinase inhibitor I, a potent inhibitor of chymotrypsin, was also found to inhibit subtilisin (Ryan, 1966). Proteinase inhibitors isolated from potato (*Solanum tuberosum*) tubers were found to inhibit proteolytic activity of microorganisms isolated from spoiled potato tubers (Senser *et al.*, 1974). Proteinase inhibitors present in green tomato fruits specifically inhibited fungal rot development in tomato fruits (Brown and Adikaram, 1983).

Additionally, Peng and Black (1976) showed that trypsin inhibitor increased more in leaves of *Phytophthora infestans*-resistant tomato cultivars than in susceptible cultivars. Similarly, Yamaleev *et al.* (1980) demonstrated stronger trypsin inhibitory activity in soft and hard wheat (*Triticum aestivum*) kernels in varieties with higher resistance against smut fungi (*Ustilaginaceae*). Roby *et al.* (1987) found that the increase in proteinase inhibitory activity was associated with *Colletotrichum lindemnthianum* infection. Another study (Rickauer, 1989) showed that increased proteinase inhibitor was induced by treating with an elicitor obtained from *Phytophthora parasitica*.

Nodule Proteinase Inhibitor

Recently, Manen *et al.* (1991) reported that a protein specifically expressed in senescent nodules of winged bean infected with *Rhizobium* is a proteinase inhibitor with trypsin inhibitory activity. An immunocytochemical study showed that this proteinase inhibitor was exclusively localized in infected senescent cells of the nodule. Since proteolytic enzymes are probably involved in nodule senescence (Vance, 1986; Peoples and Dalling, 1988), they speculated that this proteinase inhibitor could interact specifically with a proteinase (origin of plant or *Rhizobium*) and thereby, regulate its activity for maintaining the host-*Rhizobium* symbiotic relationship.

THE CAULIFLOWER MOSAIC VIRUS 35S PROMOTER

The cauliflower mosaic virus (CaMV) is a DNA virus that infects *Cruciferae* plants. Two promoters have been identified from CaMV, designated as 19S and 35S. The CaMV 35S serves as a template for translation. In addition, it also functions as an intermediate for viral DNA synthesis through a reverse transcription process.

The CaMV 35S promoter, because of its strength, has been used extensively for regulating foreign gene expression in transgenic plants. The CaMV 35S fragments containing 400-1000 bp of 35S upstream sequences have been shown to be highly active in a wide variety of plant tissues and during most stages of development when integrated into the plant genome (Odell *et al.*, 1985, 1987; Jefferson *et al.*, 1987; Sanders *et al.*, 1987; Kay *et al.*, 1987; Nagy *et al.*, 1987). It was found that the -343 to -46 upstream fragment was responsible for the majority of the 35S promoter strength (Odell et al., 1985). Two domains were also identified to confer different developmental and tissue-specific expression patterns (Benfey *et al.*, 1989). Expression from domain A (-90 to +8) was strongest in the radicle of the embryo, the radicle pole of the endosperm, and in root tissue of seedlings and mature plants. Expression from domain B was strongest in the cells adjacent the cotyledon of the endosperm, in the cotyledons of the embryo and seedlings, and in the leaves and stem of mature plants. In plants that contained both domains A and B, expression was found in all tissues and all stages of development. Specifically, strong expression was detected in the vascular tissue, pericycle, and in most other cell types in root tissues of seven-week-old plants containing both domains A and B. However, the expression patterns were not examined in root tissues of mature plants beyond seven weeks.

The CaMV 35S promoter has also been used to develop transgenic tobacco (Hilder *et al.*, 1987; Boulter, *et al.*, 1989; Johnson *et al.*, 1989), nightshade (*Solanum nigrum*) (Narváez-Vásquez *et al.*, 1992), and alfalfa (Narváez-Vásquez *et al.*, 1992) plants

expressing foreign proteinase inhibitor genes. The CaMV 35S-tomato proteinase inhibitor I fusion gene has previously been examined for its differential expression in transgenic tobacco plants (Narváez-Vásquez *et al.*, 1992). This study found that young developing leaf and floral tissues contained higher proteinase inhibitor I than other tissues. A higher percentage of proteinase inhibitor I was also shown in apical tissues of tobacco leaves as compared with basal tissues from the same leaves, suggesting that higher expression of proteinase inhibitor I was occurring in younger tissues. A cysteine proteinase inhibitor transgene regulated by the CaMV 35S promoter was also found to be differentially expressed in leaf (50 μg/g) and root (30 μg/g) tissues of tobacco plants (Masoud *et al.*, 1993). Although high expression (30 μg/g) of proteinase inhibitor I was found in root tissues of transgenic tobacco plants, temporal and tissue-specific expression of inhibitor I gene in root tissues was not examined in previous study (Narváez-Vásquez, 1992). The cellular and subcellular localization of proteinase inhibitor I and II proteins, synthesized in transgenic tomato plants, has been examined (Narváez-Vásquez *et al.*, 1993). Newly synthesized inhibitor proteins were not only deposited in the cell vacuoles, but were also secreted into the cell walls of outer epidermal and secretory cells of the root cap.

CONCLUSIONS OF LITERATURE REVIEW

Although proteinase inhibitors may function as regulators of endogenous proteinase activity, one of their primary roles in plants is clearly to inhibit proteinases of invading insects or microorganisms, thereby protecting plants from predation or infection by these pathogens (Ryan, 1973). The possible involvement of proteinase inhibitors in plant-nematode interactions has not been reported to our knowledge. Transgenic plants with overexpression of proteinase inhibitor transgenes have been shown to exhibit

enhanced defense against insect predation. However, such transgenic plants have not yet been tested for possible defense against other plant pathogens including nematodes.

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Chapter II

ROOT-KNOT NEMATODE SUSCEPTIBILITY IN CULTURED TOMATO AND TOBACCO ROOTS EXPRESSING HIGH LEVELS OF PROTEINASE INHIBITOR TRANSGENES*

ABSTRACT

Transgenic tomato (*Lycopersicon esculentum* cv. Better Boy) and tobacco (*Nicotiana tabacum* cv. Xanthi) root cultures and aseptically grown seedlings expressing high levels of proteinase inhibitors were tested for possible roles of proteinase inhibitors in defending plants against pathogenic nematodes, *Meloidogyne hapla* Chitwood, 1949 and *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949. The overexpression of tomato proteinase inhibitor I (TI-I) or II (TI-II) inhibited the growth of roots cultured on agar plates. Both lateral root formation and fresh weight were reduced in root cultures expressing proteinase inhibitor I or II transgene. The root-knot nematode susceptibility was reduced in both TI-I- and TI-II-expressing root cultures compared to untransformed root cultures of the same cultivar. Gall development was inhibited in transgenic plants compared with controls when measured at ten or twenty days after *M. hapla* inoculation. Nematode development was also delayed in proteinase inhibitor-expressing root cultures as compared with control root cultures at twenty and thirty-five days after inoculation. Similar results were shown on both tomato and tobacco seedlings cultured on agar medium.

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* Format and references of this chapter are arranged for submission to Proc. Natl. Acad. Sci. USA, therefore may be different from other chapters.

INTRODUCTION

Two groups of inhibitors of serine proteinases (inhibitor I and inhibitor II), coded by two small multigene families, have been described in tomato and potato plants (1). Proteinase inhibitor I shows strong chymotrypsin inhibitory activity, whereas proteinase inhibitor II inhibits both trypsin and chymotrypsin activities (1). Since chymotrypsin and trypsin are primary enzymes present in digestive systems in animals including insects (2-4), proteinase inhibitors may function in plant defense by interfering with digestive processes of insects (5).

Both inhibitors were found to accumulate in tomato leaves in response to wounding (6) and chewing insects. A defensive role of tomato proteinase inhibitors to insects was firmly established by Johnson and his co-workers (7). They constructed genes containing the cauliflower mosaic virus (CaMV) 35S promoter fused alternatively to open reading frames coding for tomato proteinase inhibitor I, tomato inhibitor II, or potato inhibitor II. These constructs were stably integrated into the tobacco genome by *Agrobacterium*-mediated transformation leading to overexpression of these proteinase inhibitors in the transgenic tobacco leaves. It was shown that the transgenic tobacco leaves expressing high levels of proteinase inhibitor II (>50 μg/g) were toxic to *Manduca sexta* (tobacco hornworm). Growth of *M. sexta* which fed on transformed leaves with inhibitor II was significantly inhibited as compared to growth of larvae fed on control leaves. However, the presence of tomato inhibitor I in transgenic tobacco leaves had little effect on the larvae. Their study suggested that trypsin inhibitory activity, but not chymotrypsin inhibitory activity, was mainly responsible for the inhibition of larval growth.

A strong inhibitory activity against subtilisin, a proteolytic enzyme secreted by *Bacillus subtilis*, has also been found to be associated with tomato inhibitors (1), suggested a possible defensive role for tomato proteinase inhibitors against microbial

infection. Peng and Black (8) found increased levels of a trypsin inhibitor in response to infection of resistant tomato plants by *Phytophthora infectans*. Proteinase inhibitors isolated from tomato fruits also inhibited fungal rot development (9). Senser *et al.* (10) showed that proteinase inhibitors extracted from potato tubers inhibited proteolytic activity in microorganisms from spoiled potato tubers.

We were interested to determine if proteinase inhibitors play a role in defending plants against pathogenic root-knot nematodes (*Meloidogyne* spp.). Root-knot nematodes establish a specialized and complex feeding relationship with their hosts. Infection of host root tissues by second-stage juveniles results in development of a feeding site consisting of a few nutritive giant cells from which nematodes obtain food for growth and development. Usually, host giant cell contents are partially digested extracorporeally prior to ingestion by the nematodes. Presumably, nematodes secrete hydrolytic enzymes into host cells to assist such extracorporeal digestion (11). Among many hydrolytic enzymes found in nematodes (12), proteolytic activity has been detected in juvenile exudates of the root-knot nematode, *M. incognita* (13). Trypsin-like proteinase has also been isolated, purified and characterized from second-stage juveniles of the root-knot nematode, *M. incognita* (14). Presumably, proteolytic enzymes are involved in nematode extracorporeal digestion of host cell proteins during its feeding period. However, so far it is unknown if proteolytic enzymes from root-knot nematodes are inhibited by plant proteinase inhibitors.

The trypsin-like proteinase characterized from second-stage juveniles of *Meloidogyne incognita* was similar to trypsins isolated from animals and insects in molecular weight, substrate specificity and inhibition by synthetic inhibitors (14). We therefore reasoned that if proteinase inhibitors interact with proteinases of root-knot nematodes, then overexpression of proteinase inhibitor transgenes in host plants would be expected to improve host resistance against nematodes by interfering with extracorporeal digestion of host proteins.

In this study, we employed transgenic tomato and tobacco plants with overexpression of proteinase inhibitor I or II transgene to test for the possible defensive roles of the proteinase inhibitors against nematodes. Development of root galls associated with *Meloidogyne* infection was monitored in root tissues of wild type, and transgenic tomato and tobacco plants following nematode inoculation. Nematode growth and development were also examined during the infection process.

Cultured roots and plant seedlings grown in agar medium were employed in this study. Nematode-root culture systems permit all of the required steps in the nematode infection cycle to occur aseptically (15) and ensure that other microbial pathogens do not modify important plant responses to nematode infection. Additionally, the systems permit continuous observation, and relatively easy recovery and analysis of infected root materials.

MATERIALS AND METHODS

Plant and nematode materials

Tomato (*L. esculentum* cv. Better Boy) and tobacco (*N. tabacum* cv. Xanthi) lines were generously provided by Dr. C.A. Ryan (Washington State University) as seeds. The tomato lines included T#289-E6-pJN3-Inhibitor I, T#464-D7-pGA875-Inhibitor II, and the Better Boy parental cultivar. The tobacco lines were T#8c-pJN-3-Inhibitor I, T#29-pRJ13-Inhibitor II, and Xanthi parental cultivar. Although control tomato cultivar, Better Boy, contains the *Mi* gene conferring resistance to *M. incognita* and several other *Meloidogyne* species, it is susceptible to *M. hapla*. Tobacco control cultivar, Xanthi, is susceptible to both *M. hapla* and *M. incognita*. The plasmid constructs transformed into these lines (pJN3, pGA875 and pRJ13) have been described previously by Johnson *et al.* (7). Basically, the coding sequences of tomato proteinase inhibitors or potato proteinase inhibitor are driven by a 400 bp DNA sequence from the CaMV 35S promoter (Fig. 1). Transgenic plants carrying these constructs were

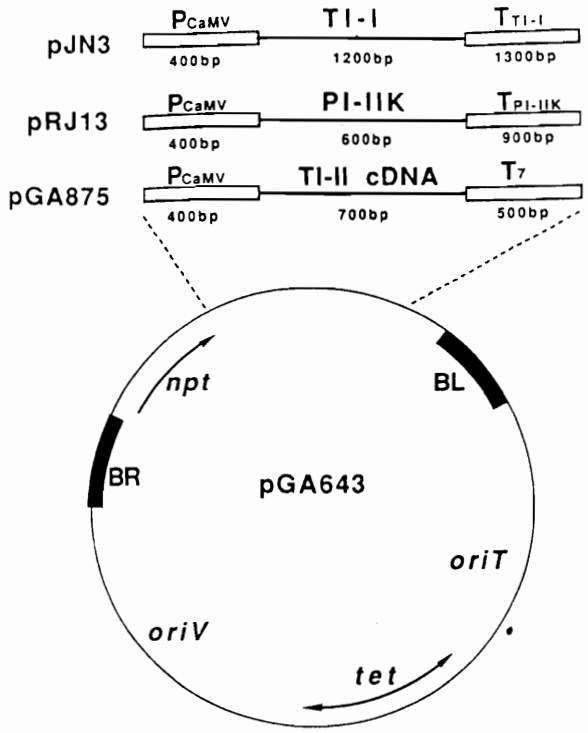

Fig. 1. Transformation plasmids pJN3, pRJ13, and pGA875. PcaMV, CaMV 35S promoter; TI-I, coding region from a tomato inhibitor I gene; T_{TI-I}, tomato inhibitor I terminator; PI-IIK, coding region from the potato inhibitor II-K gene; TI-II cDNA, coding region from a tomato inhibitor II cDNA; T7, terminator of the transcript-7 gene from *Agrobacterium tumafaciens* T-DNA; BR, T-DNA right border; BL, T-DNA left border; *npt*, a chimeric *nos-npt* (nopline synthase-neomycin phosphotransferase) fusion that serves as a selectable marker in plants; *tet*, tetracycline-resistance gene; *oriV*, pRK2 origin of replication; *oriT*, pRK2 origin of conjugative transfer (after Johnson, Narvaez, An and Ryan, 1989).

obtained by *the Agrobacterium*-mediated leaf disc transformation system (16-17). All plants used in this study were homozygous for the proteinase inhibitor I or II transgene (C. A. Ryan, personal communication). High levels of inhibitor I (125 μg/g tissue) and II (45 μg/g tissue) proteins are accumulated in root tissues of transgenic tomato plants expressing the inhibitor I and II genes, respectively (18). However, the root tissues of untransformed tomato plants contain low levels of inhibitor I and undetectable levels of inhibitor II (18). High levels of inhibitor II (35 μg/g tissue) are also present in root tissues of transgenic tobacco plants expressing inhibitor I gene (19). USDA-APHIS approval for receiving these transgenic seeds was obtained in 1991. All transgenic materials were handled in our facilities according to National Institute of Health (NIH) recombinant DNA guidelines (BL1-P). All transgenic material, and nematodes or agar medium used in association with transgenic material was autoclaved prior to final disposal.

M. hapla and *M. incognita* (race 1) nematode cultures were kindly supplied by Dr. J. D. Eisenback (Dept. of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University). Stock cultures were maintained and multiplied on tomato *(Lycopersicon esculentum* cv. Rutger) plants grown in 15 cm clay pots filled with steam sterilized soil (2 clay soil: 1 sand) in a greenhouse (25°C). The nematode populations were subcultured every 8-10 week to ensure a constant supply of nematode eggs as described (20). Plants were watered twice a day and fertilized every two weeks.

Root and seedling cultures

Root cultures: Tomato root cultures were established from Better Boy and two transgenic lines. Tomato seeds were surface-sterilized in 95% ethanol for 1 minute, in 25% commercial bleach for 10 minutes, followed by three washes with sterile water. The sterilized seeds were germinated on the surface of 1.5% water agar in the dark.

Root tips (2-3 cm) were excised and transferred to 1.5% agar medium containing Gamborg B5 salt (Appendix A) (21), Gamborg B5 vitamin mixture (Appendix A) (21) and 3% sucrose adjusted to pH 6.2 with 1N NaOH before autoclaving. Each plate contained two root tips. The petri dishes were sealed with Parafilm. Root cultures were incubated in the dark at 25°C for 2 days before inoculation with nematodes. Root cultures used for nematode inoculation were started fresh from seeds for each experiment.

Seedling cultures: Tomato and tobacco seedling cultures were established from wild-type and transgenic lines. Tomato and tobacco seeds were surface-sterilized in 95% ethanol for 1 min, in 25% commercial bleach for 10 min, followed by three washes with sterile distilled water. The sterilized tobacco seeds were germinated and grown in petri dishes containing Gamborg B5 medium. The sterilized tomato seeds were germinated and grown in large glass tubes (25X150mm) containing Gamborg B5 medium. Seedling cultures were incubated at 25°C for three weeks before inoculation with nematodes.

Preparation of nematode inoculum

Axenic nematode eggs for inoculation were produced by picking egg masses from the roots of 6- to 12-week-old nematode infected tomato plants. Egg masses were surface sterilized by treatment with 10% commercial bleach for 4 minutes with vigorous shaking. The egg suspensions were centrifuged for 1 min at 4000 rpm and the supernatant discarded. The eggs were washed three times with sterile water and resuspended in sterile tap water. The concentration of eggs was determined by counting 50 μl egg suspension under dissecting microscope. Egg suspensions were pipetted onto the surface of a culture plate or test tube. Five root cultures or seedlings of each line were inoculated with 1,000 nematode eggs suspended in 1 ml sterile tap water. All nematode-infected root cultures and seedlings were incubated at 25°C.

Estimation of root growth

To examine the root growth, root tissues were released from agar medium by boiling in water for 1 min. The fresh weight of root tissues from each plate was then determined. The lateral root formation was determined by counting root tips present in each culture plate. At least four root cultures of each line were analyzed for root growth. Student's t-tests were used to determine the significant differences in root growth between control and TI-I- or TI-II-expressing root cultures.

Estimation of gall development

To determine the gall development, infected root cultures or seedlings on agar plates were examined with a stereomicroscope over a three-week period. The number of root tips and galls formed in each root culture were determined by counting under the stereomicroscope. Root galling was expressed as the percentage of galled roots (number of galls/number of root tips) or number of galls formed per root culture. To estimate the gall development in tomato seedlings grown in test tubes, root tissues of seedlings were released from agar medium by boiling in water for 1 min and fresh weight of root tissues was determined. Root galls were separated from ungalled tissues and weighed. Galling for tomato seedlings grown in test tubes was expressed as the relative extent to which the roots were galled by nematodes (w/w) or the total gall weight per seedling. Student's t-tests were used to determine the significant differences in root galling between control and TI-I- or TI-II-expressing root cultures or seedlings.

Analysis of nematode development

To determine nematode developmental stage, the root system to be examined was recovered from agar by boiling in water for 1 min and then stained by sodium-hydrochloride-acid-fuchsin method (22). The stained roots were transferred to a Waring blender flask and made up to 200 ml with tap water. The root tissues were then

macerated at medium speed with three successive bursts of 20-second duration (23). Nematodes were separated from the root debris by sieving through a 60 (mesh) onto a 400 (mesh) sieve. The developmental stages of nematodes were determined by observation under a dissecting microscope. Second-stage juveniles, third- or fourth-stage juveniles and females were estimated according to Eisenback and Triantaphyllou (24). At least three root cultures of each line were analyzed. Student's t-tests were used to determine the significant differences in nematode development between control and TI-I- or TI-II-expressing root cultures.

RESULTS

Effect of proteinase inhibitor I or II transgene on growth of tomato root cultures

Control and proteinase inhibitor-expressing root cultures were analyzed for lateral root production and fresh weight growth (Table 1) to determine the effects of proteinase inhibitor I or II transgene on root growth. The expression of proteinase inhibitor genes in transgenic plants altered the growth of root cultures. At 10 days, a decrease in lateral root formation and fresh weight growth was observed in the TI-I-expressing root cultures compared with controls. Reductions were also found in fresh weight in TI-II-expressing root cultures at 10 days, 15 days and 20 days. This finding suggests that overexpression of proteinase inhibitor I or II transgene delayed root growth. The strongest inhibition in root growth was shown at 10 days. By 15 and 20 days this inhibition has mostly recovered.

Effect of proteinase inhibitor I or II transgene on gall development

Experiments were conducted to compare the development of root galling in control, TI-I- and TI-II-expressing transgenic root cultures infected with *M. hapla*. At least five replicates of each type were inoculated with *M. hapla*, and the roots were scored for galling at 10 days and 20 days after inoculation (Table 2). Root galling was reduced in

Table 1. Effect of proteinase inhibitor I or II transgene on tomato *in vitro* root growth[a]

Plant	Culture Period					
	10 days		15 days		20 days	
	# of tips /plate	FW[b](mg) /plate	# of tips /plate	FW(mg) /plate	# of tips /plate	FW(mg) /plate
TI-I--expressing	11±(10)[c]	10.4±(2.4)[d]	19±(17)	46.0±(26.3)	34±(23)	75.2±(41.4)
TI-II--expressing	16±(13)	11.5±(6.7)[d]	29±(25)	35.7±(18.7)[c]	37±(45)	47.3±(41.8)[c]
Control	23±(18)	25.0±(5.9)	29±(26)	66.8±(50.6)	54±(59)	110.2±(86.8)

a Mean±(standard deviation) for samples from at least four replicates
b FW - fresh weight
c Significantly different from control ($p<0.05$)
d Significantly different from control ($p<0.01$)

Table 2. Gall development in transgenic tomato root cultures inoculated with *M. hapla*[a]

	Days post-inoculation			
Plant type	10		20	
	# galls/culture	% galled roots	# galls/culture	% galled roots
TI-I-expressing	2±(2)[c]	3.0±(3.5)[b]	3±(5)[c]	12.3±(17.4)[c]
TI-II-expressing	1±(1)[b]	1.0±(1·0)[b]	19±(1)	49.5±(16.5)
Control	6±(2)	13.3±(5.3)	28±(11)	58.0±(18.2)

a Mean±(standard deviation) for samples at least three replicates
b Significantly different from control ($p < 0.01$)
c Significantly different from control ($p < 0.05$)

both TI-I- and TI-II-expressing root cultures as compared with Better Boy control cultures (Table 2). TI-I-expressing root cultures showed significantly smaller gall development at 10 and 20 days after inoculation. A significant difference in galling was also found between TI-II-expressing root cultures and untransformed control roots 10 and 20 days after inoculation. Fig. 2 shows a representative infection experiment which demonstrates the reduced root-galling caused by *M. hapla* in transformed roots as compared with wild-type roots.

Similar results were obtained in tomato and tobacco seedlings cultured on agar medium (Table 3). The root galling was reduced in both TI-I- and TI-II-expressing tomato seedlings by 15 days after inoculation with *M. hapla*. Galling was also reduced in TI-I- and TI-II-expressing tobacco seedlings by 15 days after inoculation with *M. incognita*. Fig. 3 shows the reduced galling by *M. hapla* in transformed tomato seedlings as compared with untransformed seedlings 15 days after inoculation. Thus, both proteinase inhibitor I- or II-expressing root cultures and seedlings showed reduced root galling in response to nematode infection as compared with controls.

Effect of proteinase inhibitor I or II transgene on nematode development

Nematodes were extracted from root cultures of control and proteinase inhibitor-expressing plants as described in Materials and Methods. The stages of nematode development were determined under a dissecting microscope. The high expression of proteinase inhibitor I or II transgene in tomato plants significantly delayed the development of nematodes at 20 days post-inoculation (Table 4). Only 35.1% and 51.5%, respectively, of the nematode population isolated from root cultures of TI-I- and TI-II-expressing plants were found to have developed beyond the infectious second-stage juvenile, whereas 77.2% of the nematode population from untransformed root cultures were developed beyond second-stage juveniles. By 35 days post-inoculation, nematode development was still delayed in transformed root cultures. Only 63.4% and

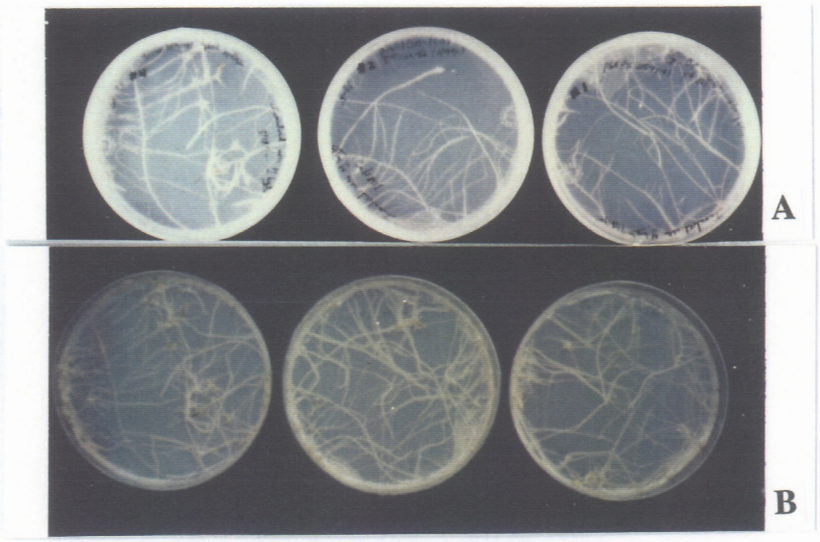

Fig. 2. Comparison of nematode disease symptom (root galling) among control and proteinase inhibitor-expressing root cultures inoculated with *Meloidogyne hapla* (1,000 eggs/plate). Observations were made 10 and 20 days after inoculation. A. 10 days after inoculation. B. 20 days after inoculation. Root galling induced by *M. hapla* was reduced in proteinase inhibitor-expressing root cultures as compared with control Better Boy root cultures.

Table 3. Gall development in transgenic tomato and tobacco seedlings inoculated with *Meloidogyne* species

Plant-type	Plant-nematode interaction*			
	Tomato-*M. hapla*		Tobacco-*M. incognita*	
	%galled roots	gall wt(g)/seedling	%galled roots	# of galls/seedling
TI-I-expressing	7.3±(10.3)	0.06±(0.10)	2.1±(3.6)	0
TI-II-expressing	11.5±(8.3)	0.06±(0.04)	10.0±(17.3)	1±(2)
Control	16.3±(8.9)	0.11±(0.09)	20.0±(19.4)	3±(4)

* Mean±(standard deviation) for samples at least two replicates. Observations were made 15 days after inoculation with eggs of *M. hapla* or *M. incognita*.

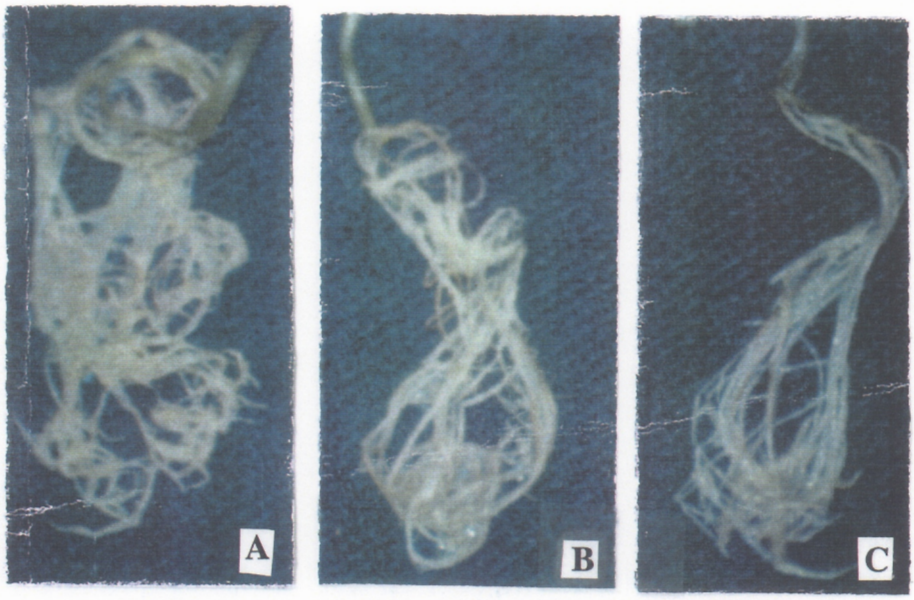

Fig. 3. Comparison of nematode disease symptoms (root galling) between control and proteinase inhibitor-transformed tomato seedlings. Aseptically grown tomato seedlings were inoculated with 1,000 eggs/seedling. Observations was made 15 days after inoculation. A, control. B, pJN3 (Inhibitor I)-transformed. C, pGA875 (inhibitor II)-transformed.

Table 4. Effect of proteinase inhibitor I or II transgene on nematode development[a]

Plant type	Days after inoculation				
	20		35		
	Developmental Stage		Developmental Stage		
	J2(%)	J3+J4+F (%)	J2	J3+J4 (%)	F(%)
TI-I -expressing	64.9±(0.4)[b]	35.1±(0.4)[b]	36.6±(12.4)[c]	53.9±(10.1)[b]	9.5±(9.2)[b]
TI-II -expressing	48.5±(1.6)[c]	51.5±(1.6)[c]	28.7±(6.9)[c]	59.7±(11.8)[b]	11.6±(6.6)[b]
Control	22.8±(9.4)	77.2±(9.4)	19.6±(5.1)	33.7±(6.9)	46.7±(6.3)

a Mean±(standard deviation) for samples of three replicates. J2, J3, J4 and F, respectively, represent second-stage, third-stage, fourth-stage juveniles and female

b Significantly different from control ($p < 0.01$)

c Significantly different from control ($p < 0.05$)

71.3% of the nematode population extracted from TI-I- and TI-II- expressing roots, respectively, had developed beyond second-stage juveniles, as compared with 80.4% for the untransformed roots.

DISCUSSION

Johnson *et al.* (7) previously demonstrated that transformed tobacco leaves with high levels (>50 μg/g tissue) of proteinase inhibitor II were toxic to *Manduca sexta* (tobacco hornworm). Growth of *M. sexta* fed on transformed leaves was significantly inhibited as compared to growth of larvae fed on untransformed leaves. Because trypsin and chymotrypsin play essential roles in the digestive processes of insects, proteinase inhibitors are thought to inhibit insect growth by interfering with its protein digestion (5). Their study demonstrated that such genetically modified plants with single gene changes provide a useful system for direct analysis of the roles of proteinase inhibitors in enhancing insect resistance and the effectiveness of using genetic engineering approaches for improving insect resistance.

In our study, transgenic tomato and tobacco plants developed by C. A. Ryan's laboratory were employed to examine the possible roles of proteinase inhibitors in plant-root-knot nematode interactions. Our study demonstrated that root-knot nematode susceptibility was reduced in transgenic plants expressing high levels of proteinase inhibitor I (125 μg/g root tissue) or II (45 μg/g root tissue). First, gall development was reduced in proteinase inhibitor I and II-expressing root cultures (Table 2, Fig. 2). Essentially similar results were obtained from tomato and tobacco seedlings aseptically grown on agar medium (Table 3, Fig. 3). Second, nematode development was significantly delayed in proteinase inhibitor I or II-expressing root cultures (Table 4).

Previous studies have reported the detection of proteolytic activity in stylet secretions of root-knot nematodes (13) and the presence of trypsin-like proteinase in second-stage juveniles of root-knot nematodes (14). In addition, giant cells from which

nematodes obtain nutrients have been shown to contain 6 times more amino acids than other root cells (25). These results suggest that nematode proteinases may play a role in digesting host proteins. Therefore, we speculated that overexpression of proteinase inhibitors in transgenic plants might interfere with nematode digestion of host proteins. This could inhibit disease progression by limiting the availability of amino acids necessary for nematode growth and development.

The data from our experiments showed that root galling was inhibited in a higher degree in inhibitor I (chymotrypsin)- expressing root cultures or seedlings than in inhibitor II (trypsin/chymotrypsin)- expressing root cultures or seedlings as compared with controls. Nematode development was also delayed to a higher degree in inhibitor I-expressing root cultures than in inhibitor II-expressing root cultures. In transgenic plant lines used in this study, expression levels of inhibitor I or II proteins were different in root tissues. The root tissues of inhibitor I-transformed plants contained higher amounts of inhibitor I (125 μg/g), compared to levels of inhibitor II (45 μg/g) (18). Correspondingly, chymotrypsin inhibitory activity was stronger in root extracts from inhibitor I-expressing plants than from inhibitor II-expressing plants. Therefore, our results suggested a relationship between levels of chymotrypsin inhibitory activity and inhibition of nematode disease development. A similar relationship between levels of inhibitor II protein and inhibition of insect (*M. sexta*) larval growth has also been observed in a previous study (7). Since inhibitor I protein contains only chymotrypsin inhibitory activity, and inhibitor II inhibits both trypsin and chymotrypsin, we can conclude that chymotrypsin inhibitory activity, but not trypsin inhibitory activity, was mainly responsible for the inhibition of nematode disease development. However, we can not rule out the possible detrimental effects of trypsin inhibitory activity on nematode development because of the low levels of inhibitor II expression in root tissues of transgenic plants.

In this study, we found that high expression of proteinase inhibitor I or II in

transgenic plants can also delay the root growth. Reduced lateral root production and fresh weight of root cultures were observed for both TI-I- and TI-II-expressing root cultures (Table 1). The physiological basis for this inhibition is unclear. One possibility is that proteinase inhibitors interact with plant endogenous proteinases, thereby affecting normal root growth and development. The possible interaction between proteinase inhibitors and endogenous plant proteinases has been suggested in a number of other studies (26-29). The inhibition of lateral root formation in our transgenic root cultures suggests that overexpression of inhibitor proteins could affect lateral root initiation or penetration process.

Since the availability of potential penetration sites for nematode juveniles was lessened in transgenic root cultures, it may be more appropriate to compare disease symptoms by the relative extent to which the roots are galled by nematodes instead of total galls formed per root system, because the percentage of galled roots is independent of the number of penetration sites. Both percentage of root galling and number of galls per root culture were used to describe the root galling in this study. Both observations showed that root galling was reduced in transgenic root cultures as compared with control root cultures, suggesting that proteinase inhibitor transgenes indeed delayed gall development by inhibiting nematode parasitism, rather than by reducing the availability of juvenile penetration sites. In addition, root galling was reduced to a higher degree than root growth in transgenic plants. At ten days after inoculation, number of root tips per culture was reduced 52% and 30%, respectively, in inhibitor-I and II-expressing root cultures. However, number of galls per culture was reduced 67% and 83%, respectively, in TI-I- and TI-II-transformed roots. Therefore, the reduction of root galling in transgenic root cultures was not caused only by reduced infection sites of nematode juveniles. This conclusion is also supported by the observation that the transgene expression delayed the development of nematode within the root. At 35 days post-inoculation, only about 10% of nematode had reached the

female stage in transgenic roots compared to almost 50% in control roots.

Results presented in this study demonstrated that transgenic root cultures provide an efficient system for direct analysis of the role of proteinase inhibitors in enhancing nematode resistance. This study support the possibility that proteinase inhibitors could be potential candidate genes for improving nematode resistance by genetic engineering technology. However, before genetically engineered proteinase inhibitor genes can be used for practical crop improvement, further experiments would be needed at the whole plant level to assess the effects of proteinase inhibitor I and II transgenes on root-knot nematode disease development.

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Chapter III

ROOT-KNOT NEMATODE RESISTANCE IN TOMATO PLANTS EXPRESSING HIGH LEVELS OF PROTEINASE INHIBITOR TRANSGENES

ABSTRACT

Genetically engineered tomato (*Lycopersicon esculentum* cv. Better Boy) plants were used to examine the effects of high levels of proteinase inhibitor I (TI-I) or II (TI-II) transgene on development of root-knot nematode (*Meloidogyne hapla* Chitwood, 1949) disease under greenhouse conditions. Nematode disease symptoms (root galling) were reduced significantly in both TI-I- and TI-II-expressing plants at five weeks after *M. hapla* inoculation. TI-I-expressing plants continued to show reduced root galling at eight weeks after inoculation, but gall reduction had disappeared in the TI-II-expressing plants. By ten weeks after nematode inoculation, neither of the transgenic lines exhibited reduced galling responses. Nematode reproduction as expressed by egg production was also inhibited in TI-I- and TI-II-expressing plants at eight weeks after inoculation; reductions of 90% and 40% in egg numbers were found in TI-I- and TI-II-expressing plants, respectively. Production of egg masses was similarly reduced in TI-I- and TI-II-expressing plants at eight weeks after inoculation. But, by ten weeks, while TI-II expressing plants still showed reduced egg numbers, the reduction in egg numbers in TI-I-expressing plants had ceased. Overexpression of proteinase inhibitors also affected root growth. Root fresh weight was reduced in both TI-I- and TI-II-expressing plants.

INTRODUCTION

Plant root-knot diseases caused by *Meloidogyne* species of nematodes are among the most economically important crop diseases limiting agricultural production. Root-knot nematodes occur throughout the world and almost all crop plants are susceptible to these pathogens (Sasser and Carter, 1985). Root-knot nematodes have broad host ranges, parasitizing monocotyledons, dicotyledons, herbaceous, and woody plants; thus once a field becomes infested, it is nearly impossible to eradicate them. Average worldwide yield losses caused by *Meloidogyne* were estimated to be about 5% (Taylor and Sasser, 1978). These losses are not evenly distributed and can exceed 90% in highly infested soils.

Limiting crop losses due to *Meloidogyne* through the use of nematicides has been effective in the past. However, several effective nematicides have recently been banned, because of their potential hazard to human health (Fassuliotis, 1985). The use of nematode-resistant cultivars has provided the best control against root-knot nematodes. However, in some crops, traditional breeding methods have not succeeded due to the lack of nematode resistance genes (Fassuliotis, 1985).

Advances in genetic engineering technology may provide new tools for developing root-knot nematode resistant cultivars that have been impossible to produce with conventional breeding methods. By transferring single genes known to enhance resistance to root-knot nematodes from one plant species to another, improved nematode resistant crops may be generated. However, before genetic engineering can be used in practical crop improvement schemes, nematode resistance genes must be identified and their role in plant defense must be tested.

Nematode proteinases are suspected to play roles in digesting host proteins during nematode feeding periods (Zinovier, 1957; Dasgupta and Ganguly, 1975). Proteinase inhibitors found in plants could be potential candidate genes for improving plant

nematode resistance by interfering with nematode digestive processes. Proteinase inhibitors have been identified as defense chemicals that protect plants against insects and microbial pathogens (Ryan, 1990) by specifically inhibiting proteinases from insects (Broadway and Duffey, 1986; Gatehouse and Boutler, 1983) and microorganisms (Garcia-Olmedo *et al.*, 1987). Introduction of the cowpea proteinase inhibitor gene into tobacco plants by genetic engineering technology has been shown to be effective in enhancing plant defense against the insect, *Heliothis virescens* (Hilder *et al.*, 1987). Genetically engineered tobacco plants with high levels of tomato and potato proteinase inhibitor II (having trypsin and chymotrypsin inhibitory activities) were toxic to larvae of *Manduca sexta* (Johnson *et al.*, 1989).

In Chapter II, the possible defensive roles of proteinase inhibitors against root-knot nematode *Meloidogyne* species were examined by using transgenic tomato and tobacco plants expressing high levels of proteinase inhibitor I (TI-I) or II (TI-II). Nematode disease symptoms were reduced in both TI-I- and TI-II-expressing root cultures infected with *M. hapla*. Nematode development was also delayed in *in-vitro*-grown transgenic root tissues. We speculated that when nematodes secreted proteinase(s) to assist extracorporeal digestion of host proteins, the high levels of proteinase inhibitors in transgenic plants inhibited nematode proteinase activity, thereby limiting nematode growth and development.

In the present study, the effects of high levels of proteinase inhibitor transgenes on nematode disease symptom development and reproduction are examined in whole plants grown under greenhouse conditions. Root galling and nematode egg production are estimated in control and transgenic plants following *M. hapla* inoculation.

MATERIALS AND METHODS

Plant and nematode materials

Tomato (*Lycopersicon esculentum* cv. Better Boy) wild type and transgenic lines have been generously provided by Dr. C. A. Ryan (Washington State University). The specific lines were T#289-E6-pJN3-inhibitor I, T#464-D7-PGA875-inhibitor II, and the Better Boy parental cultivar. Although the control tomato, Better Boy, contains *the Mi* gene conferring resistance to *M. incognita* and several other *Meloidogyne* species, it is susceptible to *M. hapla*. The transformation plasmid constructs used here (pJN3 and pGA875) have been described previously (Johnson *et al.*, 1989). Basically, the intact coding sequences of tomato proteinase inhibitor I or II are driven by approximately 400 bp of the CaMV 35S promoter (Fig. 1, Chapter II). Transgenic tomato plants were obtained by *Agrobacterium*-mediated leaf disc transformation (Horsh *et al.*, 1985; An *et al.*, 1986) and all plants tested were homozygous for the proteinase inhibitor I or II transgene (C. A. Ryan, personal communication). High levels of inhibitor I (125 μg/g tissue) and II (45 μg/g tissue) proteins are accumulated in root tissues of transgenic tomato plants expressing the inhibitor I and II genes, respectively (Narváez-Vásquez, *et al.*, 1993). However, the root tissues of untransformed tomato plants contain low levels of inhibitor I and undetectable levels of inhibitor II (Narváez-Vásquez, *et al.*, 1993). USDA-APHIS approval for receiving these transgenic seeds was obtained in 1991.

Root-knot nematode cultures of *M. hapla* were kindly supplied by Dr. J. D. Eisenback (Dept. of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University).

Growth of plants

Tomato seeds of wild type and transgenic lines were germinated and grown in a commercial soil mix (Promix, Wetsel) in a greenhouse under natural light conditions at

25°C. Plants were watered twice a day and fertilized every two weeks. All transgenic plants were handled in our facilities according to National Institute of Health (NIH) recombinant DNA guidelines (BL1-P). All transgenic plants, and nematodes or soil mix used in association with transgenic plants were autoclaved prior to final disposal.

Nematode cultures

Nematode stock cultures were maintained and multiplied on tomato *(Lycopersicon esculentum* cv. Rutger) plants grown in 15 cm clay pots filled with steam sterilized soil (2 clay soil: 1 sand) in a greenhouse (25°C). The nematode populations were subcultured every 8-10 week to ensure a constant supply of nematode eggs as described previously (Barker, 1985). Nematode infected plants were watered twice a day and fertilized every two weeks.

Nematode inoculum preparation and inoculation

The nematode egg inoculum was prepared according to Barker (1985). Tomato root tissues from a 6- to 12-week-old infected plant were collected and cut into 1-2 cm segments. Root segments were then shaken vigorously in 500 ml 10% commercial bleach solution for 4 minutes. Released eggs were collected on a 500-mesh sieve. Residual bleach was then removed by repeated washing with tap water. For nematode inoculation, three-week-old seedlings were transferred to steam-sterilized soil (2 clay soil: 1 sand) in 15 cm pots and inoculated with *M. hapla* eggs (5,000 eggs/plant) as described (Barker, 1985). Infected plants were grown in a greenhouse, watered twice a day and fertilized every two weeks.

Estimation of root-galling

Galled roots were harvested by washing under a stream of tap water. The cleaned roots were then weighed and placed in water. The number of galls was counted under a

stereoscopic microscope. Galling was expressed as the number of galls per gram of roots or per plant. Samples of at least three replicates were collected. Student's t-tests were used to determine significant differences in root galling between control Better Boy and TI-I- or TI-II-expressing plants.

Estimation of nematode egg production

Infected roots were harvested by washing under a stream of tap water. Eggs were then extracted from roots with 10% bleach as outlined by Barker (1985). The roots collected were cut into 1-2cm segments. The root segments were then shaken vigorously in 500 ml 10% commercial bleach for 4 minutes. Freed eggs were collected on a 500-mesh sieve. Extracted eggs were then counted under a stereoscopic microscope. Egg production was expressed as number of eggs per gram of roots or per plant. Samples of at least three replicates were estimated. Student's t-tests were used to determine the significant differences in egg production between Better Boy and TI-I- or TI-II-expressing plants.

Estimation of nematode egg mass production

Infected tomato roots were harvested by washing under a stream of tap water. Cleaned roots were then stained red with 0.015% phloxine B for 20 minutes as described (Daykin and Hussey, 1985). Red egg masses were then counted. Egg mass production was expressed as number of egg masses per plant or per gram of roots. Samples of at least three replicates were collected. Student's t-tests were used to determine the significant differences in egg mass production between control Better Boy and TI-I- or TI-II-expressing plants.

RESULTS

Effect of proteinase inhibitor I or II transgene on growth of plant roots

Fresh weight of roots was measured to examine the effects of proteinase inhibitor transgenes on growth of plant roots. Fresh weight of roots in transgenic and control plants was compared five and ten weeks after planting (Table 1). At five weeks after planting, 27% and 17% decreases in root fresh weights were observed for TI-I- and TI-II-expressing plants, respectively, as compared with untransformed plants. By ten weeks after planting, 24% and 55% reductions in root fresh weights were found for TI-I- and TI-I-expressing plants, respectively, as compared with controls. The overexpression of proteinase inhibitor I or II in transgenic plants, therefore, appeared to inhibit root growth.

Effect of proteinase inhibitor I or II transgene on gall development

In order to evaluate the effects of proteinase inhibitor I or II transgene on nematode disease development, the time-course of gall development was compared between control and TI-I- or TI-II-expressing plants inoculated with *M. hapla* under greenhouse conditions (Table 2). A reduction in root galling was observed in both TI-I- and TI-II-expressing plants as compared with control plants at five weeks after inoculation. By eight weeks, TI-I-expressing plants still showed reduced root galling, however, no significant differences remained in root galling between control and TI-II-expressing plants. By ten weeks post-inoculation, all differences in the number of galls per plant among the three lines had disappreared. However, TI-II-expressing plants showed increased numbers of galls per gram of roots when compared to control plants. This result suggested that overexpression of proteinase inhibitor I or II in transgenic plants can delay gall development and that gall formation in transgenic plants is inhibited during the early period of nematode infection.

Table 1. Effect of proteinase inhibitor I or II transgene on root growth (grams fresh weight)[a]

Plant	Weeks after inoculation	
	5	10
TI-I Expressing	42.9±(5.2)[b]	68.0±(0.4)[b]
TI-II Expressing	49.4±(10.0)[c]	40.4±(5.9)[b]
Control	58.8±(6.9)	88.5±(9.7)

a Mean±(standard deviation) for samples from at least three replicates
b Significantly different from control ($p < 0.05$) according to Student's t-test
c Significantly different from control ($p < 0.10$) according to Student's t-test

Table 2. Effect of proteinase inhibitor I or II transgene on gall development[a]

Plant	Weeks after inoculation					
	5		8		10	
	galls per plant	galls per g of root	galls per plant	galls per g of root	galls per plant	galls per g of root
TI-I Expressing	308±(72)[c]	7±(2)	570±(117)[c]	11±(2)[b]	748±(109)	11±(2)
TI-II Expressing	218±(99)[b]	5±(3)[c]	709±(117)	15±(3)	802±(87)	20±(1)[b]
Control	552±(300)	9±(4)	710±(97)	15±(2)	751±(81)	9±(2)

a Mean±(standard deviation) for samples from at least three replicates
b Significantly different from control ($p<0.05$) according to Student's t-test
c Significantly different from control ($p<0.10$) according to Student's t-test

In addition, the galls formed on roots of transgenic plants showed altered morphological characteristics (Fig. 1) as compared with Better Boy. Normally, *M. hapla* produces small discrete galls associated with proliferation of rootlets giving rise to a matted appearance (Fig. 1A). In contrast, TI-I- and TI-II expressing plants displayed enlarged terminal galls which were associated with the adventitious roots (Fig. 1B and 1C). At least three replicate plants of each line were observed for gall morphology. Consistent gall morphological differences were shown on root tissues of control versus transgenic plants as described above.

Effect of proteinase inhibitor I or II transgene on nematode egg production

To examine the effects of proteinase inhibitor transgenes on nematode reproduction, nematode egg production was compared among the three plant lines. Eight weeks after inoculation, reduced numbers of nematode eggs were observed on both TI-I- and TI-II-expressing plants (Table 3). Averages of 90% and 40% less eggs were produced on TI-I- and TI-II-expressing plants, respectively, as compared with control plants. While fewer eggs were produced on TI-II plants at ten weeks after nematode inoculation, as compared with control plants; more eggs were produced on the TI-I plants than on control plants.

Additionally, *M. hapla* reproduction was also measured by counting the number of egg masses produced (Table 4). This is related to the number of egg-producing females present in root tissues. Essentially a similar pattern was observed for egg mass production at eight weeks as had previously been found for eggs, indicating that the number of egg-producing females was reduced in transgenic plants. There was no difference in number of egg masses produced among three lines of plants at ten weeks after inoculation.

The number of eggs produced in each egg mass (per female) was also compared among the plants used in this study (Table 5). At eight weeks after inoculation, less

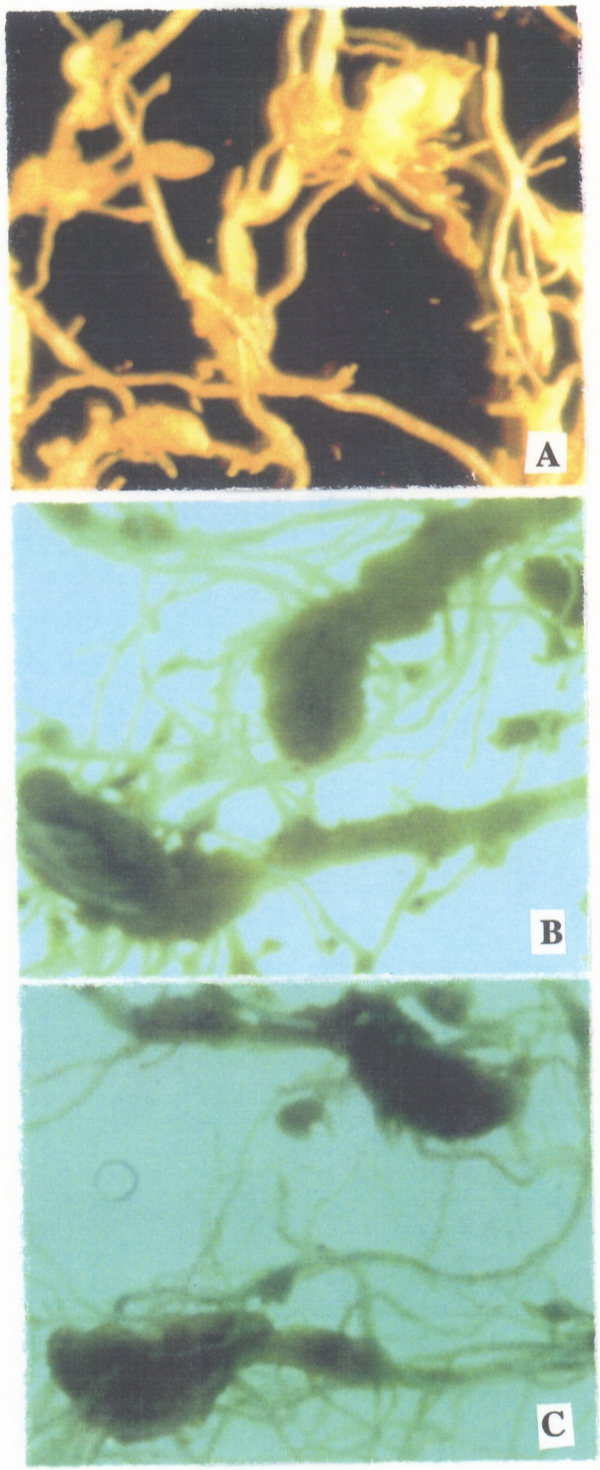

Fig.1. Morphology of root galls in control and transformed tomato plants eight weeks after *M. hapla* inoculation. A, Control; B, TI-I-expressing; C, TI-II-expressing. Enlarged terminal galls were shown on proteinase inhibitor-expressing roots.

Table 3. Effect of proteinase inhibitor I or II transgene on nematode egg production[a]

Plant	Weeks after inoculation			
	8		10	
	eggs/root	eggs/g of root	eggs/root	eggs/g of root
TI-I Expressing	12292[b]	218[b]	273000	3999
TI-II Expressing	61000	1360	76166[b]	1951
Control	98667	2026	235111	2644

a Mean for samples from at least three replicates
b Significantly different from control ($p < 0.05$) according to Student's t-test

Table 4. Effect of proteinase inhibitor I or II transgene on nematode egg mass production[a]

Plant	Weeks after inoculation			
	8		10	
	egg masses per plant	egg masses per g of root	egg masses per plant	egg masses per g of root
TI-I Expressing	82[b]	3[b]	620	14
TI-II Expressing	181	8	583	18
Control	543	14	627	15

a Mean for samples from at least three replicates
b Significantly different from control ($p < 0.10$) according to Student's t-test

Table 5. Effect of proteinase inhibitor I or II transgene on the number of eggs produced per egg mass by *M. hapla*[a]

	Weeks after inoculation	
Plant	8	10
TI-I Expressing	57±(22)	312±(88)[b]
TI-II Expressing	66±(39)	204±(73)
Control	84±(33)	219±(51)

a Mean±(standard deviation) for samples from at least three replicates
b Significantly different from control ($p < 0.10$) according to Student's t-test

eggs were produced per egg mass on TI-I- and TI-II-expressing plants than in controls. Ten weeks after inoculation, however, increases in egg production per female were found in TI-I-expressing plants as compared with controls. During the early infection period, the number of eggs produced on control and transformed plants was positively correlated with the number of egg masses ($r^2=0.9978$) and number of eggs per egg mass ($r^2=0.9697$). In other words, the decrease in egg production appeared to be due to the reduction in both egg mass and eggs per egg mass at this early stage in transgenic plants. During the later stage, however, total egg production was correlated only with the amount of eggs produced per egg mass ($r^2=0.9902$). The increased egg production in TI-I-expressing plants was mainly due to an enhanced egg production per egg mass (per female) at ten weeks after inoculation.

This experiment showed that overexpression of proteinase inhibitor I or II in transgenic plants inhibited nematode egg production during the early stage, but not in the late stage of nematode infection.

Effect of temperature on egg production

An experiment was performed to examine the reproductive responses of *M. hapla* on transgenic and control plants at different temperatures. By eight weeks after inoculation (Table 6), egg production was reduced in TI-I- and TI-II-expressing plants when they were incubated at 24°C. However, increased amount of eggs were produced on TI-I-expressing plants eight weeks after inoculation when they were incubated at 31°C. Thus at this higher temperature, the inhibition of nematode egg production in transgenic plants was not even observed at early stages of nematode infection.

Table 6. Effect of temperature on *M. hapla* egg production eight weeks post-inoculation[a]

Plant	Temperature			
	24°C		31°C	
	eggs/plant	eggs/g of root	eggs/plant	eggs/g of root
TI-I Expressing	5542[c]	170[c]	298400[b]	24331[b]
TI-II Expressing	15444	603	88267	4759
Control	64084	1488	82667	6793

a Mean for samples from at least four replicates
b Significantly different from control ($p < 0.05$) according to Student's t-test
c Significantly different from control ($p < 0.10$) according to Student's t-test

DISCUSSION

A previous study (Johnson *et al.*, 1989) had shown that transgenic tobacco leaves expressing high levels of tomato proteinase inhibitor II (> 50 μg/g tissue) inhibited growth of the insect, *Manduca sexta* (tobacco hornworm). Because trypsin and chymotrypsin play essential roles in the digestive processes of insects, proteinase inhibitor II was thought to inhibit insect growth by interfering with its ability to digest plant proteins.

In Chapter II, genetically engineered tomato and tobacco plant lines developed by C. A. Ryan's laboratory were employed to test the possible roles of proteinase inhibitor transgenes in plant-root-knot nematode interactions. Aseptically grown root and seedling cultures on agar medium were used for this nematode infection study. Results presented in Chapter II clearly demonstrated that overexpression of proteinase inhibitor I (125 μg/g tissue) or II (45 μg/g tissue) transgene in root tissues enhanced plant resistance to nematode infection. Both gall formation and nematode development were inhibited in proteinase inhibitor-expressing root cultures or seedlings as compared with controls.

The present report extends this study to the whole plant level by infecting intact greenhouse grown transgenic tomato lines with *M. hapla*. Similar to our previous study in Chapter II, we found that root growth was inhibited in both TI-I- and TI-II-expressing plants (Table 1) confirming that overexpression of proteinase inhibitors in transgenic plants affected the normal growth and development of root tissues. This inhibition of root growth probably accounted for the enlarged terminal galls in transgenic plants infected with nematodes (Fig. 1). Normally, terminal galls move up as roots elongate. However, terminal galls stay the original position when root elongation is inhibited.

Both root gall formation (Table 2) and nematode egg production (Table 3 & 4) were reduced in proteinase inhibitor-expressing plants during the early nematode infection period. As discussed in Chapter II, the reduced root mass in transgenic plants was not responsible for the reduced root gall formation. At five weeks after inoculation, root fresh weight per plant was reduced 27% and 16%, respectively, in TI-I- and TI-II-expressing plants. However, gall formation per plant was inhibited 44% and 61%, respectively, in inhibitor I- and II-transformed plants. In addition, the number of galls per gram of root tissues, which is independent of root mass per plant, was also reduced in trangenic plants. Therefore, the delayed nematode disease symptom development was mainly caused by the direct inhibition of nematode parasitism rather than the reduced root growth in transgenic plants during the early stages of nematode infection. At later stages, both transgenic lines ceased to exhibit reduced galling and nematode reproductive responses. In addition, nematode egg production on TI-I-expressing plants acturally appeared to be enhanced at ten week after nematode inoculation. Furthermore, transgenic plants did not show reduced nematode egg production during the early infection stage at high temperature (31°C) (Table 6).

The reasons for the disappearance of enhanced nematode resistance in transgenic plant lines at late nematode infection stages and at high temperature are unclear. A possible explanation could lie in the expression pattern of the CaMV 35S promoter used for the gene constructs. To successfully employ proteinase inhibitor genes for nematode defense, it would be crucial to use a promoter that can strongly express the proteinase inhibitors in root tissues at all stages of the infection process, especially in cells utilized for nematode feeding.

Previous studies (Odell *et al.*, 1985, 1987; Nagy *et al.*, 1987; Kay, *et al.*, 1987; Jefferson *et al.*, 1987) showed that the 400 to 1000 base pairs of CaMV 35S upstream fragments were highly active in a wide variety of plant tissues and during most stages of development when integrated into plant genome. However, the expression of CaMV

35S promoter was routinely determined only in young seedlings, rather than in mature plants in these studies. Benfey *et al.* (1989) demonstrated that the -343 to +8 upstream fragment of CaMV 35S promoter was active in the vascular tissue, the pericycle, and most other cell types in root tissues of seven-week-old mature plants. However, the expression was not examined in mature plants after seven weeks. In addition, the plants used in their study were maintained in tissue culture medium, but not in soil or under greenhouse conditions.

The CaMV 35S-tomato proteinase inhibitor I gene construct used in this study had been previously examined for its differential expression in transgenic tobacco plants (Narváez-Vásquez *et al.*, 1992). This study found that young developing leaf tissues and floral tissues contained higher proteinase inhibitor I. A higher percentage of proteinase inhibitor I was also found in apical tissues of tobacco leaves, compared with basal tissues from the same leaves, indicating stronger expression of proteinase inhibitor I in younger tissues.

Most recently, the cellular localization of proteinase inhibitor I and II proteins, synthesized in transgenic plants was examined (Narváez-Vásquez, *et al.*, 1993). It was found that newly synthesized inhibitor proteins were not only accumulated in the cell's vacuoles as in wild-type plants, but were also secreted into the cell walls of outer epidermal and secretory cells of root cap through the secretory pathway. It was suggested that such extracellular proteinase inhibitor proteins in root tissues may function in protecting the growing meristems from insects or microorganisms in soil. However, the presence of extracellular inhibitor proteins may not help provide protection against root-knot nematode infection, because of the specific feeding relationships between nematodes and host cells. Thus, the extracellular deposition of inhibitor proteins in older root tissues of transgenic plants might also cause the loss of nematode resistance responses during later stages of nematode infection.

Further research will be necessary to precisely monitor the expression of proteinase inhibitors, driven by CaMV 35S promoter, in root tissues of transgenic plants inoculated with nematodes. Exact localization of proteinase inhibitors within root tissues needs to be determined. Additionally, more information is needed to understand the adaptive biology of parasitic nematodes under host nutritional stress.

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Chapter IV

TEMPORAL AND TISSUE-SPECIFIC EXPRESSION OF CaMV 35S-TOMATO PROTEINASE INHIBITOR GENES IN ROOT TISSUES OF TRANSGENIC PLANTS INFECTED WITH NEMATODES

ABSTRACT

The expression levels of proteinase inhibitors in root tissues were examined in transgenic tomato (*Lycopersicon esculentum* cv. Better Boy) plants inoculated with the endoparasitic nematode *Meloidogyne hapla* Chitwood, 1949. High expression of proteinase inhibitor I was confirmed in root tissues of inhibitor I-transformed and nematode infected plants at eight weeks after inoculation. Similarly, high levels of inhibitor II were observed in root tissues of inhibitor II-transformed plants infected with nematodes. The different developmental expression of inhibitors in nematode-infected root tissues was observed and correlated with nematode disease development. Higher levels of proteinase inhibitors were found in root tissues at six weeks than at eight weeks after inoculation. Nematode disease development was inhibited in transgenic plants six weeks after inoculation and this inhibition ceased by eight weeks after inoculation. Additionally, transgenic plants carrying CaMV 35S-ß-glucuronidase reporter (GUS) gene fusion were used for analyzing the tissue-specific expression of GUS gene regulated by the CaMV 35S promoter. High expression of GUS was found in all tissues of young roots. However, much less expression of GUS was observed in older roots. Among the nematode-infected roots examined, GUS expression was only shown on root tips, and no expression was observed in gall tissues containing feeding nematodes. The results of this study suggest that the CaMV 35S promoter may not be suitable for genetically engineering nematode-resistant plants, because of its differential

developmental and tissue-specific expression in root tissues of transgenic plants infected with nematodes.

INTRODUCTION

The cauliflower mosaic virus (CaMV) 35S promoter has been extensively used for regulating foreign gene expression in transgenic plants. The CaMV 35S fragments containing 400-1000 bp of 35S upstream sequences have been shown to be highly active in a wide variety of plant tissues and during most stages of development when integrated into plant genomes (Odell *et al.*, 1985, 1987; Jefferson *et al*, 1987; Sanders *et al.*, 1987; Kay *et al.*, 1987; Nagy *et al.*, 1987). The regulatory elements for developmental and tissue-specific expression of this promoter have been identified and characterized (Benfey *et al.*, 1989; Fang *et al.*, 1989). Benfey *et al.* (1989) demonstrated that the -343 to +8 upstream element of the CaMV 35S promoter was highly expressed in the vascular tissues, pericycle and most other cell types of root tissues of transgenic plants grown in tissue culture medium. However, in these studies the expression of the CaMV 35S promoter was not determined in plants older than seven weeks. The CaMV 35S promoter has also been used to develop transgenic tobacco (*Nicotiana tabacum*) plants expressing foreign proteinase inhibitor genes (Hilder *et al.*, 1987; Johnson *et al.*, 1989). Such proteinase inhibitor-expressing tobacco plants exhibited increased resistance against insects (Hilder *et al.*, 1987; Johnson *et al.*, 1989). Because proteinases play essential roles in the digestive processes of insects (Applebaum, 1985; Broadway, 1989; Houseman *et al.*, 1989), proteinase inhibitors are thought to help defend plants by interfering with insect feeding (Ryan, 1990).

Recently, we employed transformed tomato plants with fused CaMV 35S-proteinase inhibitor genes to test the possible roles of proteinase inhibitors against pathogenic root-

knot nematodes (Chapter III). We found that nematode disease development was inhibited during early infection stages in transgenic plants. However, this inhibition ceased during later infection stages. These results led us to question the suitability of the CaMV 35S promoter used for regulating proteinase inhibitors in the gene constructs.

The successful exploitation of proteinase inhibitor transgenes for nematode defense requires the use of a promoter that can strongly and continuously drive the expression of proteinase inhibitors in root tissues, especially in cells specifically adapted for nematode feeding. The CaMV 35S-tomato proteinase inhibitor I fusion gene had previously been examined for its differential expression in transgenic tobacco plants (Narváez-Vásquez *et al.*, 1992). That study found that young developing leaf and floral tissues contained higher proteinase inhibitor I levels than other tissues. A higher amount of proteinase inhibitor I was also shown in apical tissues as compared with basal tissues of tobacco leaves, suggesting that higher expression of proteinase inhibitor I was occurring in younger tissues. High expression (30 μg/g) of proteinase inhibitor I was also found in root tissues of young transgenic tobacco plants. However, developmental and tissue-specific expression of inhibitor I gene in root tissues were not examined in previous studies.

In the present study, the expression levels of proteinase inhibitors were measured in root tissues of transgenic tomato plants infected with root-knot nematodes. In addition, the developmental expression of proteinase inhibitors in nematode-infected roots was monitored and correlated with nematode disease development. Furthermore, transgenic plants carrying the CaMV 35S-ß-glucuronidase (GUS) fusion gene were used to analyze the tissue-specific expression of the CaMV 35S promoter.

MATERIALS AND METHODS

Plant and nematode materials

Tomato wild type (*L. esculentum* cv. Better Boy) and transgenic lines were generously provided by Dr. C.A. Ryan (Washington State University). The specific lines were T#289-E6-pJN3-inhibitor I, T#464-D7-pGA875-inhibitor II, and the Better Boy parental cultivar. Although control tomato cultivar, Better Boy, contains the *Mi* gene conferring resistance to *M. incognita* and several other *Meloidogyne* species, it is susceptible to *M. hapla*. The transformation plasmids used (pJN3-TI-I and pGA875-TI-II) have been described previously (Johnson *et al.*, 1989). Basically, the intact coding sequences of tomato proteinase inhibitor I (TI-I) or II (TI-II) are driven by approximately 400 bp of the CaMV 35S promoter (Fig. 1, Chapter II). Transgenic tomato plants were obtained by *Agrobacterium*-mediated leaf disc transformation (Horsh *et al.*, 1985; An *et al.*, 1986) and all plants tested were homozygous for the proteinase inhibitor transgenes (C.A. Ryan, personal communication). USDA-APHIS approval for receiving these transgenic seeds was obtained in 1991.

Transgenic tobacco (*N. tobacum* cv. Xanthi) and tomato (*L. esculentum* cv. Vender) plants transformed with plasmid construct SLJ1911 (Fig. 1) were obtained from Dr. Carole Cramer (Dept. of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University). This gene construct contains a CaMV 35S promoter (394 bp) fused to ß-glucuronidase (GUS) reporter gene.

All transgenic plants were handled in our facilities according to National Institute of Health (NIH) recombinant DNA guidelines (BL1-P). All transgenic plants, and nematodes or soil mix used in association with transgenic plants were autoclaved prior to final disposal.

Root-knot nematode *M. hapla* cultures were kindly supplied by Dr. Jon Eisenback (Department of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic

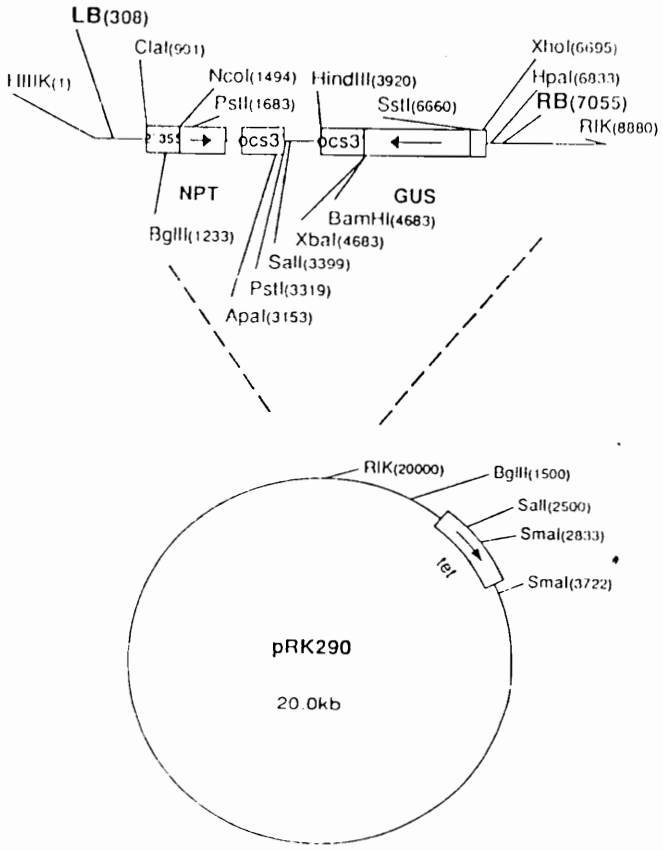

Fig. 1. Transformation plasmid SLJ1911. 394bp of CaMV 35S promoter was fused to beta-glucuronidase (GUS) reporter gene (Plasmid was constructed in the laboratory of Dr. Jonathan Jones, Johnlnnes Inst. UK and provided to the laboratory of Dr. Carole Cramer.

Institute and State University). Stock cultures were maintained and multiplied on tomato (*L. esculentum* cv. Rutger) plants grown in 15 cm clay pots filled with steam sterilized soil (2 clay soil: 1 sand) in a greenhouse. The nematodes were subcultured every 8-10 week to ensure a constant supply of nematode eggs as described (Barker, 1985). Plants were watered twice a day and fertilized every two weeks.

Nematode inoculation

Seeds were germinated and grown in a commercial soil mix (Promix, Wetsel) in a greenhouse under natural light conditions. About three-week-old seedlings were transferred to steam-sterilized soil (2 clay soil: 1 sand) in 15 cm pots and inoculated with *M. hapla* eggs (5,000 eggs/plant). Nematode egg inoculum was prepared according to Barker (1985) as follow: Infected tomato roots of 6- to 12-week-old plants were collected and cut into 1-2 cm segments. Root segments were then shaken vigorously in 500 ml 10% commercial bleach solution for 4 minutes. Freed eggs were collected on a 500-mesh sieve. Residual bleach was then removed by repeated washing with tap water. Plants inoculated with nematodes and grown in a greenhouse were watered twice a day and fertilized every two weeks.

Estimation of root-galling

Galled roots were harvested by washing under a stream of tap water. The cleaned roots were then weighed and placed in water. The number of galls was counted under a stereoscopic microscope. The galling was expressed as the number of galls per plant.

Estimation of nematode egg production

Infected roots were harvested by washing under a stream of tap water. Eggs were then extracted from roots with 10% commercial bleach as outlined by Barker (1985). The roots collected were cut into 1-2 cm segments. The root segments were then

shaken vigorously in 500 ml 10% commercial bleach for 4 min. Freed eggs were collected on a 500-mesh sieve. Extracted eggs were then counted under a stereoscopic microscope. Egg production was expressed as number of eggs per plant.

Preparation of root extracts

Root extracts were prepared following the procedure of Johnson *et al.* (1989) with modifications. All steps were carried out at 0-4°C. Root tissues (5 g) collected from middle part of root systems were ground with a mortar and pestle after adding 10 ml of an extraction buffer containing 0.1% ascorbic acid, 0.1% cysteine, 0.5 M sucrose, and 0.1 M Tris (pH 7.0). The resulting macerates were centrifuged at 10,000xg for 10 min. The supernatants were collected and again centrifuged at 10,000xg for 10 min. The solubilized proteins were then fractionated by adding solid ammonium sulfate with stirring to 80% saturation. After 1 hour, the precipitates were collected by centrifugation at 2,500xg for 10 min and dissolved in 1 ml distilled water. The solution was stored at -20°C until it was assayed for inhibitor activity.

Total protein contents of root tissues were determined by the method of Bradford (1976) with bovine serum albumin as a standard.

Activity assays for proteinase inhibitors

Root extracts were assayed spectrophotometrically for proteinase inhibitory activity by measuring their inhibition (%) of trypsin and chymotrypsin activities. Trypsin and chymotrypsin activities were determined using the method of Hummel (1959) with the substrates tosyl-L-arginine methyl ester (TAME) and benzoyl-L-tyrosine ethyl ester (BTEE), respectively.

Assay for proteinase inhibitor activity was initiated by incubating root extracts with 1 μg trypsin or 3 μg chymotrypsin for 5 min at 25°C. The mixtures were then assayed for trypsin and chymotrypsin activity as described (Hummel, 1959). In trypsin assay, 1

ml of 1.04 mM TAME in 0.05 M Tris-HCl buffer (pH 8.0) buffer was mixed thoroughly with 1 ml Tris-HCl (pH 8.0) buffer and 0.1 ml sample mixture. The changes in absorbance were read at 247 nm at 25°C at 1 min intervals. The chymotrypsin activity was measured using 1 mM BTEE as a substrate in 60% methanol. 1 ml substrate was mixed with 1 ml 0.1 M Tris-HCl (pH 7.8), 0.1 M $CaCl_2$ buffer and 0.1 ml sample. The absorbance was determined at 256 nm at 25°C at 1 min intervals.

Immunodiffusion assays for proteinase inhibitors

Immunological radial diffusion assays were used for determining proteinase inhibitors I and II levels in root tissues of transgenic plants infected with *M. hapla*, using purified proteinase inhibitor I and II as standards (Ryan, 1967). Leaf and root samples from three replicates of each line were collected and freeze dried in our laboratory and sent to Dr. Clarence Ryan's laboratory (Washington State University) for the immunodiffusion assays (Ryan, 1967). Leaves were collected from middle part of plants, and root tissues were collected from middle part of root systems.

Indirect enzyme-linked immunosorbent assay (ELISA) of proteinase inhibitors

The procedure for indirect ELISA was essentially outlined by Converse and Martin (1990) (Appendix B), by using polystyrene ELISA plates. Root tissues (2 g) collected from middle part of root systems were extracted in 2 ml extraction buffer (0.05 M sodium carbonate pH 9.6). The root extracts (100 μl) were then added to the wells of microplates. After incubation and washing steps, rabbit anti-potato inhibitor I or anti-potato inhibitor II serum (gifts from Clarence Ryan, Washington State University) diluted in conjugate buffer (1:10,000 dilution) was added, incubated and washed. Finally, goat anti-rabbit antibody conjugated to alkaline phosphatase and then the substrate p-nitrophenyl phosphate were added to wells. Absorbance (E_{405}) was read at

405 nm on a ELISA reader. ELISA values (E_{405}) were used directly to compare proteinase inhibitor levels in root extracts. This experiment was performed under the guidance of Dr. Sue Tolin (Dept. of Plant Pathology, Physiology, and Weed Science, Virginia Polytechnic Institute and State University).

Histochemical analysis of ß-glucuronidase (GUS) expression

Histochemical localization of GUS *in-situ* was performed with GUS chromogenic substrate 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (X-gluc). Root samples from transgenic plants carrying CaMV 35S-GUS fusion gene were collected and washed with tap water. Cleaned roots were then placed in 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM substrate as described by Jefferson (1987). Root tissues were vacuum-infiltrated three times, 1 min each and then incubated at 37°C overnight. Stained roots were washed and photographed for GUS expression. Nematodes within root tissues were localized by the sodium-hypochloride-acid-fuchsin staining method (Daykin and Hussey, 1985).

RESULTS

Expression of proteinase inhibitors in transgenic tomato plants infected with *M. hapla*

To examine the tissue-specific expression of proteinase inhibitors in transgenic plants infected with nematodes, leaf and root extracts from control and transgenic tomato plants infected with *M. hapla* eight weeks after inoculation were assayed by immunoradial diffusion for the presence of inhibitor I and II proteins (Table 1). As expected, leaves of TI-I-transformed plants contained higher levels of inhibitor I than either control or TI-II-transformed plants. Similarly, leaves of TI-II-transformed plants showed higher inhibitor II levels than those of untransformed and TI-I-transformed plants. High expression of proteinase inhibitor I and II was also shown in root tissues

Table 1. Presence of proteinase inhibitors in leaves and roots from control and transformed plants 8 weeks post-inoculation with *M. hapla*[a]

Plant	Inhibitor I (μg/g DW[b])		Inhibitor II (μg/g DW)	
	leaves	roots	leaves	roots
Control	641 ± (25)	134 ± (127)	121 ± (55)	0
TI-I-expressing	1604 ± (116)	1525 ± (306)	156 ± (16)	0
TI-II-expressing	704 ± (56)	47 ± (66)	946 ± (108)	233 ± (81)

a Mean ± (standard deviation) for samples from at least three replicates
b DW - dry weight

of TI-I-transformed and TI-II-transformed plants respectively. However, lower levels of proteinase inhibitor II were expressed in roots than in leaves of the TI-II-transformed plants, indicating the differential expression of proteinase inhibitor II in leaf and root tissues of transgenic plants infected with nematodes. Endogenous expression of inhibitor II was not detected in control and TI-I-transformed root tissues infected with *M. hapla*. Only minimum expression of inhibitor I was detected in control and TI-II-transformed roots infected with *M. hapla*.

Root extracts from tomato plants were also assayed for their inhibitory activities against trypsin and chymotrypsin proteinases. Root extracts from tomato plants transformed with inhibitor I inhibited chymotrypsin (Fig. 2) but not trypsin activity (data not shown). Root extracts from tomato plants transformed with inhibitor II inhibited both trypsin and chymotrypsin activity (Fig. 3). Root extracts from control root tissues contained neither trypsin nor chymotrypsin inhibitory activity (Fig. 2 and Fig. 3). These results demonstrated that the proteinase inhibitors expressed in transgenic plant roots were biologically active and functional inhibitors of trypsin or chymotrypsin proteinases.

Therefore, high expression of proteinase inhibitors was confirmed by immunodiffusion assays in root tissues of transgenic plants infected with *M. hapla* eight weeks after inoculation, and these were functional inhibitors of trypsin and chymotrypsin enzymes.

Developmental expression of proteinase inhibitors in root tissues of transgenic plants infected with *M. hapla*

Expression of proteinase inhibitor I or II in root tissues of transgenic plants infected with *M. hapla* was monitored to examine the possible correlation between nematode resistance responses and expression levels of inhibitors. ELISA values (E_{405}) for proteinase inhibitors were determined in root extracts from 6- and 8-week-old *M.*

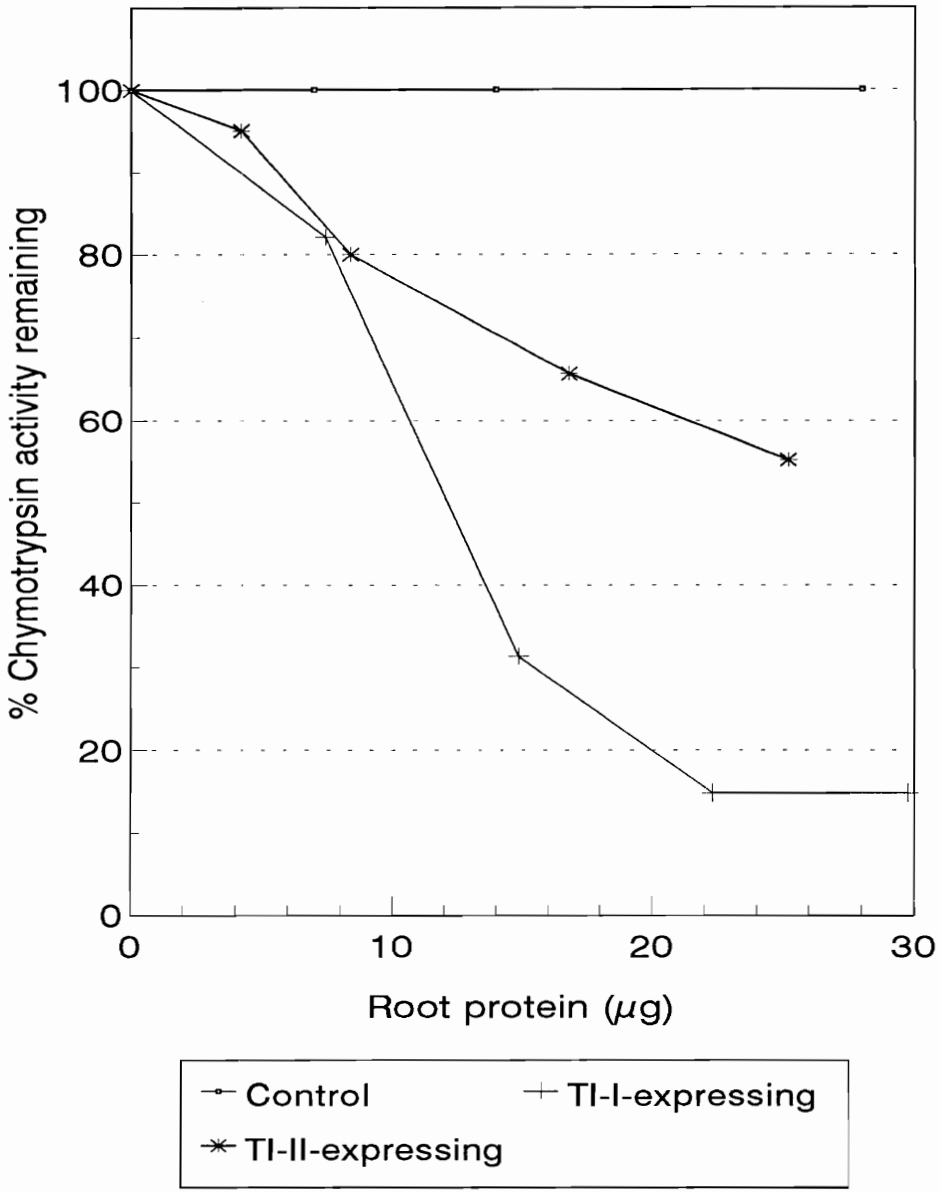

Fig. 2. Inhibition of chymotrypsin activity by tomato root extracts from control, TI-I-expressing and TI-II-expressing plants inoculated with *M. hapla* eight weeks after inoculation. Root extracts were prepared as described in Materials and Methods. Root extracts were incubated for 5 min with 3μg chymotrypsin. Chymotrypsin activity against BTEE was then assayed to determine the remaining activity as detailed in the text. Each point represents the mean of two duplicate assays.

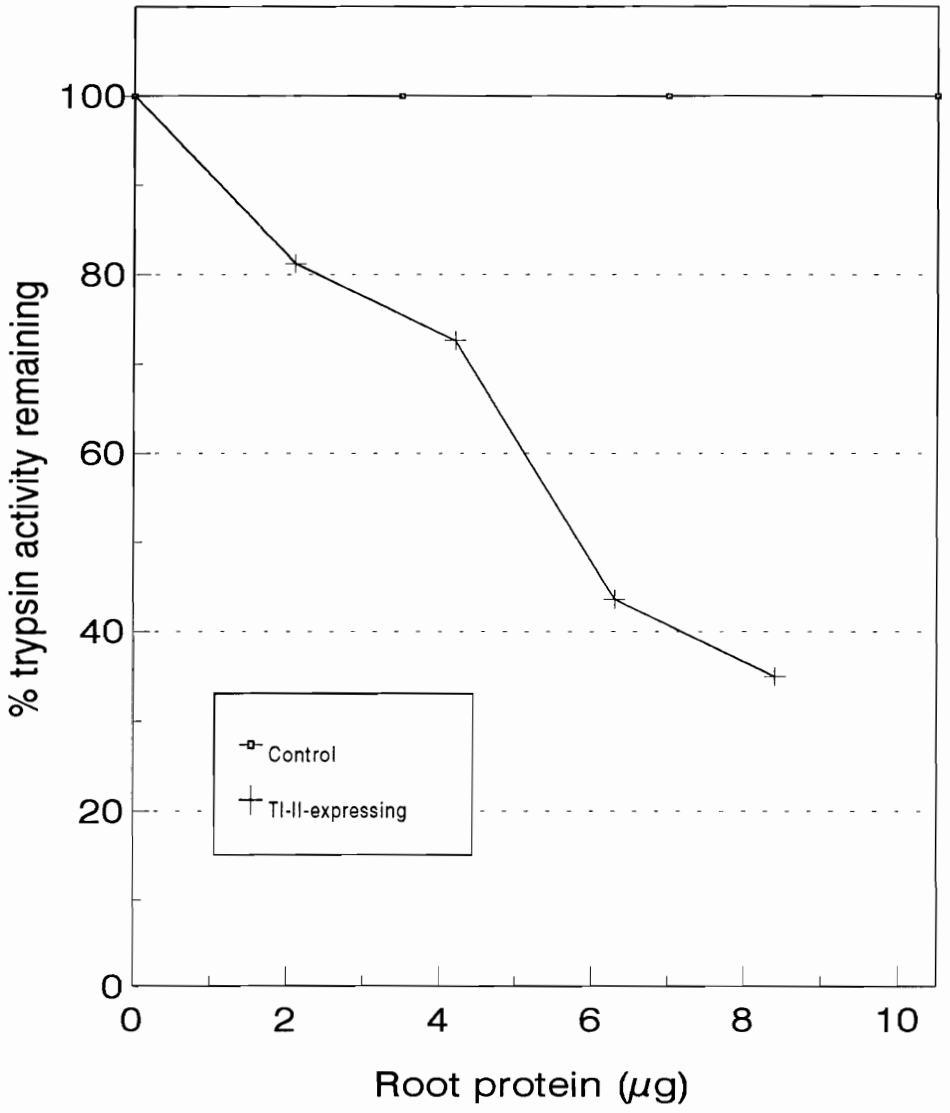

Fig .3. Inhibition of trypsin activity by root extracts from control and TI-II-expressing plants. Root extracts were prepared as described in Materials and Methods. Root extracts were incubated for 5 min with 1μg trypsin. Trypsin activity against N-tosyl-L-arginine methyl ester was then assayed to determine the remaining activity as detailed in the text. Each point represents the mean of two replicate assays.

hapla-infected control and transgenic tomato plants (Table 4). We found that ELISA values for inhibitor I or II were higher in root extracts from 6-week-old than those from 8-week-old infected transgenic plants. For transgenic plants without nematode inoculation, ELISA values were similar in root extracts from 6- and 8-week-old transgenic plants. This expression pattern of proteinase inhibitors was correlated with nematode resistance responses. Both root galling (Table 2) and nematode egg production (Table 3) were inhibited in transgenic plants as compared with control plants at six weeks after inoculation. However, this inhibition ceased by eight weeks after inoculation.

This experiment showed that nematode disease development was correlated with inhibitor levels in root tissues of transgenic tomato plants. When higher levels of proteinase inhibitors were expressed in root tissues, nematode disease development was inhibited. This early inhibition ceased when expression of inhibitors was reduced in root tissues at eight weeks after inoculation.

Localization of GUS expression in root tissues

Transgenic tobacco and tomato plants containing the CaMV 35S-reporter (GUS) fusion gene were used for determining the localization of GUS gene expression regulated by the CaMV 35S promoter within root tissues (Fig.4). High levels of GUS expression were shown in all root tissues of transgenic tobacco plants at five weeks of age (Fig. 4a). However, much less expression of GUS was found in root tissues of fully mature tobacco plants (one year old) (Fig. 4b). This suggests that the CaMV 35S promoter confers differential gene expression in root tissues at different developmental stages. GUS expression was also examined in root tissues of 35S:GUS transgenic tomato plants infected with *M. hapla* eight weeks after inoculation. Among five rootlets examined, three were shown to express GUS gene in the root tips (Fig. 4c). However, GUS expression was not shown in gall tissues containing feeding nematodes (Fig. 4d).

Table 2. Root galling (galls/plant) in transgenic tomato plants infected with *M. hapla*[a]

Plant type	weeks after inoculation	
	6	8
TI-I-expressing	432±(60)	793±(44)
TI-II-expressing	496±(31)	816±(80)
Control	721±(54)	812±(41)

a Mean±(standard deviation) for samples of three replicates

Table 3. Egg production of *M. hapla* (eggs/plant) on transgenic tomato plants expressing proteinase inhibitors[a]

	weeks after inoculation	
	6	8
TI-I-expressing	57200	480000
TI-II-expressing	86500	625000
Control	120000	600000

a Mean for samples of two replicates.

Table 4. Enzyme-linked immunosorbent assay (ELISA) values (E_{405})[a] of proteinase inhibitors in root tissues of plants inoculated with *M. hapla*[b]

Plant type	uninoculated		inoculated	
	weeks after inoculation		weeks after inoculation	
	6	8	6	8
TI-I-expressing	0.461±(0.027)	0.478±(0.027)	1.279±(0.001)	0.266±(0.015)
TI-II-expressing	0.213±(0.079)	0.297±(0.026)	0.826±(0.102)	0.327±(0.022)
Control (TI-I)	0.109±(0.011)	0.109±(0.121)	0.214±(0.021)	0.196±(0.010)
Control (TI-II)	0.165±(0.041)	0.183±(0.090)	0.301±(0.014)	0.379±(0.028)

a Absorbance read at 405 nm on a ELISA reader for 100 µl extracts. Root extracts were prepared by grinding 2 g root tissues in 2 ml extraction buffer as described in the text.

b TI-I-expressing plants were assayed for proteinase inhibitor I and TI-II expressing plants were assayed for proteinase inhibitor II. Mean±(standard deviation) for samples of two replicates.

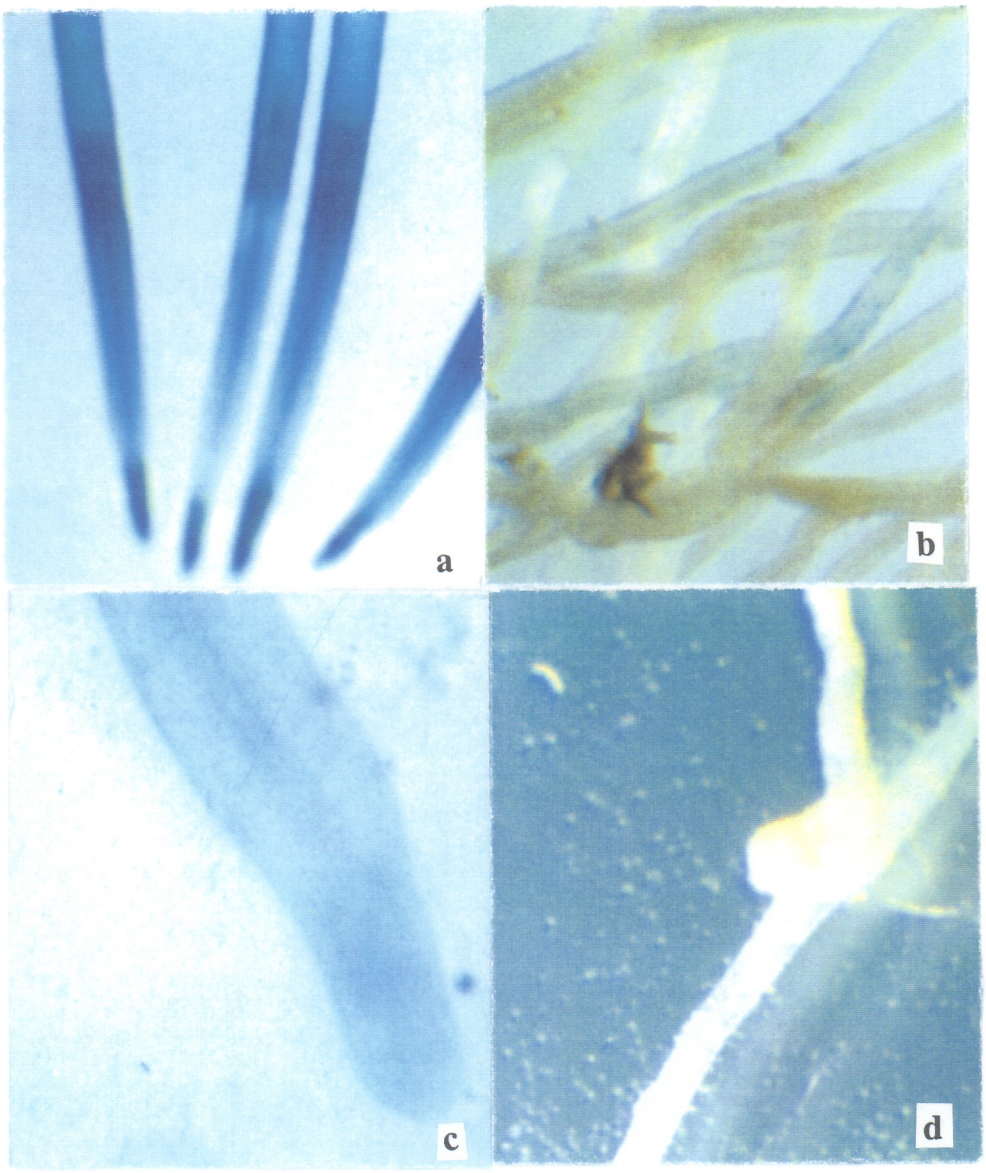

Fig. 4. Histochemical localization (blue) of GUS, driven by CaMV 35S promoter in transgenic tomato and tobacco plants. a. Roots of young tobacco plants (five-week-old). b. Roots of greenhouse grown tobacco plants (one-year-old). c. Nematode-infected tomato root eight weeks after inoculation. Root tip shows GUS expression. d. Nematode infected tomato root eight weeks after inoculation. Gall tissues do not show GUS expression.

This study clearly showed that the CaMV 35S promoter conferred different developmental and tissue-specific expression in root tissues of transgenic plants infected with root-knot nematodes.

DISCUSSION

Previous studies (Hilder *et al.*, 1987; Johnson *et al.*, 1989) have shown that tobacco plants expressing foreign proteinase inhibitor genes, driven by the CaMV 35S promoter, exhibited enhanced resistance against insects, by inhibiting the activity of proteolytic enzymes of insects. Our previous results (Chapter II) showed that tomato root cultures and aseptically grown seedlings expressing high levels of inhibitor I or II proteins regulated by the same CaMV 35S promoter exhibited increased resistance against pathogenic root-knot nematodes. We speculated that overexpression of inhibitor proteins could delay nematode disease development by inhibiting nematode proteinase(s). However, when transgenic plants were tested for nematode resistance under greenhouse conditions, they exhibited enhanced nematode resistance only during the early nematode infection stages, but lost this resistance during the later stages (Chapter III). These earlier observations led us to seek possible reasons for the disappearance of enhanced nematode resistance responses in transgenic plants during later nematode infection stages. One possibility could lie in the expression pattern of proteinase inhibitors in root tissues of transgenic plants infected with nematodes.

In the present study, therefore, we addressed this problem by examining the developmental and tissue-specific expression of proteinase inhibitors in transgenic plants infected with nematodes. Nematode-infected root tissues of inhibitor I-expressing plants contained high levels of inhibitor I protein (1525 μg/g dry tissue). Relatively lower levels of inhibitor II proteins (233 μg/g dry tissue) were found in nematode-infected root tissues of transgenic plants expressing inhibitor II (Table 1). The

differential expression levels of inhibitor I (125 μg/g fresh tissue) or II (45 μg/g fresh tissue) protein were also found in root tissues of each transgenic line without nematode inoculation (Naváez-Vásquez *et al.* 1993). Proteinase inhibitors present in root tissues infected with nematodes were also biologically active in that they were shown to inhibit trypsin or chymotrypsin activity, and were, therefore, functional in root tissues of nematode-infected plants (Fig. 2 & 3). Different chymotrypsin inhibitory activities observed in root extracts from various transgenic lines also was found to reflect the variable expression levels of inhibitor proteins in root tissues (Fig. 2). That is, root extracts from TI-I expressing plants showed stronger inhibitory activity against chymotrypsin than those from TI-II-expressing plants. In addition, different developmental expression of proteinase inhibitors was also found in root tissues of transgenic plants infected with nematodes (Table 4). These expression patterns were positively correlated with the nematode resistance responses. Therefore, we reasoned that the lower expression of inhibitors in root tissues of transgenic plants might cause the loss of nematode resistance response during the late infection stages. Such expression differences by the CaMV 35S promoter in root tissues had not been reported in previous studies. An additional experiment showed that the GUS transgene driven by the CaMV 35S promoter was highly expressed in young root tissues of transgenic tobacco plants, and that this expression pattern was similar to the observation made in a previous study (Benfey *et al.*, 1989). However, much less expression of GUS was found in root tissues of older plants and no specific pattern was observed. In addition, gall tissues containing feeding nematodes did not show GUS expression at eight weeks after inoculation. Thus the absence of transgene expression driven by the CaMV 35S promoter in gall tissues containing feeding nematodes might also account for the loss of nematode resistance during later infection stages. Recently, it was found that nematodes down-regulate some promoters including CaMV 35S promoter (Goddljn, *et al.*, 1993). Using transgenic *Arabidopsis* and tobacco plants carrying CaMV 35S-GUS construct, it

was shown that CaMV 35S was silenced in nematode feeding structures within days after nematode infection

We can conclude from this study that the differential developmental and tissue-specific expression of proteinase inhibitor genes, driven by the CaMV 35S promoter, could result in the disappearance of increased resistance in transgenic plants during late nematode infection stages. Thus the CaMV 35S might not be a suitable promoter for engineering nematode resistant plants.

Recently, Narváez-Vásquez *et al.* (1993) examined the cellular localization of proteinase inhibitor I and II proteins in root tissues of transgenic tomato plants. They found that newly synthesized inhibitor proteins were not only deposited in the vacuoles of cells as in wild-type plants, but were also secreted into the cell walls of outer epidermal and secretory cells of root cap through the secretory pathway. Although such extracellular accumulation of inhibitor proteins may function in defending plants from insects and microorganisms in soil, it may not help provide protection against endoparasitic nematode infection. Therefore, it is also possible that the extracellular accumulation of inhibitor proteins in root tissues of transgenic plants might accounted for the loss of resistance during later stages of nematode infection.

To successfully employ proteinase inhibitor genes for nematode defense, an appropriate promoter must be found which can strongly and continuously express the proteinase inhibitor genes in root tissues, especially in cells used for nematode feeding. Alternatively, nematode-inducible promoter may be used so that tissue-specific expression of proteinase inhibitors can be induced only by nematode infection, which is more preferred in practice. Recently, a novel gene was identified and characterized from root tissues of potato plants infected with potato cyst nematodes (*Globodera rostochiensis* Wollenweber, 1923) (Gurr *et al.*, 1991). This gene was found to be specifically expressed in nematode feeding cells. It was suggested that this gene encodes a protein responsible for establishment and maintenance of nematode feeding

sites. The isolation of this gene offers possibilities for engineering nematode resistant plants. Specifically, the promoter of this gene could be used to regulate nematode resistant gene expression in transgenic plants. In another study, the HMGR gene (coding for enzyme 3-hydroxy 3-methylglutaryl CoA reductase) promoter has been found to be specifically expressed in gall tissues induced by root-knot nematodes (Cramer, *et al.*, 1993). Thus this promoter may also be a potential candidate for engineering nematode resistant plants by employing proteinase inhibitor genes. Additionally, an extensin (coding for a major structural cell wall protein) was found to be induced very early upon nematode infection (Niebel *et al.*, 1993). The discoveries of these nematode-inducible genes offers possibilities for engineering nematode resistant plants. Most recently, Opperman *et al.* (1994) found that expression of TobRB7, a gene expressed only in tobacco roots , was induced during feeding site development. Thus this gene promoter may also be a potential candidate for engineering nematode resistant plants by employing proteinase inhibitor genes.

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Chapter V

THE ROLES OF PROTEINASES AND PROTEINASE INHIBITORS IN TOMATO ROOT-KNOT NEMATODE INTERACTIONS

ABSTRACT

Root-knot nematodes (*Meloidogyne hapla* Chitwood, 1949) at different developmental stages were examined for the presence of proteolytic activity. Both trypsin and chymotrypsin activity were detected in extracts of second-stage juveniles. Only trypsin activity was found in female extracts. Neither trypsin nor chymotrypsin activity was detected in nematode eggs or the exudates of second-stage juveniles. Both tomato proteinase inhibitor I and II were elevated in root tissues infected by nematodes. The results of this study showed that nematode proteinases and plant proteinase inhibitors are present during the nematode infection process, and thus, could be involved in host-nematode interactions.

INTRODUCTION

Proteinase inhibitors are relatively small proteins commonly found in the plant kingdom and other organisms. Because plant proteinase inhibitors have been found to inhibit proteolytic enzymes from insects (Broadway and Duffey, 1986; Gatehouse and Boulter, 1983) and microorganisms (Garcia-Olmedo *et al*, 1987), they are thought to play a role in defending plants against insect predation and microbial infection. Evidence has also accumulated to support the hypothesis that proteinase inhibitors are among a class of defensive chemicals in plants which are induced in response to insect attack and microbial infection (Ryan, 1990).

A defensive role for proteinase inhibitors against insects has recently been confirmed by employing genetically engineered plants expressing high levels of proteinase

inhibitor transgenes. Hilder *et al.* (1987) demonstrated that tobacco plants expressing a foreign cowpea trypsin inhibitor gene were more resistant to feeding by larvae of *Holiothis virescens* than control plants. Johnson *et al.* also (1989) showed that tobacco leaves expressing high levels of tomato or potato proteinase inhibitor II (having both trypsin and chymotrypsin inhibitory activity) were toxic to larvae of *Manduca sexta*. These studies found that growth of larvae fed on transgenic leaves was greatly inhibited as compared to larvae fed on non-transgenic leaves with normal levels of proteinase inhibitors.

The possible involvement of proteinase inhibitors during the plant-nematode parasitic relationship has not been previously reported. Root-knot nematode *Meloidogyne* species are obligate plant pathogens. *Meloidogyne* species establish a specialized and complex feeding relationship with their hosts. Infection of host tissues by *Meloidogyne* second-stage juveniles results in development of a feeding site consisting of several nutritive giant plant cells from which nematodes obtain food for growth and development. Usually, host giant cell contents are partially digested extracorporeally prior to ingestion by the feeding nematode. Presumably, nematodes secrete hydrolytic enzymes into host cells to assist such extracorporeal digestion (Hussey, 1989). Among many hydrolytic enzymes found in nematodes (Esbenshade and Triantaphyllou, 1985), a trypsin-like proteinase has been isolated, purified and characterized from second-stage infectious juveniles of the root-knot nematode *M. incognita* (Dasgupta and Ganguly, 1975). This enzyme showed similarities with vertebrate and invertebrate animal trypsins including those of insects. Proteolytic activity has also been reported in larval exudates of root-knot nematodes (Zinovier, 1957). But this observation was not confirmed subsequently (Dasgupta and Ganguly, 1975). The presence of proteinase inhibitors specific for these nematode proteinases has not been demonstrated in plants.

The present study was initiated to address the uncertainty regarding the role of proteinases and proteinase inhibitors in plant-nematode interactions. We investigated the proteolytic activity in *M. hapla* at different stages of development including nematode eggs, second-stage juveniles and adult females. Additionally the occurrence of tomato proteinase inhibitors was evaluated in galled roots infected with *M. hapla*.

MATERIALS AND METHODS

Plant and nematode materials

Tomato (*Lycopersicon esculentum* cv. Better Boy) seeds were generously supplied by Dr. C.A. Ryan (Washington State University). Although the tomato cultivar Better Boy contains the *Mi* gene conferring resistance to *M. incognita* and several other *Meloidogyne* species, it is susceptible to *M. hapla*. Root-knot nematode *M. hapla* cultures were kindly provided by Dr. J.D. Eisenback (Dept. of Plant Pathology, Physiology, and Weed Sciences, Virginia Polytechnic Institute and State University). Stock cultures were maintained and multiplied on tomato (*L. esculentum* cv. Rutger) plants grown in 15 cm clay pots filled with steam sterilized soil (2 clay soil: 1 sand) in a greenhouse. The nematode populations were subcultured every 8-10 week to ensure a constant supply of nematode eggs as described (Barker, 1985). Plants were watered twice a day and fertilized every two weeks.

Nematode inoculation

Tomato seeds were germinated and grown in a commercial soil mix (Promix, Wetsel) in a greenhouse under natural light conditions at 25°C. About three-week-old seedlings were transferred to steam-sterilized soil (2 clay soil: 1 sand) and inoculated with *M. hapla* eggs (5,000 eggs/plant). Inoculated plants were watered twice a day and fertilized every two weeks. Nematode egg inoculum was prepared as described (Barker,

1985). Infected tomato roots of 6- to 12-week old plants were harvested and cut into 1-2 cm segments. The segments were then shaken vigorously in 500 ml 10% commercial bleach for 4 minutes. Released eggs were collected on a 500-mesh sieve and the residual bleach was removed by repeated washing with tap water.

Preparation of root extracts

Root samples were collected from the middle part of root systems of control and nematode-infected plants. Infected root samples contained 10 galls/g tissue and 15 galls/g tissue six and eight weeks after inoculation, respectively.

Root extracts were prepared following the procedure of Johnson *et al.* (1989) with modifications. All steps were carried out at 0-4°C. Harvested root tissues (2 g) were ground with a mortar and pestle after adding 2 ml of an extraction buffer containing 0.1% ascorbic acid, 0.1% cysteine, 0.5 M sucrose, and 0.1 M Tris (pH7.0). The resulting macerates were centrifuged at 10,000Xg for 10 min and the supernatants were again centrifuged at 10,000xg for 10 min. The supernatants were stored frozen at -20°C until used for protein assays.

Preparation of nematode extracts

Nematode eggs: Egg masses were collected from infected roots and surface sterilized in 0.5% inhibitane diacetate for 4 hours at room temperature followed by three washes with sterile water. The egg masses were then homogenized by a tissue homogenizer in 0.05 M Tris-HCl buffer, pH 8.0 at 4°C and centrifuged at 12,000g for 10 minutes at 4°C. The supernatants containing egg extracts were stored frozen at -20°C until used.

Second-stage juveniles: Egg masses were collected from galled tomato roots infected with *M. hapla* and surface sterilized as described above. Axenized egg masses were allowed to hatch in sterile water and resulting second-stage juveniles were harvested daily. Once harvested, the nematodes were surface-sterilized in 500 ppm of

streptomycin sulfate and then rinsed three times with sterile water. Second-stage juveniles were homogenized in 0.05 M Tris-HCl (pH 8.0) by sonication for 10 min at 4°C. Homogenates were centrifuged in microfuge tube at 13,000 rpm for 10 min at 4°C. The resulting supernatant extracts were stored frozen at -20°C for later use.

Nematode females: *M. hapla* females were collected by the method of Hussey (1970). Galled roots were macerated and softened by pectinase and then the released nematode adult females were collected on a 20-60-mesh sieve series as described by Hussey (1970). Females collected by this technique were essentially debris-free. Females were surface sterilized for 15 minutes in 0.05% inhibitane and washed three times with sterile water. The females then were homogenized with a tissue homogenizer in 0.05 M Tris-HCl buffer (pH 8.0) for 10 min at 4°C. The resulting homogenates were centrifuged at 12,000g for 10 min at 4°C. Supernatant extracts were stored frozen at -20°C until used.

Measurement of protein contents

Soluble protein contents of root and nematode extracts were determined by the method of Bradford (1976) with bovine serum album used as a standard.

Proteinase assay

Trypsin and chymotrypsin activity in nematode extracts were determined spectrophotometrically using the method of Hummel (1959) with the substrates tosyl-L-arginine methyl ester (TAME) and benzoyl-L-tyrosine ethyl ester (BTEE), respectively.

In the trypsin assays, 1 ml of 1.04 mM TAME in 0.05 M Tris-HCl buffer (pH 8.0) buffer was mixed thoroughly with 1 ml Tris-HCl (pH 8.0) buffer and 0.1 ml sample solution. The changes in absorbance were read at 247 nm at 25°C. One trypsin unit was defined as that enzyme activity producing an increase in absorbance of 0.2 in 30 min at 25°C. Specific activity was expressed as trypsin units per mg of protein.

The chymotryptic activity of nematode extracts was measured using 1 mM BTEE as a substrate in 60% methanol. 1 ml of substrate was mixed with 1 ml 0.1 M Tris-HCl (pH 7.8), 0.1 M $CaCl_2$ buffer and 0.1 ml sample. The absorbance was determined at 256 nm. One chymotrypsin unit was defined as that enzyme activity producing an increase in absorbance of 0.2 in 30 min at 25°C. Specific activity was expressed as chymotrypsin units per mg of protein.

Determination of proteinase inhibitors by indirect enzyme-linked immunosorbent assay (ELISA)

Root samples were collected from the middle part of root systems of control and nematode-infected plants. Root samples contained 10 galls/g tissue and 15 galls/g tissue six and eight weeks after inoculation, respectively.

The procedure for indirect ELISA was essentially as outlined by Converse and Martin (1990) with modifications (Appendix B). Root tissues (2 g) were ground in extraction buffer (0.05 M sodium carbonate pH 9.6). Root extracts (100 μl) were added to each well of a microplate. After incubation and washing steps, rabbit anti-potato inhibitor I or anti-potato inhibitor II serum (gifts from Dr. C.A. Ryan, Washington State University) diluted in conjugate buffer (1:10000) was added, incubated and washed. Finally, goat anti-rabbit antibody conjugated to alkaline phosphatase and substrate p-nitrophenyl phosphate were added to the wells. Absorbance was read at 405 nm on an ELISA reader. Purified proteinase inhibitor I and II (gifts from Dr. C.A. Ryan, Washington State University) were used as standards. These assays was performed under the guidance of Dr. Sue Tolin in her laboratory.

RESULTS

Proteinase activity in nematode extracts

Nematode proteinases are involved in the nematode feeding process, nematodes at different developmental stages were examined for the presence of proteolytic activity (Table 1). Neither tryptic nor chymotryptic activities were detected in the egg extracts. Both proteinase activities were, however, detected in second-stage juvenile extracts. Only trypsin-like proteinase activity was found in nematode female extracts.

Proteinase activity in second stage juvenile exudates

Experiments were conducted to determine if second-stage juveniles release proteinases in water (Table 1). About 23,000 surface sterilized nematode second-stage juveniles were incubated aseptically in 5 ml sterile water at 4°C for 48 hours. Then the second-stage juveniles were removed by centrifugation and incubation medium was assayed for proteolytic activity as described (Dasgupta and Ganguly, 1975). In our assay conditions, neither tryptic nor chymotryptic activity was detected in larval incubation medium.

Soluble protein contents in root tissues infected with *M. hapla*

Root extracts from nematode-infected and uninfected plants were compared for the soluble protein contents. Nematode-infected root tissues contained higher soluble protein contents than control uninfected root tissues (Table 2). The plant and nematode components were not separated in nematode infected root tissues. Therefore, soluble proteins measured include the components from both plants and nematodes.

Table 1. Proteinase activity (units[a]/mg protein) in *Meloidogyne hapla* extracts[b]

Extracts	Substrates	
	BTEE (chymotrypsin)	TAME (trypsin)
Eggs	n.d.[c]	n.d.
J_2[d]	7.29±(9.72)	31.9±(4.6)
females	n.d.	42.6±(21.3)
J_2 exudates	n.d.	n.d.

a One unit of activity against BTEE and TAME is arbitrarily defined as the amount of enzyme which produced a $\triangle O.D._{256}$ and $\triangle O.D._{247}$ of $\triangle 0.2/30$ min at 25°C.
b Mean± (standard deviation) for samples of two replicates
c n.d. not detected
d J_2 second-stage juveniles

Table 2. Soluble protein content (μg/ g FW[a]) of root extracts from tomato plants infected with *M. hapla*[b]

Weeks after inoculation			
6		8	
uninoculated	inoculated	uninoculated	inoculated
120±(60)	640±(114)	360±(44)	440±(2)

a FW - fresh weight
b Mean±(standard deviation) for samples of two replicates

Proteinase inhibitors in nematode-infected tomato roots

To determine the occurrence of proteinase inhibitors in response to nematode infection, proteinase inhibitor I and II were examined in *M. hapla*-infected tomato root tissues by using indirect enzyme-linked immunosorbent assay (ELISA) (Table 3). Proteinase inhibitor I was not detected in uninfected tomato roots. Only minimum amounts of proteinase inhibitor II were found in uninoculated tomato roots. Nematode-infected root tissues contained substantially higher proteinase inhibitor I and II levels as compared with uninfected root tissues at both six and eight weeks after inoculation.

DISCUSSION

Previous study has shown that trypsin and chymotrypsin proteinases play essential roles in insect feeding and digestion (Broadway, *et al.*, 1986). However, the potential roles of trypsin and chymotrypsin in nematode feeding process are unclear. In this study, root-knot nematodes at different developmental stages were examined for the presence of tryptic and chymotryptic activities. Both trypsin- and chymotrypsin-like activity were detected in second-stage juvenile extracts. However, only trypsin activity was found in *M. hapla* female extracts. Unlike a previous observation in *M. incognita* (Dasgupta and Ganguly, 1981), no proteolytic activity was detected in *M. hapla* eggs under our assay conditions. Nematode second-stage juveniles and females are major feeding stages of the nematode life cycle (Eisenback and Triantaphyllou, 1991). Therefore, proteolytic enzymes made by second-stage juveniles and females may play roles in nematode feeding by digesting host proteins.

Additionally, we failed to detect proteolytic activity in second-stage juvenile exudates. One possible reason is that nematodes secrete enzymes only in response to a stimulus from host cells (Bird, 1966; Deubert and Rohde, 1971) and therefore failed to release enzymes in the absence of host plants. Additionally, our incubation conditions (4°C) might not be optimal for this nematode metabolic activity.

Table 3. Concentration (ng/FW[a] g) of proteinase inhibitors in tomato root tissues inoculated with *M. hapla*[b]

	Weeks after inuculation			
	6		8	
	uninoculated	inoculated	uninoculated	inoculated
TI-I[c]	n.d.[e]	114.4±(20.42)	n.d.	61.4±(2.9)
TI-II[d]	11.4±(1.6)	125.7±(4.19)	40.0±(2.2)	218.6±(40.7)

a FW - fresh weight
b mean±(standard deviation) for samples of two replicates
c TI-I - proteinase inhibitor I
d TI-II- proteinase inhibitor II
e n.d. - not detected

Our previous study (Chapter II & III) showed that nematode disease development was inhibited in a greater degree in inhibitor I-transformed plants than in inhibitor II-transformed plants during early stages of nematode infection. We speculated that this differential inhibition was mainly due to the different expression levels of inhibitor I (125 μg/g tissue) and II (45 μg/g) proteins in root tissues of transgenic plants. Because inhibitor I inhibits only chymotrypsin activity and inhibitor II inhibits both trypsin and chymotrypsin, we suggested that chymotrypsin inhibitory activity, but not trypsin activity, was mainly responsible for the inhibition of nematode disease development during early stages of nematode infection. However, we could not rule out possible detrimental effects of trypsin inhibitory activity on nematode development because of low expression levels of inhibitor II protein in root tissues of transgenic plants. Results of this present study indeed showed that the second-stage juveniles of *M. hapla* contained high trypsin activity, suggesting that trypsin proteinase may be one of the important enzymes during the juvenile feeding period. We expect that transgenic plants containing higher levels of inhibitor II protein would be expected to improve plant resistance against nematodes. In our previous study, we also showed that transgenic plants containing inhibitor I or II protein exhibited reduced resistance response against nematodes during later stages of nematode infection as compared with early stages of nematode infection. Especially, nematode resistance was totally lost in transgenic plants expressing inhibitor I protein during later stages of nematode infection. We attributed the loss of nematode resistance to the reduced levels of inhibitor proteins in root tissues of transgenic plants during later stages of nematode infection (Chapter IV). Results in the present study show that nematode females contain only trypsin, but not chymotrypsin activity. Therefore, it is possible that inhibitor I protein is not effective against nematode females during later stages of nematode infection, whereas inhibitor II is still effective against nematode females containing only trypsin activity. Thus, the data obtained in this study could partially explain why nematode resistance is lost to

different degrees in transgenic plants expressing inhibitor I verses II proteins during the later stages of nematode infection.

In this study, we also found that nematode-infected root tissues contained higher soluble protein contents. Nematode components might account for the increased concentrations of these soluble proteins in the galled roots. Another possibility is that gall tissues of host plants contain higher soluble proteins to satisfy nematode nutritional needs.

Both proteinase inhibitors I and II were induced in root tissues in response to nematode infection. Low levels of inhibitor I (144.4 ng/g tissue) and II (125 ng/g tissue) were detected in root tissues six weeks after inoculation. Similar levels of inhibitor I (61.4 ng/g tissue) or II (218.6 ng/g tissue) were also found in root tissues eight weeks after inoculation. Therefore, the endogenous levels of inhibitor I or II protein are extremely low as compared with expression levels of inhibitor I (125 μg/g tissue) or II (45 μg/g) transgene regulated by the CaMV 35S promoter. However, we do not know the specific localization of the proteinase inhibitors within the infected root tissues. It is possible that inhibitor proteins are only induced in specific cells or tissues after nematode infection. Recently, Manen *et al.* (1991) reported that a proteinase inhibitor with trypsin inhibitory activity was specifically expressed in senescent cells of winged bean nodules infected with *Rhizobium*, suggesting the tissue-specific expression of proteinase inhibitors in nodules induced by symbiotic *Rhizobium*. It would be interesting to know if proteinase inhibitors are expressed in specific cells (especially giant cells) of root galls induced by root-knot nematodes.

This study showed that proteinases and proteinase inhibitors are involved in plant-nematode interactions. Further studies are necessary to characterize the nematode proteinases and identify the specificity of these proteinase inhibitors toward nematode proteinases.

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Chapter VI

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The results of this dissertation clearly demonstrated that plant proteinase inhibitors play roles in plant-root-knot nematode interactions. Proteinase inhibitors were shown to be induced in tomato root tissues in response to root-knot nematode infection. In addition, high expression of proteinase inhibitor I or II transgene, driven by CaMV 35S promoter, conferred enhanced nematode resistance of root and seedling cultures. Intact transgenic plants grown in the greenhouse also exhibited increased resistance against nematodes during early infection stages. Although this inhibition of disease development in transgenic plants ceased during later stages, this could have been caused by the reduced levels of proteinase inhibitors present in root tissues during the later infection period. A significant finding was that the transgene (GUS) driven by the CaMV 35S promoter was not expressed in gall tissues containing feeding nematodes, suggesting that the CaMV 35S promoter used for these and previous transgene constructs may not be as a suitable promoter for engineering nematode resistant plants.

Future directions

To further elucidate the roles of proteinase inhibitors in plant-nematode interactions, and employ the proteinase inhibitor genes in practical crop improvement schemes, the following studies must be conducted in future research:

1. To identify, purify and characterize the nematode proteinases at different nematode developmental stages in order to understand the roles of proteinases in nematode feeding and digestive processes.
2. To examine the specificity of available plant proteinase inhibitors toward nematode

proteinases so that plant proteinase inhibitor genes which specifically inhibit nematode proteinases can be found and used for engineering nematode resistant plants.

3. To study the tissue-specific expression of proteinase inhibitors in response to nematode infection. Especially to examine if proteinase inhibitors are specifically induced in giant cells which are used as feeding cells by nematodes, and whether this induction vary in compatible versus incompatible interactions.
4. To seek suitable promoters for engineering nematode resistant plants, either constitutive or nematode-inducible promoters. To ensure that such promoters can confer high expression of proteinase inhibitor transgenes in nematode infected root tissues.

Overall, plant proteinase inhibitor genes are potential candidate genes for engineering nematode resistant plants. The results obtained from this study provide some necessary information for such potential applications.

Appendix A. Formula of Gamborg B5 solid medium

components	mg/L
INORGANIC SALTS	
Ammonium Sulfate	134.0
Boric Acid	3.0
Calcium Chloride (Anhydrous)	113.2
Cobalt Chloride $6H_2O$	0.025
Cupric Sulfate $5H_2O$	0.025
Ethylenediaminetetraacetic Acid (Disodium Salt)	37.25
Ferrous Sulfate $7H_2O$	27.80
Magnesium Sulfate (Anhydrous)	122.1
Manganese Sulfate H_2O	10.0
Molybdic Acid (Sodium Salt) $2H_2O$	0.25
Potassium Iodide	0.75
Potassium Nitrate	2500.0
Sodium Phosphate H_2O	150.0
Zinc Sulfate H_2O	2.0
VITAMINES	
Myo-inosital	100.0
Nicotinic Acid	1.0
Pyridoxine HCl	1.0
Thiamine HCl	10.0
Agar	15000.0

Appendix B

DETECTION OF PROTEINASE INHIBITORS BY INDIRECT ELISA

Day 1

1. Root samples (2 g) are ground in 2 ml extraction buffer with a mortar and pestle at 4°C. The macerates are then centrifuged at 10,000 g for 10 min at 4°C. The supernatants were collected.
2. Add 100 μl root extracts to each well of a microplate. Cover plate with a plastic bag.
3. Incubate plate overnight at 4°C.

Day 2

1. Shake out antigen. Rinse initially by filling well, starting from bottom row of a tilted plate, with tap water from a fine stream from the faucet. Shake out immediately and blot plate on paper towel.
2. Rinse plate thoroughly. Fill wells of plate with PBS-Tween from rinse bottle. Let stand 3 minutes. Shake out and blot. Repeat with 2 additional rinses. Leave PBS-Tween in the plate until ready to add the primary antibody.
3. Add 100 μl of antibody (rabbit anti-potato inhibitor I or II serum) diluted in conjugate buffer (1:10,000) to each well. Cover plates with plastic. Incubate at 37°C for 3 hours.
4. Rinse 3 times with PBS-Tween as above, Leave PBS-Tween in wells until ready to add enzyme-conjugate. Wells should not dry out.
5. Add 100 μl goat anti-rabbit antibody conjugated to alkaline phosphatase (goat anti-rabbit, F'Ab2, 1:1000) to each well.
6. Incubate for 3 hr at 37°C.
7. Shake out contents of plate. Rinse 3 times with PBS-Tween.
8. For each plate, prepare 25 ml of fresh substrate buffer. Place 20 ml of it into a small Erlenmeyer flask; add 0.02 g p-nitrophenyl phosphate (from pre-weighed aliquots in the freezer), to give 1 mg/ml, and stir magnetically until completely dissolved. Using a multichannel pipettor, quickly add 200 μl substrate to each well.
9. Incubate at room temperature. After 15 and 30 min, read absorbance at 405 nm on ELISA reader.

Buffer and Reagents for Indirect ELISA

PBS buffer: 5xsolution (pH 7.4). 40 g NaCl, 1.0 g KH_2PO_4, 10.75 g Na_2HPO_4 (anhydrous) and 1.0 g KCl per liter.

Rinsing buffer (pH 7.4): 0.5 ml Tween, 1 liter of 1xPBS buffer.

Extraction buffer: 1.59 g sodium carbonate, 2.93 g sodium bicarbonate, adjusted to pH 9.6, 10 g polyvinylpyrrolidone (PVP) per liter.

Conjugate buffer: 1XPBS+Tween + 2 g bovine serum albumin + 20 g polyvinylpyrrolidone per liter.

Substrate buffer: make fresh. 4.85 g diethanolamine in 40 ml distilled water, adjust pH to 9.8 with 1N HCl. Add additional water to 50 ml.

Substrate: Dissolve 0.02 g para-nitrophenyl phosphate in 20 ml substrate buffer for one plate.

VITA

Xiaorong Zhang was born in Jilin, Jilin Province, People's Republic of China on December 17, 1962. She graduated from Beijing Normal University in 1983 with a Bachelor of Science degree in Biology. She was awarded a Master of Science degree in Plant Physiology from the Institute of Botany, Chinese Academy of Science in 1986. She entered Northern Arizona University in 1987 as a ph.D student and then transferred to Virginia Polytechnic Institute and State University in the fall of 1989. She is a member of American Society of Plant Physiologists.