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Go Where the Science Leads You

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Abstract

This article relates how my lifelong passion for nematology evolved and the philosophy that drove my research program, including maintaining a balance between applied and basic research, and key collaborations I have had with other researchers. Although the driving force behind my basic research was to advance our understanding of the molecular mechanisms of nematode parasitism of plants, the underlying theme was how to apply the new basic knowledge of nematode biology to provide better control of these economically important crop pathogens in grower fields. There are high expectations that new nematode control strategies will result from science-based solutions that can be delivered through biotechnology-derived crops and provide an unprecedented opportunity for limiting nematode damage to multiple crops.

INTRODUCTION

The invitation from the editor to write a prefatory chapter for the *Annual Review of Phytopathology* and share some of the ideas and experiences I have had during my career in nematology was unexpected and required some reflection as to how to approach this rare opportunity and honor. Each year when a new volume of the *Annual Review of Phytopathology* is published, I am drawn immediately to the prefatory to read about the author's personal experiences and philosophy that guided his or her career in plant pathology. Therefore, I have utilized that approach in writing this chapter and have used as a title one of the driving forces influencing the direction of my research career: Go where the science leads you. In today's world, where all aspects of science are advancing at an incredible rate and new opportunities are constantly becoming available to scientists, we must avail ourselves of these new advances and go where the science leads us. My research goals led me to acquire new knowledge and technologies in electron microscopy, immunology, and molecular biology in order to answer the fundamental questions I was asking about nematode parasitism of plants. I cannot think of a more exciting and fulfilling career than working in agriculture for the benefit of society and also having the opportunity to explore the new directions in which science has been evolving and to adapt the new technologies to my research program to answer previously intractable questions.

I grew up in a small town, Mt. Pleasant, Ohio, and spent most summer days and weekends on our family farm, which was started by my great-great grandfather Penrose Hussey in 1847. My parents were both school teachers until my father returned to the farm, where he and his brother raised Herford cattle and tended a commercial apple and peach orchard. Although I frequently saw them spraying the apple trees with chemicals to control apple scab and probably other diseases, I never associated that practice with plant pathology until I was in college. Of the many things I learned from my parents, the willingness to work hard to accomplish your goals and the value of education have guided me throughout my career.

WHY NEMATOLOGY

In high school, I had developed a keen interest in science and was interested in becoming a biology teacher when I matriculated at Miami University in Oxford, Ohio. Majoring in biology education required that I enroll in courses in the Department of Botany, and one of the courses was taught by William "Prof" Wilson, who took an interest in me as a student and convinced me to change my major to botany. Two things occurred during my junior year at Miami to direct me toward plant pathology and, ultimately, nematology. Although there wasn't a course in plant pathology taught in the Department of Botany, Prof Wilson, who had a PhD in plant pathology from the University of Illinois, developed a one-semester graduate student seminar course covering topics in plant pathology. One of the seminars presented was on the root-knot nematode (*Meloidogyne* spp.), which was my first introduction to the plant-parasitic nematode that would later become the primary focus and passion of my research career. I also was beginning to consider what career path to follow after graduation and realized employment opportunities were limited with a BA degree in botany, so I considered attending graduate school. Prof Wilson encouraged me to pursue a graduate degree in plant pathology and suggested that I apply to the Department of Botany at the University of Maryland, which had several excellent plant pathologists on the faculty, including a nematologist, Lorin Krusberg. What appealed to me when considering this path was that because I originally had a strong interest in general biology, which includes both animals and plants, I could still pursue this interest by studying nematodes that parasitized plants. Plus, with my farm background, the idea of working in a profession that would benefit agriculture also appealed to me. Deciding on this path, I accepted a teaching assistantship

in the Department of Botany at Maryland and immediately became a graduate student of Krusberg's, majoring in botany (plant pathology) with a minor in biochemistry. Krusberg was an outstanding nematologist and mentor and had been invited to author the first review on plant-parasitic nematodes in Volume 1 of the *Annual Review of Phytopathology* in 1963. My background in general plant pathology came from excellent courses taught by Bill Klarman, Hugh Sisler, and Ken Corbett. My dissertation research focused on resistance in garden pea to *Ditylenchus dipsaci* and distinguishing two populations of the nematode based on electrophoretic isoenzyme profiles. It was during my MS and PhD programs that I developed a fascination for plant-parasitic nematodes.

As I was completing my PhD degree, Krusberg suggested I consider applying for a postdoctorate position available in the Department of Plant Pathology at North Carolina (NC) State University working with Joe Sasser, a world authority on root-knot nematodes. Krusberg had received his PhD from NC State, and the rich history of nematology and the cadre of outstanding nematologists on the faculty in the Department of Plant Pathology convinced me to apply for the position. This position also gave me the chance to work on the nematode that first piqued my interest in nematology at Miami. Because the root-knot nematode was the principal nematode studied by most of the nematologists at NC State, I also had the opportunity to be exposed to many different areas of research on a nematode that attacks most major crops worldwide. I was offered the postdoctorate position to conduct studies using electrophoretic analysis of proteins and serology as tools for the identification and characterization of *Meloidogyne* species. Biochemical approaches were beginning to provide valuable information on the systematics and phylogeny of nematodes and had the potential to augment the morphological and anatomical characters in the identification and characterization of species and races of plant-parasitic nematodes. Indeed, isoenzyme patterns of single adult female root-knot nematodes have proven to be a valuable and practical assay for expediting accurate species identification of root-knot nematodes and are now routinely used in nematode diagnostic laboratories for this purpose (27, 58). Also critically beneficial for me at NC State was that I took every opportunity to interact with and learn from each of the nematologists and, for those that conducted field research, I visited their field plots with them and at times assisted in setting up their experiments. This experience proved to be very valuable for developing my own research program later because I had had limited exposure to applied research at Maryland. When Sasser's United States Department of Agriculture (USDA) grant ended, Ken Barker hired me on his USDA grant to study the interaction of nematodes and *Bradyrhizobium* species on several legumes. In these studies, the impact of plant-parasitic nematodes on nodulation and nitrogen fixation in soybean was shown to vary according to the nematodes' modes of parasitism (4, 47). Undoubtedly, the mentoring by Sasser and Barker contributed immensely to my having a successful career in nematology. Their counsel and encouragement and their distinguished careers as nematologists provided exemplary guidance for a young nematologist. I also had the privilege to substitute for Sasser as the NC State representative at the annual meetings of the Southern Regional Nematology Technical Committee, where I had the opportunity to meet and work with other leading nematologists in the south.

While at NC State, I was able to develop a new method for collecting inoculum of root-knot nematodes that greatly facilitated and expanded the research that could be conducted with this agriculturally significant pathogen. The new method utilized sodium hypoclorite (NaOCl) to dissolve the gelatinous matrix of egg masses on galled root systems so the eggs could be easily collected by sieving. I originally developed this method to collect eggs for a study of the lipid and fatty acid composition of different stages of the root-knot nematode

(61). Because this method proved to be an efficient way to collect root-knot nematode eggs for inoculum, Barker and I conducted a study to compare the eggs collected by NaOCl with other types of root-knot nematode inocula. At that time, freshly hatched second-stage juveniles or egg masses handpicked off of galled roots were used as inoculum for root-knot nematodes and both types of inoculum had limitations. However, using NaOCl was an easy and rapid way to collect large quantities of eggs that could be standardized and uniformly distributed around root systems for reproducible inoculations. After our study establishing the percentage of NaOCl and the exposure time that could be utilized without affecting egg viability or juvenile infectivity was published (46), the NaOCl method soon became the standard method, with currently more than 1000 citations, for preparing inoculum and for comparing reproduction of root-knot nematodes on different hosts or cultivars (50).

During this period, Barker had been working closely with the Agronomic Division in the NC Department of Agriculture in laying the foundation for developing a Nematode Advisory Service for the farmers in North Carolina. This aspect of Barker's research program focused on developing damage thresholds for the major nematode pathogens on crops grown in North Carolina and also involved training two Agronomic Division technicians in nematode identification. Barker's research had advanced to the point where the NC Department of Agriculture decided to establish a Nematode Advisory Service in the Agronomic Division, and I was hired by Don Eaddy, Director of the Agronomic Division, to implement the state service.

GEORGIA ON MY MIND

After I began working for the NC Department of Agriculture, I received an unexpected call from Wiley Garrett, chairman of the Division of Plant Pathology at the University of Georgia, asking me to apply for a nematology position that would be opening in his department with the pending departure of George Bird to Michigan State University. Because my career goal was to obtain a University research and teaching position, I decided to apply for the position, which involved one-third instruction and two-thirds research. I felt very fortunate to have been offered the position so that I could continue my nematology career at the University of Georgia, which I began on January 1, 1974, as an Assistant Professor. The position was open ended in that there was no crop assignment, but I was expected to develop a broad-based independent nematology research program.

I now had the exciting challenge of developing my own nematology research program. My family farm background, my exposure to basic research at Maryland, and my breadth of experiences at NC State all had a significance influence on how I developed my research program at the University of Georgia. Foremost, I wanted to have a balance between applied and basic research, and I strived to maintain a balance by routinely conducting greenhouse and field studies along with the basic laboratory studies throughout most of my career. Although the driving force behind my basic research was to advance our understanding of the molecular mechanisms of nematode parasitism of plants, the underlying theme was how new basic knowledge of nematode biology could be applied toward the control of these economically important crop pathogens in grower fields. Having an applied component to my research program provided a constant reminder for me that discoveries from my basic research ultimately needed to be translated into nematode control strategies of value to growers. Another very important aspect of both my applied and basic research programs was the collaborations I established with other scientists, and I have been fortunate to be able to collaborate with several distinguished researchers. These collaborations were intra- and interdepartmental at the University of Georgia, involved former postdocs that have faculty positions at other Universities, and were international in scope as well. I considered these collaborations important for broadening my research program and at the same time

providing the intellectual stimulation for maintaining a dynamic research program.

The nematologist I replaced at the University of Georgia was part of a multidisciplinary research team conducting field studies on cotton stunt or up-and-down cotton growth (15). Soil compaction and plant-parasitic nematodes, in particular *Hoplolaimus columbus* and *Meloidogyne incognita*, were considered important factors contributing to this disease syndrome. With the use of larger tractors, multiple moldboard plows, and heavy disk harrows on farms in southeastern Georgia, soil compaction became a widespread problem, affecting early season growth and subsequent plant development and yield. Subsoiling under the row and bedding had been proven to be beneficial for cotton growth and was becoming a common tillage practice utilized by cotton growers. With funding available from the Cotton Commodity Commission, I began my research career at Georgia by working with the cotton stunt research team and subsequently initiated studies on the effects of subsoiling on nematode populations associated with cotton.

A compacted soil typically has a sand texture plow-pan layer between a sandy loam top soil and sandy clay subsoil that cotton roots can not penetrate. The cotton roots are restricted to the shallow top soil layer with the tap roots often growing horizontally along the surface of the impermeable plow-pan layer. This restricted root growth significantly limits plant access to water and nutrients in the lower soil zones and greatly suppresses plant growth and yield. Our studies showed subsoiling alone offsets nematode damage and increased cotton yields during the first year of the tillage treatment, but the greatest yield responses to subsoiling during the second year occurred in plots fumigated with a nematicide (43). However, the nematicide response was not as dramatic in cotton compared with that which occurred with soybean (77), suggesting that cotton was able to compensate for nematode damage to some degree if the tap root was able to penetrate the moist subsoil. Although subsoiling slightly increased nematode population densities at the deeper soil depths, the development of deeper root systems as a result of subsoiling was beneficial not only by increasing moisture and nutrient availability to plants, but also by promoting root penetration into the subsoil, where nematode population densities were low.

My participation in the multidisciplinary cotton team led to a collaboration with Ron Roncadori on the interaction of vesicular-arbuscular mycorrhizal fungi (VAMF) and plant-parasitic nematodes on cotton and other crops. The obligate symbiotic VAMF are ubiquitous in the rhizosphere and often coexist with plant-parasitic nematodes in roots, with each having a characteristic but opposite effect on plant vigor. The universal presence of VAMF in soil of major crop production areas and their beneficial effect on plant health primarily through improved phosphorus nutrition present an interesting concept—one of a symbiont altering biological stress caused by nematode pathogens. We wanted to determine whether VAMF, which improve plant health directly through symbiosis, can affect plant growth indirectly by offsetting damage caused by nematode pathogens. To address this question, we conducted extensive greenhouse and field microplot studies on the interaction of VAMF with nematodes having different modes of parasitism on several crops, including cotton, tomato, soybean, and peach. These studies showed that, although each nematode-VAMF-plant species combination may be unique and generalizations regarding such interaction are difficult, the single most common effect of VAMF on nematode-susceptible plants was promoting tolerance (little suppression of growth and yield of nematode-infected plants) to plant-parasitic nematodes by improving root growth and function (57, 85). In other words, plants heavily colonized by VAMF were able to grow well in spite of the presence of damaging levels of plant-parasitic nematodes. Later studies suggested that improved nutrition through the increased uptake of phosphorus was the principal contributor to the increase in tolerance of vesicular-arbuscular mycorrhizal plants to parasitic nematodes (88, 90, 92). This proof

that VAMF can also influence plant health by affecting the plant's reaction toward nematodes increased our awareness for the need to better understand this most common and universal type of symbiosis in our agroecosystem. Although we completed our studies in 1996, we understood that the potential role of the VAMF in altering plant stress caused by parasitic nematodes remained to be determined under natural field conditions before their ultimate impact on crop yield could be fully assessed. Nevertheless, exploiting VAMF as biocontrol agents against nematodes was still problematic because it was impractical to produce sufficient quantities of these fungi for economical field inoculations. Still, VAMF's ubiquitous nature and their ecological importance may play a vital role in preventing significant yield suppression by plant-parasitic nematodes on certain crops.

COLLABORATING WITH A PLANT BREEDER

When I arrived in Georgia, soybean acreage was rapidly increasing and reached its peak in 1980 at 2.5 million acres, whereas "king" cotton had declined to 170,000 acres that year. The increase in soybean acreage was followed by an increase in nematode diseases, particularly root-knot nematodes. In 1978, Roger Boerma, a distinguished soybean breeder in the University of Georgia's Department of Agronomy, approached me about the possibility of screening large numbers of breeding lines for root-knot nematode resistance in the greenhouse. The availability of resistance to root-knot nematodes in soybean was important for cultivars adapted to the southern United States because of the wide distribution of the pathogen and its potential for suppressing yields. Furthermore, planting of resistant cultivars was the only economical means of managing root-knot nematodes on soybean. Because the NaOCl method for collecting eggs easily could provide large quantities of inoculum, Boerma and I conducted greenhouse studies to determine the optimum inoculum concentration and to evaluate different *M. incognita* collections for screening soybean genotypes for root-knot resistance (48). These studies started a collaboration with Boerma that lasted my entire career and was an extremely satisfying experience for me. As a result of this collaboration, I switched my crop focus from cotton to soybean.

Having the support of your college administration is always important for a research program, and Boerma and I had great support from the administrators of the College of Agriculture. When the soybean cyst nematode (SCN, *Heterodera glycines*) was first found in Georgia in 1979, Bill Flatt, the dean of the College of Agriculture, charged Boerma and me with developing soybean cultivars with SCN resistance to grow in Georgia, and he asked us to prepare a proposal for funding by the College. In the proposal, we outlined three possible levels of SCN research and funding, and in the following week, Dean Flatt approved the full level of funding we requested, which included a permanent technician position on experiment station funds.

Because nematologists frequently are asked by breeders to screen breeding lines for nematode resistance, I have been asked by other nematologists what made my collaboration with Boerma so successful. If the number of breeding lines to be screened is large, and in our case more than 22,000 lines were screened annually for resistance to five nematode species and races, this obviously would be very time consuming and would require a major portion of a technician's time at the expense of conducting research in our program. Although we were fortunate that Dean Flatt provided support for our collaboration, the key that made this collaboration productive and lasting was that we developed a truly collaborative research program that extended beyond simply screening breeding lines for nematode resistance in the greenhouse. Boerma and I were both interested in conducting in-depth research dealing with many aspects of soybean-nematode interactions. For that reason, the major thrust of our collaboration was devoted to in-depth research projects.

One of the initial studies we conducted was to evaluate soybean genotypes for tolerance to SCN under different field environments. Resistance and crop rotation were the principal control strategies for limiting yield losses to SCN, but the effective use of resistant cultivars was complicated by the variability in SCN populations. Our goal was to identify tolerant susceptible cultivars that would have little yield suppression while limiting the selection pressure on SCN for the development of resistance-breaking races. When used in rotation with resistant cultivars, tolerant susceptible cultivars have the potential for stabilizing yields on SCN-infested land. We identified only moderate levels of tolerance in the soybean cultivars tested, and none yielded as well as resistant cultivars, indicating that tolerance could not be used as a replacement for resistance in an SCN management scheme (14). The mechanism by which soybean plants were able to tolerate nematode damage was not well understood, although a rhizotron study indicated compensatory regeneration of roots was an important characteristic of SCN tolerance (70). Other in-depth collaborative research projects involved greenhouse and field microplot studies on the interactions between different nematode species that could be found in soybean fields [*M. incognita* and *H. glycines* (72, 73), and *M. incognita* and *Pratylenchus brachyurus* (35)], resistance responses of soybean to root-knot nematodes (36, 37, 65, 71, 79, 80), and inheritance of resistance to root-knot nematodes (66–69). In the early 1990s, Boerma recognized the importance of molecular markers for enhancing the rate of genetic gain for the advancement of his soybean breeding program, and he went where the science led him. Boerma adapted the new molecular technologies for marker-assisted selection to his breeding program and became a leader in using molecular markers for the selection of nematode resistance and other soybean traits. This new research thrust led to the utilization of molecular markers for screening for root-knot nematode resistance (34, 63, 91) and reniform nematode resistance (33) in soybean. Overall, our collaboration involved twelve graduate students between the two programs and resulted in the release of twenty-three soybean cultivars and germplasms with resistance to multiple nematode species and races.

AN INTEREST IN MICROSCOPY

Ever since I enrolled in the excellent plant anatomy and histology courses taught by Charles Heimsch at Miami, I have been interested in using microscopy to study the cellular responses of plants to nematode parasitism. This interest was applied to the study of the histopathology of roots of susceptible and resistant soybean infected with root-knot nematodes (81) and the host-parasite interactions of numerous other nematode species for use in teaching nematology. My interest in using light microscopy to study nematode-host interactions led to a desire to investigate nematode biology in more detail at the ultrastructural level. Because I had no experience with transmission electron microscopy, I sought the advice of Charles Mims, an outstanding electron microscopist in our department, and we collaborated on several interesting ultrastructural studies involving nematodes.

One particularly fascinating study involved the ectoparasitic ring nematode (*Criconemella xenoplax*), a common and economically important pathogen on peanut, peach, and turf. When visiting Clemson University, I met with Wickes Westcott, who was studying the feeding behavior of the ring nematode in monoxenic culture on root explants of several plant species (96). While observing the root explant cultures under a stereomicroscope, I became fascinated watching ring nematodes feeding at the root surface with their stylet inserted into the root cortex and their metacorpal pump chamber pulsating as they were ingesting nutrients from a parasitized cell. I returned to Georgia with monoxenic cultures of ring nematodes with a goal of conducting an ultrastructural study of what appeared to be a remarkable nematode-host relationship. At the time, the feeding of ring nematodes, as with other ectoparasitic

nematodes, was considered to be very destructive, resulting in cell death from the removal of the cytoplasm. However, our ensuing studies showed that the ring nematode induced adaptive cellular changes rather than the expected destructive cellular changes with a single root cortical cell being modified into a food cell for sustained feeding by the nematode (54). Remarkably, the plasma membrane of the parasitized cell was not penetrated when the nematode stylet was inserted through the cell wall but became invaginated around the stylet and remained intact except for a minute pore created at the stylet orifice. The nematode was able to feed continuously, sometimes for days, from the cytoplasm of the food cells without killing the cell because the plasma membrane formed a tight seal with the wall of the stylet orifice, resembling a patch-clamp electrode. Plasmodesmata in some food cells were dramatically modified to facilitate an increased flow of solutes from neighboring cells into the food cell. Penetration of the cell wall by the stylet induced the deposition of callose around the stylet, which was a common wound response to feeding by sedentary nematodes (55). I spent many days on a Zeiss EM 10A transmission electron microscope looking at sequential serial sections of ring nematodes feeding on root cortical cells and was very fortunate to observe median longitudinal sections through the stylet of two feeding nematodes, one during the secretion phase and another during the ingestion phase of the feeding cycle (54). I can still remember how exciting it was when I saw that first median section through the stylet of a ring nematode in the process of feeding from the cytoplasm of a modified root cortical cell! The feeding behavior and the highly specialized cellular adaptations induced by ring nematodes indicated that ring nematodes have a highly evolved feeding relationship with their host plants. Their nondestructive feeding behavior supports observations that high population densities of ring nematodes are required for root damage and subsequent suppressed plant growth and yield to occur.

In another interesting ultrastructural study, we focused on the development of feeding tubes formed within the cytoplasm of parasitized root cells from stylet secretions injected by the root-knot nematode. These remarkable structures are used by sedentary endoparasitic nematodes for efficiently withdrawing soluble nutrients from specialized feeding cells (giant-cells) by functioning as a filter for selective solute uptake (13, 53, 84, 89). The unique tubular structures formed by *M. incognita* females were long tubes with an electron dense wall that had a very regular crystalline structural pattern (53). A portion of the endomembrane system of the feeding cell (giant-cell) formed a compact membrane system around a newly formed tube, which suggests it was involved in synthesizing and/or transporting nutrients to the feeding tube for ingestion by the nematode. The feeding cycle of a root-knot nematode necessitates that the stylet be retracted from the giant-cell and a new feeding tube be formed when the nematode reinserts its stylet into one of the giant-cells radiating around its head to initiate a new feeding cycle (53). I used many different fixatives, host species, and tissue preparations in an attempt to rapidly kill adult females in the process of feeding from a giant-cell and was greatly disappointed when I was never able to observe a root-knot nematode with its stylet inserted through the wall of a giant-cell.

SECRETS IN SECRETIONS

From the time I first worked with the sedentary endoparasitic root-knot nematode at NC State, I have been fascinated by the biology of these highly specialized nematodes that are capable of parasitizing thousands of plant species, making them one of nature's most successful parasites. It is remarkable that these biotrophic parasites can have such a broad host range when they require a very intimate and complex feeding relationship with their host plants in order to complete their life cycle. For a successful host-parasite relationship, root-knot nematodes must transform root vascular cells into the very specialized and metabolically active giant-cells by modulating complex changes in root cell morphology, function, and gene expression

(16, 49). The cells fed on by infective second-stage juveniles are induced to undergo repeated synchronized nuclear division uncoupled from cytokinesis to become multinucleate while enlarging dramatically with the cytoplasm increasing in volume and density. The walls of the giant-cells also are dramatically remodeled to form extensive branching ingrowths bound by the plasma membrane to facilitate an influx of solutes into the feeding cell, a feature typical of transfer cells (59, 74). Root-knot nematodes are able to orchestrate the redifferentiation of the root cells into giant-cells by dramatically inducing transcriptional changes in the parasitized cells by upregulating some genes while silencing other genes (31). These unique giant-cells represent one of the most elaborate responses to be elicited in plants by any organism.

What makes a nematode a plant parasite? What are the molecular signals secreted through the nematode's stylet that trigger giant-cell formation? These were the questions that drove my basic research on root-knot nematodes. Understanding the genetic adaptations underlying the evolution of parasitism by nematodes is not only fascinating biology, it also has the potential to reveal novel and specific targets for disrupting the parasitic process to achieve nematode control. As plant-parasitic nematodes evolved from their free-living ancestors to be able to exploit plants as food, in addition to developing a protrusible feeding stylet, their esophageal gland cells, a single dorsal and two subventral, greatly enlarged as the cells adapted for enhanced secretory activity directly involved in plant parasitism (5, 45). Detailed ultrastructural studies in my lab and elsewhere revealed that each esophageal gland in root-knot nematodes was a single cell specialized for exporting secretory (effector) proteins into the lumen of the esophagus so they can be injected through the nematode's feeding stylet into host tissue (26, 52). The secretory effector proteins in root-knot nematodes evolved to enable the parasite to regulate, directly or indirectly, specific host genes to reprogram normal root cells for the formation of the specialized giant-cells. The genes expressed in the esophageal gland cells encoding secretory effector proteins with a direct role in parasitism have been termed parasitism genes (21, 51).

The earliest evidence that root-knot nematodes secreted saliva through a hollow, protrusible feeding stylet directly into host cells was provided in 1937 by Linford (64), who suggested that these secretions were responsible for the formation of the giant-cells. He proposed that the saliva was produced in the three gland cells in the esophagus of the root-knot nematode. From 1967 to 1969, Alan Bird published a series of articles (8–12) on the morphology and function of the esophageal gland cells in root-knot nematodes. Ultrastructural and morphological changes in the gland cells were correlated with developmental phases in the nematode's life cycle, indicating various roles for the gland secretions during different stages of parasitism. His studies also showed secretory granules contained only proteins, and the protein absorption pattern in the subventral glands differed between the preparasitic and parasitic second-stage juveniles, suggesting that the secretory components changed with the onset of parasitism. While at NC State, I became intrigued by Bird's studies, and as a result, the nature of the secretions produced in the root-knot nematode esophageal gland cells became the principal focus of my fundamental research program at Georgia.

When I started my research project on root-knot nematode stylet secretions, very little was known about the nature of the secretions even though they had been a topic of discussion for decades. Investigations into the nature of the secretions had been hampered by the difficulties in working with root-knot nematodes because of their obligate endoparasitic lifestyle, small size, complicated genetics, long life cycles, and the minute amount of stylet secretions they produced in vitro. I was fortunate that advances in immunology and molecular biology were beginning to provide new tools that would allow us to investigate the secretions in more detail than was previously achievable.

Our first project on root-knot nematode secretions focused on the development and

utilization of monoclonal antibodies (MAbs) to characterize the secretions produced in the esophageal gland cells. This research was initially supported by Agrigenetics Research Corporation, which was exploring genetic engineering technologies for crop improvement. We believed that MAbs, because of their epitope specificity, would be valuable tools for identifying and characterizing nematode secretions. The first MAbs specific for antigens in the esophageal glands of a plant-parasitic nematode were generated by Atkinson et al. (2), who showed that different antigens were synthesized in the subventral and dorsal gland cells of SCN. We were able to raise MAbs that were specific for antigens present in secretory granules within both types of esophageal gland cells in three species of root-knot nematodes and that did not bind to antigens in SCN (44). The initial results with our MAb research led to my first proposal on nematode secretions funded by the USDA National Research Initiative (NRI) competitive grants program in 1988, and I was fortunate to have had continuous NRI funding for this research through 2009. After we had generated a panel of MAbs that bound to the secretory granules in the esophageal glands cells, we anticipated that the MAbs would be valuable tools for identifying and purifying specific secretions involved in nematode parasitism of plants and lead to the subsequent cloning of the encoding parasitism genes. One particular interesting MAb ($6D_4$) we generated bound to a secretory protein localized to a specific domain of the secretory granules produced in the subventral gland cells and also in the dorsal gland cell and stylet secretions of adult female *M. incognita* (56). Although immunoaffinity purification and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated the secretory protein was a high-molecular-weight glycoprotein, we were never able to further characterize the secretion. We also used several novel immunization strategies to generate additional MAbs, which enabled us to conclusively demonstrate that secretions synthesized in the subventral gland cells could be secreted through the nematode's stylet and thereby have a role in parasitism, a topic being debated at that time (18, 19, 45).

Our antibody research on nematode secretions led to an interaction in 1988 with the nematology group, led by Fred Gommers, at Wageningen Agricultural University in the Netherlands, which was also beginning to use MAbs to study nematode secretions. My lab hosted several student interns from Wageningen over the years, and I was invited to present seminars and serve on the examining committees of two doctoral students at Wageningen. This interaction led to an important international collaboration on nematode parasitism genes that began in 1993 with the first meeting to discuss research progress being held in Georgia in 1994 and thereafter, followed by annual meetings rotated among the participating labs that continue today. The many interesting discussions we had at the early meetings were invaluable to the advances made in the discovery of nematode parasitism genes. The initial collaboration on parasitism genes also involved the labs of Jaap Bakker and Arjen Schots at Wageningen Agricultural University, Rick Davis at NC State University, Thomas Baum at Iowa State University, and the French nematology group led by Pierre Abad at the French National Institute for Agricultural Research (INRA) in Antibes (21). The Dutch group, working with the potato cyst nematode (PCN, *Globodera rostochiensis*), generated a MAb (MGR 48) that bound to an antigen in the secretory granules within the subventral glands of PCN and also the subventral glands of SCN (22). In collaboration with Davis, the MGR 48 antibody was used to affinity purify the antigens from the subventral glands in PCN and SCN for peptide sequencing, which led to the cloning of beta-1,4-endoglucanase (cellulases) genes from both nematode species (87). The endoglucanase genes represented the first parasitism genes encoding esophageal gland cell secretory proteins to be cloned from a plant-parasitic nematode and the first endoglucanase genes to be cloned from an animal. The observation that cellulases were secreted through the stylets of

infective second-stage juveniles to facilitate partial cell wall degradation during the nematodes' intracellular migration through the root cortex unequivocally established a prominent role in plant parasitism for secretions from the subventral glands (94). An interesting finding was that the cyst nematode cellulases had the greatest similarity to bacterial cellulases and weak similarity to other eukaryotic genes, which suggested these parasitism genes may have been acquired from prokaryotic microbes via horizontal gene transfer to an ancestor bacterivorous nematode of plant-parasitic nematodes (87, 97). The acquisition of cellulase genes from bacteria could possibly represent the first step in the evolution of plant parasitism given that plant cell walls had to be breached before nematodes could feed on the cytoplasm. This led to speculation that horizontal gene transfer from prokaryotes may have been a common mechanism by which plant-parasitic nematodes acquired their parasitism genes, but only a few of the different classes of parasitism genes subsequently discovered have similarity to prokaryote genes, with the majority coding for cell wall–modifying proteins (32). With all the effort put into using MAbs to characterize nematode secretions, the endoglucanases ended up being the only secretion successfully identified with antibodies.

In 1996, the United Soybean Board (USB) funded a national biotechnology project for the purpose of organizing a broad-based focus group with interest in the molecular aspects of the SCN-soybean interaction in order to establish long-term coordination and exchange of ideas among the SCN research community. This action was prompted by the movement of SCN into the northern soybean growing regions, making SCN the major limiting factor of soybean production in the United States. The project goal was to develop new forms of genetic resistance to SCN that could be used in future soybean cultivars. Funding by USB allowed us to expand our parasitism gene research to include SCN and enabled Davis at NC State, Baum at Iowa State, and me to begin a long-standing collaboration on characterizing SCN parasitism genes. The collaboration with Davis and Baum, who previously had been postdocs in my lab working on root-knot nematode parasitism genes, has been a very special and productive association. Having the opportunity to collaborate with these two outstandng scientists was one of the highlights of my career. Our interuniversity collaboration has been very successful because of the cooperation and communication among our labs, which is facilitated by holding annual meetings rotated among our universities. This allows the members of our labs to meet to discuss research progress, share methods and materials, and develop future research plans.

During this period, major advancements were being made in molecular biology that enabled the characterization of expressed genes from submicrogram quantities of starting material. With the limited success we had using MAb to identify nematode secretions, we decided we must go where the science was leading us and begin using the current molecular tools to directly clone the parasitism genes expressed in the esophageal gland cells. RNA fingerprinting, differential screening of cDNA libraries, and cDNA-AFLP analysis of different nematode life stages enabled a number of labs to clone parasitism genes expressed within the esophageal gland cells of root-knot and cyst nematodes (23, 24, 62, 83). Even whole nematode expressed sequence tag (EST) analysis from preparasitic second-stage juvenile cDNA libraries was being exploited to identify gland-expressed genes. This approach had limited potential because it primarily identified only parasitism genes whose translation products obviously were related to parasitism, e.g., cell wall–modifying enzymes (17, 82). Although these new molecular approaches successfully identified several parasitism genes, it became evident that the most efficient and fruitful strategy for large-scale cloning of parasitism genes would require direct analysis of the transcriptome of the esophageal gland cells.

An important development for my research program occurred in 1997 when the Georgia Research Alliance funded a new state-of-the-art

research facility on campus to house the Center for Applied Genetic Technologies, and I was fortunate to be able to purchase a complete microinjection station as part of the equipment for the facility. I was inspired to purchase the microinjection station by studies showing that the model nematode, *Caenorhabditis elegans*, could be genetically modified by microinjection of transgenes, even though at the time I wasn't certain how I would use the microinjection station in my research on root-knot nematodes. That quickly changed when we read an article describing a method that coupled a microaspiration technique for collecting the contents of single living plant cells with a protocol for isolating the mRNA populations for creating cell-specific cDNA libraries by reverse transcription polymerase chain reaction (RT-PCR) (60). That article spurred us to determine the feasibility of microaspirating the cytoplasm from the nematode esophageal gland cells so that we could directly analyze gene expression in the specific tissues known to be the source of the secretions mediating plant parasitism (86). We eventually developed a protocol for cloning parasitism genes by pooling mRNA isolated from cytoplasm microaspirated directly from the transcriptionally active gland cells of a range of parasitic stages dissected from host roots and using the gland cell mRNA in RT-PCR to create gland cell–specific cDNA libraries. The Karrer et al. (60) method and the microinjection station enabled the breakthrough that was needed to obtain a comprehensive profile of root-knot and cyst nematode parasitism genes.

The first parasitism genes identified from cytoplasm microaspirated from esophageal gland cells were cloned from SCN using a yeast signal sequence trap method (93) and suppression subtractive hybridization (29). The key criteria we utilized to identify parasitism genes among the numerous gland cell–specific cDNA clones were the presence of a secretion signal, the absence of transmembrane-spanning helices in the predicted protein, and the confirmation of gene expression in the esophageal gland cells by in situ mRNA hybridization. Screening a gland cell cDNA library using a signal peptide-selection vector expressed in yeast yielded three cDNA clones that encoded putative extracellular proteins that specifically hybridized to transcripts within the dorsal gland cell of parasitic stages of SCN (93). In the suppression subtractive hybridization study, three genes of the predicted extracellular cDNA clones identified were specifically expressed in the dorsal gland cell and one in the subventral gland cells of SCN (29).

The high quality and complexity of the cDNA libraries generated from the cytoplasm microaspirated from the esophageal gland cells of SCN inspired us to utilize a more comprehensive approach in order to obtain a more complete profile of the parasitism genes. Our strategy of macroarraying a gland cell cDNA library onto nylon membranes for indexing and subtracting with an intestinal cDNA library allowed us to efficiently identify a battery of unique parasitism gene candidates (30). By combining EST analysis of the gland cell cDNA library with high-throughput in situ expression localization of clones encoding secretory proteins, 51 new SCN gland-expressed parasitism genes were identified, with 80 percent of the clones being specifically expressed within the dorsal gland cell. This was the first comprehensive profile of parasitism genes obtained for a parasitic nematode. Remarkably, more than 70 percent of the parasitism genes discovered were completely novel sequences without homology to any sequences in existing databases. A similar EST analysis of a gland cell–specific cDNA library from *M. incognita* identified 37 unique parasitism genes with 65 percent being expressed within the dorsal gland cell (39), and an additional 11 parasitism genes were identified by solid-phase subtraction hybridization of a second gland-specific cDNA library from *M. incognita* with the majority of these parasitism genes being expressed in the subventral glands (40). As with the SCN parasitism genes, more than 70 percent of the root-knot nematode

parasitism genes were novel sequences. The differences in the number and function of the parasitism genes expressed within the dorsal and subventral gland cells of these sedentary nematodes was reflective of the role the two types of glands have in the parasitic cycle (45). Another interesting discovery was that, even though SCN and root-knot nematodes have similar parasitic lifestyles, there were few similarities among their parasitism genes, indicating these sedentary endoparasitic nematodes employed different molecular strategies to induce and maintain their specialized feeding cells.

The large battery of parasitism genes that were cloned from cyst and root-knot nematodes was revealing what makes a nematode a plant parasite. Undoubtedly, one of the most surprising and interesting findings from the discovery of the parasitism genes was the large number of the genes that have no homology with sequences in the existing databases. These pioneer parasitism genes appeared to represent genes unique for nematode parasitism of plants, a hypothesis supported by the complex and unique interactions that the sedentary endoparasites have with their host plants. Although determining the roles of the pioneer genes in parasitism awaits functional analysis, several SCN pioneer parasitism genes encode effector proteins with functional nuclear localization signals that target the proteins to the nuclei of the parasitized plant cells where they may directly alter gene expression (25, 30). Detailed studies of one pioneer root-knot nematode parasitism gene (*16D10*) revealed it encodes a mature 13-amino acid secretory peptide that interacts with SCARECROW-LIKE transcription factors in parasitized root cells and dramatically increases root growth, suggesting this secreted effector peptide may represent an early signaling event in giant-cell formation (41). This was the first evidence that a nematode-secreted parasitism gene product may regulate host gene activity by directly binding to an intracellular plant transcription factor. The parasitism genes that did encode effector proteins with predicted functions provided further insights into the various molecular strategies these parasites use to manipulate plant cell biology. The infective second-stage juveniles of root-knot and cyst nematodes employ a suite of various cell wall–modifying enzymes to facilitate their penetration and migration in roots (reviewed in Reference 32). Two peptides secreted by SCN are a remarkable example of functional mimics of plant CLAVATA3/ERS-like peptides that appear to interact in signaling pathways that affect plant cell differentiation and thus could play a role in parasitic modification of root cells into the feeding site (syncytium) (30, 75, 93, 95). This was the first report of a CLAVATA3/ERS-like peptide discovered outside of the plant kingdom! Other parasitism genes of SCN encode for a novel ubiquitin extension protein and additional members of the ubiquitination pathway that may have regulatory roles in feeding cell development (30). An SCN-secreted cellulose-binding protein binds to a plant pectin methylesterase, which could condition host cell walls for parasitism (38). Also, an SCN parasitism gene encodes a protein, similar to annexins in host plants, that binds to an oxidoreductase that could enhance plant susceptibility to the nematode (78). These are just a few examples of the remarkable molecular tools revealed through the discovery of parasitism genes that these nematodes evolved to establish a successful parasitic relationship with their host plants (7, 20, 32). Although the discovery of these parasitism genes represents a major step toward understanding the biology of plant parasitism by sedentary endoparasitic nematodes, they may not represent the complete repertoire of parasitism genes used by these pathogens. The recent completion of the genome sequences of two root-knot nematode species has revealed that these parasites possess a larger suite of cell wall–modifying enzymes than previously described (1, 76).

As I stated earlier, our long-term goal of advancing our understanding of the molecular genetics of nematode-plant interactions was to provide new basic knowledge that can

be translated into new strategies for better management of these crop pathogens in the future. Nematode management continues to be a significant challenge for growers, with the most effective nematicides having been withdrawn from the market and natural resistance being available in only a few crop species. Given that nematode control options have not advanced in decades, I believe opportunities for effective new control strategies must result from science-based solutions that can be delivered through biotechnology-derived crops (3). We first explored the possibility of developing novel root-knot resistance by engineering tobacco plants with the ability to produce an antibody (plantibody) that specifically binds to a *M. incognita* stylet secretion (6). Although a functional antibody that binds to the antigen in the esophageal glands and stylet secretions was produced in the transgenic tobacco plants, the expression of the antibody had no effect on root-knot nematode parasitism of the transgenic plants. At the time, the secretion recognized by the antibody had not been fully characterized nor was its role in parasitism known, which was reflected in the antibody having no effect on nematode infection. The discovery of gene silencing by RNA interference (RNAi) in *C. elegans* (28), however, did provide the technology for us to explore the prospect of genetically engineering plants to disrupt the parasitic cycle by silencing essential parasitism genes in root-knot nematodes. We engineered plants to express double-stranded RNA complementary to parasitism gene *16D10* in root-knot nematodes, which resulted in the *16D10* parasitism gene being silenced in nematodes infecting the transgenic plants and dramatically disrupting their parasitic cycle (42). Furthermore, given that the *16D10* parasitism gene was conserved in *Meloidogyne* species (41), the transgenic plants were highly resistant to the four most common and damaging root-knot nematode species, providing an effective range of resistance not conditioned by any natural root-knot resistance gene. It is truly exciting to contemplate that this new RNAi technology has the potential to revolutionize root-knot nematode management by developing novel durable broad-spectrum resistance to *Meloidogyne* species and also provide a strategy for developing root-knot-resistant crops for which natural resistance genes have not been found.

CLOSING REMARKS

It was at NC State that I developed a passion for nematology, and root-knot nematodes in particular, that I never lost, even to this day. I feel very fortunate that my career in nematology spanned the period when groundbreaking advances were being made in molecular biology that could be applied to my research program to answer previously intractable questions about nematode parasitism of plants. In 1989, I wrote a review on nematode disease-inducing secretions for the *Annual Review of Phytopathology* (45), and it is exciting to see the tremendous advances that have been made in identifying the parasitism genes encoding the secretions and what they are revealing about nematode parasitism of plants (20). The discovery that sedentary endoparasitic nematodes produce in their esophageal gland cells an arsenal of secretory proteins with functions in parasitism has been one of the greatest conceptual advances in nematology over the past decade. The parasitism genes discovered revealed that plant-parasitic nematodes have evolved the remarkable ability to manipulate the plant cell cycle, mimic plant signaling peptides, modify cell walls, alter cell metabolism, modulate protein degradation, and evade plant defenses. The molecular tools that make a nematode a plant parasite are being identified, and we are on the cusp of knowing how the sedentary endoparasites establish the unique feeding cells in susceptible hosts required for their growth and development. The landmark advances being made in understanding the molecular basis of nematode parasitism of plants provide optimism that this new knowledge will enable novel approaches to be applied to the pressing problems we have in managing plant-parasitic nematodes in grower fields.

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The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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