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NEMATODE-INDUCED PLANT GENE EXPRESSION AND RELATED CONTROL STRATEGIES

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Summary – Sedentary endoparasitic nematodes such as *Meloidogyne* spp. establish an intimate and complex relationship to the host plant. Very little is understood of the molecular and cellular mechanisms involved in establishment and maintenance of a feeding site, or in host resistance to nematode attack. It is known that many plant genes in both susceptible and resistant hosts are either induced or altered in their expression during nematode infection. For example, genes encoding proteins such as chitinases and glucanases may be induced during a defense response. In addition, the expression of other genes, such as those encoding structural proteins or constitutive enzymes may be enhanced to accommodate the increased cellular metabolism occurring during nematode infection. Identification of host genes altered in their expression patterns may lead to identification of DNA sequences directing expression in nematode infection sites. We have isolated one such sequence. The availability of promoters capable of directing expression of a foreign gene in the infection site will allow the development of molecular-based nematode control strategies.

Résumé – *Expression de gènes induite par les nématodes chez les végétaux et stratégies de contrôle en découlant* – Les nématodes endoparasites sédentaires tels les *Meloidogyne* établissent avec leur plante hôte une relation étroite et complexe. Peu de choses sont connues sur les mécanismes impliqués dans l'établissement et le maintien d'un site de prise de nourriture, de même que dans la résistance de l'hôte aux attaques du nématode. Il est connu que, chez les plantes tant sensibles que résistantes, l'expression de nombreux gènes végétaux est induite ou modifiée au cours de l'infestation par le nématode. Par exemple, les gènes codant les protéines telles la chitinase ou la glucanase peuvent être induits lors d'une réaction de défense. De plus, l'expression d'autres gènes, tels ceux codant les protéines de structure ou les enzymes constitutives, peut être renforcée pour s'adapter à l'augmentation du métabolisme cellulaire se produisant pendant l'infestation. L'identification de gènes appartenant à l'hôte et modifiés dans leur mode d'expression peut conduire à l'identification de séquences d'ADN dirigeant l'expression dans les sites d'infestation par les nématodes. Nous avons isolé une telle séquence. La disponibilité de promoteurs capables de diriger l'expression d'un gène étranger dans un site d'infestation permettra la mise au point de stratégies de contrôle ayant une base moléculaire.

Key-words : Transgenic plants, host resistance, molecular biology.

Plant parasitic nematodes account for over $ 75 billion in crop losses worldwide on a yearly basis (Sasser & Freckman, 1987). The sedentary endoparasitic forms, such as *Meloidogyne, Globodera,* and *Heterodera* spp., are responsible for the majority of this damage. These nematode species establish and maintain an intimate and complex relationship with their host plants. Unlike many other plant pathogens, sedentary endoparasitic nematodes induce a fixed feeding site within the host root that must provide nourishment for the duration of the nematode life cycle. Inherent in this interaction is the fact that the feeding site cells must remain healthy and metabolically active in order for the nematode to reproduce. The nature of the interactions and the mechanisms involved in feeding site formation and maintenance have been the subject of intensive investigation for over 50 years. Despite the significance of the problem and the effort applied towards elucidating its causes, very little substantive information exists regarding biochemical and developmental alterations of plant cells by endoparasitic nematode species.

Management of sedentary endoparasitic nematodes has centered on three tools : crop rotation, application of chemical nematicides and host resistance. Crop rotation may be an extremely effective way to manage some plant parasitic nematode species. For example, resistant soybean-susceptible soybean-corn rotations have been used to reduce damage caused by the soybean cyst nematode, *H. glycines* (Schmitt & Noel, 1984). In many other cases, however, the length of the rotation and the host ranges of the nematode preclude effective management without use of supplemental nematicide applications. Chemical nematicides have been used very effectively in the past. During the last 15 years, many nematicides have gradually been either removed from the market or drastically restricted in their use due to

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environmental or toxicity concerns. In many cases there is no alternative control measure to replace the nematicide, forcing growers to either switch crops or suffer greater nematode losses.

Genetic host resistance is the most cost effective and environmentally sound method for management of plant parasitic nematodes (Cook & Evans, 1987). Resistance to root-knot nematodes is available in a limited number of host species (Fassuliotis, 1979). Resistance to the various species of cyst nematodes is also available (Cook & Evans, 1987). In most cases, though, resistance may be restricted to single species, or even races, of a particular nematode genus. Resistant crop cultivars respond to infection by the activation of a number of inducible responses that are thought to be disease resistance mechanisms (Bowles, 1990). One mechanism commonly employed is the hypersensitive response (HR) (Kiraly, 1980). The HR is a rapid localized necrosis of cells at the place of feeding site initiation, limiting the parasitic ability of the nematode. Very little is understood of the molecular and cellular mechanisms involved in plant resistance to nematodes. It is known that many plant genes are up-regulated during a resistance reaction, including genes encoding proteins such as glucanases, chitinases, and other enzymes commonly referred to as pathogenesis related proteins (Bowles, 1990). There have also been reports of induction of proteinase inhibitor genes (Bowles *et al.,* 1991), however no functional role in nematode resistance has been assigned to any of these proteins.

Advances in basic understanding of both plant molecular biology and plant-pest interactions have made it possible to design genetically engineered crop plants resistant to particular pest species. There are now numerous examples of transgenic crop cultivars carrying foreign gene sequences conferring resistance to pathogens. The earliest and most widespread of these approaches is that of integrating viral coat protein genes into the host genome to confer protection from infection. This phenomenon was originally demonstrated in tobacco, where plants genetically engineered to carry and express the Tobacco Mosaic Virus coat protein gene were shown to resist systemic infection by the intact virus (Able *et al.,* 1986). This result has been extended to cover cross protection from other viruses and is currently a standard approach to the development of virus resistant crop cultivars. The mechanisms involved in viral coat protein mediated cross protection remain poorly understood at the present time.

Transgenic crops have also been developed that carry resistance to insect pests. These plants carry and express the Δ-endotoxin (BT) gene from the bacterium, *Bacillus thuringiensis* (Barton *et al.,* 1987). When ingested, the protein toxin binds to the gut membrane and causes paralysis, leading to the demise of the insect. Proper expression and function of the gene in plants has required significant remodeling of the gene sequence to compensate for plant gene expression strategies (Perlak *et al.,* 1991). Although employment of this strategy has resulted in the development of insect resistant crop cultivars, it has some drawbacks. Constitutive expression of any toxin in a host species places strong selective pressures on the pest population, and the host plant suffers negative energy balances when any foreign gene sequence is expressed constitutively. Global gene expression insures that many non-target species also will be exposed to the transgenic protein. There are currently no reported examples of genetically engineered host resistance to plant parasitic nematodes.

Feeding site formation

As previously mentioned, sedentary endoparasitic nematodes establish elaborate feeding sites within the host root. Nematode feeding sites may range from slightly altered cortical cells to vascular cells that are developmentally altered in their fate and function (Jones, 1981). Of the many types of feeding sites formed, those induced by the root-knot (*Meloidogyne* spp.) and cyst (*Globodera* and *Heterodera* spp.) nematodes are the most elaborate and economically important. There are also many species of plant parasitic nematodes that do not form specialized feeding sites. These forms, both ectoparasitic and migratory endoparasitic, will require a somewhat different approach for management using genetically engineered crop plants. This review will focus on management of sedentary endoparasitic nematodes.

Root-knot nematodes have an very broad host range, encompassing over 2000 plant species (Sasser, 1980). Most or all major cultivated crops are attacked by at least one of the greater than 50 species of *Meloidogyne* (Sasser & Carter, 1985). The four most common species, *Meloidogyne arenaria, M. hapla, M. incognita,* and *iM. javanica,* account for greater than 90 % of the reported occurrences (Sasser & Carter, 1985). There is genetic resistance to certain species available in some hosts. For example, resistance in tobacco is conferred by a single gene *(rk),* resulting in a hypersensitive response when infected by races 1 or 3 of *M. incognita* (Sasser, 1980; Slana & Stavely, 1981). The gene is not effective against the other races of *M. incognita,* nor against any other *Meloidogyne* species. Tomato plants carrying the *Mi* gene may be resistant to *M. arenaria, M. incognita,* and *M. javanica,* but not *M. hapla* (Gilbert & McGuire, 1956). The gene appears to be quite stable although there may be breakdown under temperature stress. In other crop species, resistance to root-knot nematode is generally limited to a single species.

The feeding site, or giant cells, of the root-knot nematode represents the most complex interaction known between a plant host and a pathogen. The infective second-stage juvenile penetrates the host root, generally near the root tip, and migrates to the developing vascular cylinder. Glandular secretions are injected via the nematode stylet into several cells surrounding the head, resulting in the induction of giant cell formation (Hussey, 1989). The five to seven giant cells formed within the vascular cylinder become the permanent feeding site for the developing root-knot nematode. The giant cells undergo repeated nuclear divisions without cytokinesis. The multiple nuclei are enlarged and lobate, and may contain fourteen to sixteen times more DNA than do normal root tip nuclei (Wiggers *et al.*, 1990). The giant cells become greatly enlarged, and increased numbers of cellular organelles are observed to accumulate. The cytoplasm becomes very dense and granular, and the central vacuole is greatly reduced in size. The giant cells are metabolically highly active, acting as nutrient sinks to provide food to the developing nematode (Huang, 1985). One characteristic feature of root-knot nematode giant cells is the highly invaginated and thickened cell wall, similar to cell walls observed in transfer cells (Jones, 1981). The nematode is absolutely dependent upon the giant cells since it loses the ability to move soon after infection. The life cycle of the root-knot nematode can be completed in approximately 30 days, and as many as 5-7 generations may occur during a growing season.

There are two major genera of cyst nematodes; *Globodera* (round cysts) and *Heterodera* (lemon shaped cysts). Cyst nematode species tend to have a much narrower host range than do root-knot nematodes. There are numerous species of economic importance, including the potato cyst nematode (*Globodera pallida* and *G. rostochiensis*), the soybean cyst nematode *(Heterodera glycines)*, and the sugar beet cyst nematode *(H. schachtii)*. There is host resistance available to several species of cyst nematodes, but as with the root-knot nematodes it is often specific for races of the nematodes. For example, there are sixteen putative races of *H. glycines*, and there are soybean cultivars that carry varying degrees of resistance to certain races (Riggs & Schmitt, 1988). Since these races may occur as mixtures in a field, it is sometimes difficult to obtain good nematode management with resistance alone. In some cases, there is not good natural host resistance available for the major cyst nematode pests of a given crop.

The feeding site of cyst nematodes is referred to as a syncytium. Although morphologically similar to the giant cells of root-knot nematode, syncytia are probably induced and formed by very different mechanisms (Jones, 1981). Although syncytia are multinucleate, this does not occur by repeated nuclear divisions without cytokinesis. Instead, the multinucleate state of syncytia results from the coalescence of several cells (Jones & Northcote, 1972). The nuclei are enlarged and lobate, but DNA synthesis in the syncytia does not appear to be related to development of the nematode (Endo, 1971). As observed in giant cells, syncytia have thickened and invaginated cell walls, increased numbers of organelles, and dense cytoplasm. In both giant cells and syncytia, the number of plasmadesmata between the feeding site and the surrounding root cells are greatly reduced (Jones, 1981). The enlarged feeding sites are dependent upon constant stimulation from the nematode or they will begin to degrade.

Table 1. *Possibilities for plant gene expression patterns during a susceptible interaction with root-knot (or cyst) nematodes.*

I. Up-regulated gene expression
  A. Enzymes
    1. Catalase
  B. Structural proteins
    1. Extensins
    2. Membrane channels
  C. Unknown functions
    1. pMR 1

II. Down-regulated gene expression
  A. Maturation
  B. Senescence
  D. Unknown functions

III. Timing/pattern of gene expression
  A. Developmental
  B. Regulatory
  C. Unknown functions

IV. Not affected

The elaborate changes that occur to plant cell metabolism and development during nematode infection indicate that plant gene expression patterns must be substantially altered. There are a number of potential alterations in gene activity during nematode infection (Table 1). One may envision that, in all cases, certain constitutive enzymes and structural proteins may be up-regulated in order to support the increased cellular metabolic activity related to nematode feeding. Although few transcripts have been identified to date, this class of genes might be the most straightforward to identify. There have been a few preliminary reports of structural or enzyme genes that are up-regulated during either root-knot or cyst nematode feeding site establishment. For example, it has been reported that catalase synthesis is increased in potato during infection by *G. rostochiensis* (Niebel *et al.*, 1992). Structural protein genes related to the extensin family have been isolated from root-knot nematode infected tomato roots in a screen designed to identify up-regulated genes (van der Eycken *et al.*, 1992). One gene (pMR1) with unknown function has been isolated from potato roots undergoing infection by *G. rostochiensis* (Gurr *et al.*, 1991). For the most part, however, few plant genes up-regulated during nematode infection have thus far been characterized.

There are a number of types of genes that may be altered in their expression patterns in a different manner from up-regulation. The most obvious is the example of genes that may be down-regulated or even turned off entirely. There are no examples from the literature of such genes, but it may be speculated that defense genes may be actively suppressed by the nematode during a compatible interaction. It is also possible that genes involved in maturation and senescence of root tissue may be down-regulated during nematode parasitism. Not all genes that are affected during nematode infection and feeding site establishment are necessarily up- or down-regulated. Certain regulatory genes involved in the interaction may have their expression altered in a temporal sense. These genes may be expressed for a longer duration of time in order to accommodate the increased metabolic needs of the developing feeding site. Alternatively, some genes may be expressed at inappropriate times compared to normal root cell differentiation and development. It is also likely that genes not normally expressed in root cells at all may be utilized during giant cell or syncytia formation. The substantial alteration of root vascular system cells towards the nematode's needs guarantees that many plant genes have their normal expression patterns either quantitatively or qualitatively shifted to meet the demands of the nematode.

Molecular approaches to designing nematode resistant crops

There are several approaches to the design of nematode resistant transgenic crop cultivars. Perhaps the simplest to implement is the expression of a nematode toxin gene under the control of a constitutive promoter. As mentioned, this type of strategy has been used to design plants carrying the BT gene that are resistant to insect pests (Barton *et al.*, 1987). If a suitable toxin gene could be identified, this same approach might be used to make nematicidal plants. Unfortunately, very few protein toxins that act specifically upon plant parasitic nematodes have been indentified. A number of protein toxins known to be active against vertebrates also act upon nematodes (Opperman, unpubl.) but are undesirable due to the broad spectrum of activity they exhibit. Several invertebrate-specific toxins have been identified, but they have not yet been tested against nematodes. For example, the TxP-I toxin from female mites of the species *Pyemotes tritici* is extremely potent against a wide range of insect species, causing immediate muscle paralysis upon exposure to very low dosages (Tomalski & Miller, 1991). Nematode natural enemies may be a source of similar types of toxin molecules. The fungal endoparasite *Nematoctonus* spp. secretes a nematoxic substance during spore germination that causes rapid paralysis and death of the nematode (Stirling, 1992). The predatory nematode *Seinura* injects a toxin into its nematode prey, causing immobilization within seconds (Stirling, 1992). Although the nature of these molecules is unknown, they are potentially useful in the design of nematicidal transgenic plants. If the toxin is a peptide, it would be possible to engineer it into plants to function in a manner similar to BT-transformed plants. Other fungal and bacterial antagonists are known to produce toxic substances to nematodes, but very little information is available on the composition of these compounds.

A different approach to designing nematicidal plants is to transform plants with nematode genes that may disrupt development if expressed in the feeding cells. For example, collagenase is one possible molecule that could be utilized in this way. The nematode cuticle serves as the structural exoskeleton of the organism, and also forms the stylet (feeding apparatus) and the lining of the esophagus and intestine (Bird & Bird, 1991). One of the major structural components of the nematode cuticle is collagen, a polymer unique to animal species. If genes encoding nematode collagenases could be isolated, they may be useful to design transgenic plants. The expression of this gene should have no effects on plants, which do not contain collagen. If transgenic plants expressing collagenase were active, the nematode might have a difficult time overcoming the resistance. Any mechanism to destroy collagenase would result in disruption of normal molting. Likewise, alterations in nematode collagen molecular structure would have similar results. It is very unlikely that the nematode could quickly evolve resistance to this particular molecule.

One of the greatest advantages to these strategies is that, theoretically, any nematode which feeds for a prolonged period upon cells containing the toxin moieties is doomed. There are several disadvantages to this approach, however. The constitutive expression of any " toxin " gene may place upon the pest population very strong selective pressure for resistance. Secondly, peptide toxins useful in this approach typically are narrow in their toxic spectra, as is the case with the BT toxins. Another disadvantage of this approach is the negative energy balance the host plant suffers when there is no pest pressure. Finally, the global constitutive expression of toxin genes guarantees that non-target species, including humans, will be exposed to the protein products.

Genetically engineered host resistance

Regulating expression of foreign genes conferring pest resistance to particular target organs, or pest-induced expression, might overcome some of the disadvantages described above. Of the numerous molecular studies of plant organ-specific expression, most have focused upon tissues derived from the shoot meristem; the organ-specific and light regulation of genes expressed in leaves, genes expressed during flower devel-

the most extensively characterized (Conkling *et al.*, 1990; Yamamoto *et al.*, 1990, 1991). The *TobRB7* gene encodes a protein of 250 amino acids and has conserved structural domains with several membrane spanning proteins (Yamamoto *et al.*, 1990). *In situ* hybridization studies have revealed that expression of *TobRB7* is limited to the root meristematic and immature vascular cylinder regions (Yamamoto *et al.*, 1991). The high level of expression in these tissues coupled with the lack of expression in mature tissue suggests a developmental role for the *TobRB7* protein. In order to identify sequences controlling the root-specific expression patterns, fusions were made between a deletion series of the 5′ flanking region and the bacterial reporter gene, β-glucuronidase (GUS) (Jefferson, 1987). The pattern of the GUS gene expression driven by the full-length flanking sequence was indistinguishable from that obtained by *in situ* hybridization. The deletion series experiments revealed that *cis*-acting elements necessary for root-specific expression of *TobRB7* are located between -636 and -299 nucleotides from the site of transcription initiation (Yamamoto *et al.*, 1991). Significantly, all gene expression was lost when the promoter was deleted to the -299 position (Δ 0.3).

Root-knot nematode infection of transgenic tobacco plants carrying the deletion-reporter constructs demonstrated that *TobRB7* is one of the plant genes that is affected by nematode parasitism. By 4 days after infection, significant levels of GUS activity in and around the developing feeding site were seen (Opperman *et al.*, 1994). As the root continues to grow, the tissue around the root-knot nematode infection site matures. Although *TobRB7* would not normally be expressed in this region, during *Meloidogyne* development GUS is observed to accumulate throughout the nematode life cycle. This spatial and temporal shift in gene expression suggests that nematode infection has caused significant alterations in the control of plant gene expression.

This is further demonstrated by results obtained with the deletion series of *cis*-acting sequences. As indicated, root-specific control regions are located between 636 and 299 bases 5′ of transcription initiation, and no expression is observed with deletions containing only the Δ 0.3 region. Infection with *Meloidogyne* spp., however, significantly alters this pattern. In those plants carrying the Δ 0.3 construction, GUS accumulation was limited to the developing giant cells and appeared to be regulated by the nematode infection (Opperman *et al.*, 1994). Because the *TobRB7* protein is predicted to be structural, it is likely that it is at or near the end of the gene expression hierarchy during giant cell formation. Therefore, it seems likely that expression of the truncated Δ 0.3 construct during nematode infection is not a direct interaction, but rather a result of the myriad cellular events that occur during pathogenesis. Further experiments have revealed that all races and species of *Meloidogyne* thus far examined induce expression of the Δ 0.3 reporter, but the tobacco cyst nematode, *G. tabacum* does not (Opperman *et al.*, 1994). The apparent specificity for induction by root-knot nematode is indicative of a somewhat different mechanism for giant cell *versus* syncytia formation. Most significantly, the nematode responsive element of the *TobRB7* promoter is not the same as the root-specific element, and can be uncoupled (Opperman *et al.*, 1994).

The identification of control elements such as the Nematode Responsive Element of *TobRB7* is a significant step in the move towards transgenic nematode resistant crops. The ability to limit expression of foreign gene sequences to the developing nematode feeding site will allow the greatest flexibility and power in designing control strategies. In addition, it will overcome many of the problems associated with less specific constructs. In the future, plants carrying inducible hypersensitivity cassettes may be designed that are resistant to all species of *Meloidogyne*. Indeed, preliminary results from our laboratories suggest that this strategy will be successful (Opperman & Conkling, unpubl.). As more information is obtained regarding other genera of plant parasitic nematodes, new methods of multiple nematode resistance will be possible. Of particular significance is the concept of using genes necessary in a normal susceptible interaction. The use of these types of sequences coupled to proteins detrimental to the nematode feeding site should make it very difficult for resistance to the control strategy to occur. The ultimate durability of transgenic nematode resistance may depend upon the specificity of the regions controlling gene expression.

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