John Jones
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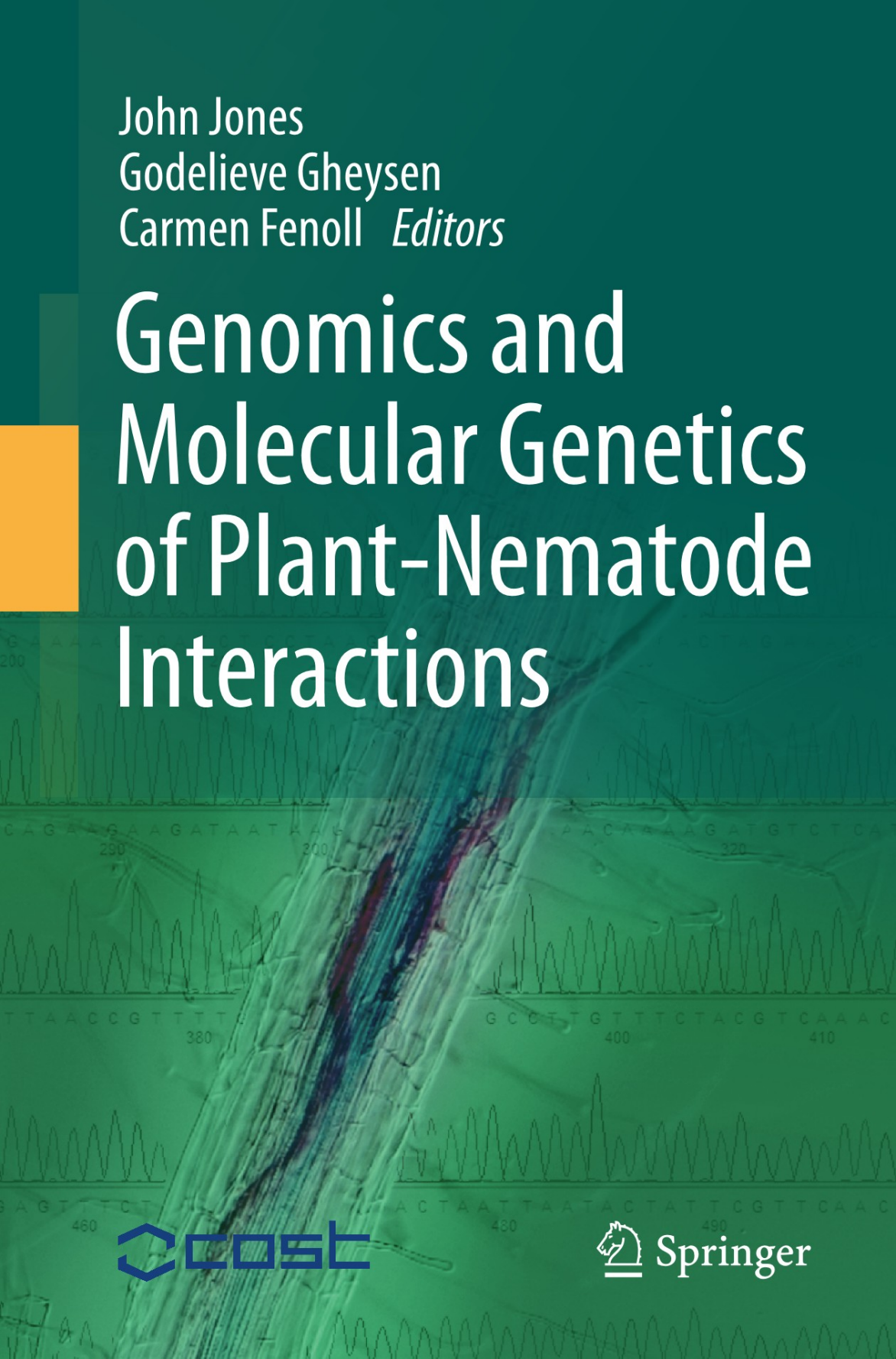
Genomics and Molecular Genetics of Plant-Nematode Interactions



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Editors

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COST Description

COST—the acronym for European Cooperation in Science and Technology—is the oldest and widest European intergovernmental network for cooperation in research. Established by the Ministerial Conference in November 1971, COST is presently used by the scientific communities of 35 European countries to cooperate in common research projects supported by national funds.

The funds provided by COST—less than 1% of the total value of the projects—support the COST cooperation networks (COST Actions) through which, with EUR 30 million per year, more than 30,000 European scientists are involved in research having a total value which exceeds EUR 2 billion per year. This is the financial worth of the European added value which COST achieves.

A "bottom up approach" (the initiative of launching a COST Action comes from the European scientists themselves), "à la carte participation" (only countries interested in the Action participate), "equality of access" (participation is open also to the scientific communities of countries not belonging to the European Union) and "flexible structure" (easy implementation and light management of the research initiatives) are the main characteristics of COST.

As precursor of advanced multidisciplinary research COST has a very important role for the realisation of the European Research Area (ERA) anticipating and complementing the activities of the Framework Programmes, constituting a "bridge" towards the scientific communities of emerging countries, increasing the mobility of researchers across Europe and fostering the establishment of "Networks of Excellence" in many key scientific domains such as: Biomedicine and Molecular Biosciences; Food and Agriculture; Forests, their Products and Services; Materials, Physical and Nanosciences; Chemistry and Molecular Sciences and Technologies; Earth System Science and Environmental Management; Information and Communication Technologies; Transport and Urban Development; Individuals, Societies, Cultures and Health. It covers basic and more applied research and also addresses issues of pre-normative nature or of societal importance.

Web: http://www.cost.eu

Foreword

This book reflects two decades of collaborative research on plant nematode interactions with a core of European teams that were brought together in the early nineties. When asked to write the foreword for this book, I wanted to document the origin of this group as it demonstrates that chance happenings can shape the future.

In the pioneering years of genetic engineering, I was working as a plant physiologist at the first plant biotech company in the Netherlands, Mogen International. In 1988 the Dutch potato processing industry approached us to solve their number one headache; potato cyst nematodes. It was a 4 year program with a team of 3–4 scientists to engineer resistance into their main cultivars. For a small biotech company that had no income except from investors, this was a big contract and we were keen to sign it in 1989.

To put this into historic perspective, these were the days where consumers had no clue yet about genetically modified food and we could routinely express a viral coat protein in tobacco and show systemic virus resistance… but anything else was far from routine or could even be classified as science fiction. We had just barely demonstrated transgenic potato plantlets and the first frail GM plants were cultivated in our high containment space age growth chambers, dazzling every visitor. We thought we were the new masters of the universe, carrying an unbelievable new toolbox that was growing every month with breathtaking inventions like PCR, reporter genes, genome sequencing, DNA synthesizers, etc. So, the fact that no one in the company had ever seen a live nematode before (my main background was in root physiology, but all the others were top-ranking molecular biologists) was considered a minor issue by management and by our contractors. They had faith in the scientists and the new toolbox. In retrospect, the naivety with which we entered this project was laughable, but without dreams there is no progress. It turned out to become the most fascinating period of my scientific career.

Parasitic nematodes are a problem in every country but are notoriously difficult to study. As a research subject they present many obstacles, delaying scientific progress to a pace that is no longer acceptable in the competitive world of grants and careers. On the other hand, the economic damage is significant everywhere and each country had at least one group of specialists to study their local threats and maintain a level of expertise, with relatively secure national funding. Therefore

the European landscape was scattered with small but dedicated research groups, looking for ways to reduce chemical treatments, running breeding programs or just fascinated by the complex interactions between multicellular organisms. With one or two exceptions, molecular techniques were not widely used by these groups.

In the beginning of the project at Mogen, I visited many of these groups to get a better feeling for the state-of-the-art. Mogen in those days was just about 25 scientists, but being an "industry representative", I was met with scepticism and also with curiosity, as we were definitely a new kid-on-the-block. There was no budget for collaborations so I had very little to offer and my hosts kept their cards close to their chests. Seeing the work done by these groups, it gradually began to dawn on me that studying the life cycle of cyst nematodes on potato plants *in vitro* would already be a challenge, let alone interfering with its life cycle. A turning point in those visits was a trip to Kiel in Germany to meet Professor Urs Wyss. His enthusiasm was inspiring to say the least. After a crash course on nematode behaviour with his magnificent videos, I noticed that the Kiel group was able to grow cyst nematodes routinely on rapeseed *in vitro*, and from a root physiologist's point of view, these roots looked excellent, a far cry from the stunted potato roots we were growing at Mogen. The Kiel group offered me a few of their Petri dishes to bring back to Holland to use as a starter culture and to allow me get hands-on experience growing nematodes. No paperwork, no lawyers, no signatures, this was mutual trust only.

For other projects, I was growing *Arabidopsis* plants. At this time *Arabidopsis* was rapidly becoming the gold standard for plant molecular biologists, attracting the best and the brightest in plant science across the globe. One of the few areas where this model was not considered seriously was Phytopathology; *Arabidopsis* appeared to be resistant to most pathogens. Deviating way off from my project (I wouldn't dare tell my industrial partners that I was working on anything other than potato and I didn't dare tell my colleagues I was infecting *Arabidopsis* with a pathogen, which would have been considered a rather stupid venture in those days), I set up several experiments with *Arabidopsis* to see if I could get juveniles harvested from rapeseed cultures to infect the roots. I checked progress outside lab hours or during coffee breaks when I had the lab for myself. On several occasions I noticed behaviour similar to that which I had seen on the videos from Urs Wyss and realized that the worms recognized the presence of roots. Over the next few days I could even see movement within the translucent roots, indicating that the nematodes had managed to penetrate. I did not dare tell anyone yet. I remember vividly the first day I saw syncytia developing, the most prominent syncytia I had ever seen as they were developing in these really tiny roots. It was obvious that the nematodes were changing root growth in a way I had only seen with nitrogen fixing Rhizobia. But more astonishing, this was a pathogen infecting the model-plant *Arabidopsis*! Even before informing my colleagues, I phoned Urs to tell him what I had done. The message didn't really sink in and I took the next plane to Kiel, a bunch of *Arabidopsis* reviews in my bag, preparing a lecture on the model plant during the flight, it was my turn to inspire Urs and his group. The message did get through this time. We could jump on the fast train of *Arabidopsis* research. I left them with seeds and detailed protocols to repeat this in their lab.

A few more groups got involved and they all got the protocols to grow nematodes on *Arabidopsis*. Preparations were made to get European funding through a Concerted Action, bringing together 16 groups from all over Europe. All groups had basic funding already and we only applied for money to increase collaborations. *Arabidopsis* would be the common theme, a worthless weed so there were no issues about valuable crop species, exclusive fields and other potential roadblocks for such a large project. I could convince our industry partners that this was definitely a faster track to reach useful results that, at a later stage, we could transfer to potatoes. So fortunately, they stayed on board and I was allowed to continue. Brussels approved the program in 1992. For such a large group, it was a modest amount of money but just for travelling expenses, it was a staggering figure. With all expenses paid, any scientist from any of those groups could travel to any other group for the next 4 years and we organized large annual meetings where even the most junior members were able to attend. Obviously, collaborations flourished and gathered momentum with hundreds of exchange visits across the continent. The group had reached a critical mass that was unheard of in this field, resulting in excellent scientific publications in high ranking journals, patent applications, newspaper coverage, professorships, and last but not least, it attracted new scientists and students.

There were times where we thought that breakthroughs were close, as we were able to target gene expression directly in the syncytia and could beat the parasite using its own tricks; triggering plant promoters that were now coupled to toxic genes. But nature proved to be far more complex and within the time span of the Concerted Action, nobody came close to showing resistance even though the first field tests were done in 1995. The final annual meeting was staged in Toledo, Spain, and although we did not reach our ambitious milestones, it was clear that research on plant-nematode interactions had made a great leap forward. It was no longer a completely black box. The irony now is that to date, not one *Arabidopsis* ecotype could be identified with natural resistance against nematodes and this line of work still solely relies on crop species.

Even though I moved on to another job at that time, the momentum of this group remained and follow-up EU projects were prepared, submitted and granted throughout the following 15 years. People come and go and move on with their lives, but this book demonstrates that the backbone of our first Concerted Action is still prominently visible. No less then 21 of the 24 chapters include labs or scientists from the original group and the critical mass has been kept together for all these years. This is a vital ingredient for a niche in science that involves so many disciplines and focuses on such a complex biological interaction.

The book reviews progress that is impressive. Whole genome sequences of important plant-parasitic nematodes, application of new molecular tools for *Arabidopsis,* microanalysis of feeding cells, unravelling (suppression of) the host immune reaction, hormone regulation, cell cycle- and cell wall interference, cytoskeleton design, new breeding strategies and a series of field trials with GM-crops are all milestones within their specialized areas. Of course, *Arabidopsis* can not claim all the credit for this progress, but to have a non-commercial common interest was

essential to start the initial collaboration and became the basis for the long term collaborations of which this book is the concrete proof.

It has been a privilege and a pleasure to work with this group of dedicated and enthusiastic scientists.

Peter C. Sijmons
Szienz

Preface

These are extraordinary times to be a biologist. The advent of new DNA and RNA sequencing technologies that allow massive amounts of sequence to be generated at a very low cost means that the opportunities offered by application of genomics tools are now available to researchers working with almost any organism. This is in stark contrast to the situation just a few years ago where almost the only genome sequences that were available were those of a few carefully chosen model organisms. With so much data available, the best way to drive biological discovery forward and ensure that practical developments emerge is to work in teams rather as individuals.

The potential benefits of closer co-operation between researchers seeking to exploit this new genome information were recognised by COST who, in 2006, approved funding for COST Action 872 "Exploiting genomics to understand plant-nematode interactions". The aim of this Action (as lifted from the original proposal) was "to develop a co-ordinated approach to exploitation of genomics information that is appearing for plant parasitic nematodes and host crops".

Plant parasitic nematodes cause economic losses to crops throughout the world. The need for new control strategies for plant nematodes has become more pressing in recent years as many of the most effective nematicides have been withdrawn from use, or scheduled for withdrawal, on environmental grounds. In addition, increased international trade and movement of materials means pressure on quarantine organizations to keep new pests and diseases out of new areas. The difficulties faced by workers in this sector are reflected by the introduction and apparent establishment since 1999 of the pine wilt nematode, *Bursaphelenchus xylophilus* into the EU.

Although they are damaging pests, many plant parasitic nematodes have fascinating interactions with their hosts. Plant nematodes can be ectoparasites, browsing on cells at the root surface, or can be endoparasites that invade the host plant and migrate through host tissues. The most complex interactions are those between the sedentary endoparasites and their hosts, including the most economically important nematodes—the root knot and cyst forming nematodes. These induce feeding structures (giant cells or syncytia) which are kept alive for several weeks in order to supply the nematodes with the nutrients they need to reach maturity. This is a degree of biotrophy that is almost unparalleled by any plant pathogen. In order to induce the formation of the feeding site the nematodes induce huge changes in plant gene

expression including changes in the cell cycle and other fundamentally important developmental processes. Uncovering the mechanisms behind feeding site induction and suppression of host defences offers huge scientific opportunities.

Nematodes, of course, do not have it all their own way. Natural resistance against many nematode species is available and there is much work ongoing aimed at understanding resistance mechanisms and identifying resistance genes. One of the immediate outputs of genomics programmes is a full list of potential targets for new control strategies against nematodes using chemical or GM approaches. Much progress has been made—particularly in the latter area.

The purpose of this book is to showcase the developments in plant-nematode interactions over the last few years and to summarise the impact that genomics has had on our field. We have also tried to include sufficient background information in Part I to make the book accessible to relative newcomers to the field. We hope that this will make it useful to new students and postdocs entering this area for the first time as well as to more established researchers.

We would like to acknowledge the impact that COST funding has had on plant nematology in Europe over the last four years. Funds from COST have allowed researchers to meet each year and forge new partnerships that will tackle important areas in this field. Funding has been made available to early career stage researchers to attend these meetings, undertake exchange visits and attend training events. COST funding has therefore had an impact on the skill development of many young plant nematologists.

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About the Authors

John Jones did a first degree in Zoology at the University of Newcastle upon Tyne and then a PhD in nematode sense organ structure and function at University College of Wales Aberystwyth and Rothamsted Research. After a brief diversion working on moths and zebrafish in the USA, John moved to SCRI in 1993 and has worked on plant-nematode interactions here ever since. Current interests include PCN genomics and suppression of host defences by biotrophic nematodes. John also has a guest professor position at Ghent University. John contributes to the teaching of plant nematology at Ghent, Dundee and St Andrews Universities and is Chair of COST Action 872.

Godelieve Gheysen received an M.Sc. degree in zoology and a PhD degree in plant molecular biology from Ghent University, Belgium. Her PhD research was on plant transformation by *A. tumefaciens*. In 1991 she started her own research group on the molecular analysis of plant-nematode interactions. This research focuses on (1) the plant response in the compatible interaction at the molecular and cytological level (2) characterisation of the parasitism genes of nematodes (3) using this knowledge to engineer nematode resistance in plants. Since 1993, she has been a professor in molecular genetics at Ghent University and teaches several classes including molecular biology, plant biotechnology and plant-nematode relations.

Carmen Fenoll obtained her PhD in Biological Sciences at the Universidad Autónoma de Madrid, working on photosynthetic bacteria. She moved to plant molecular biology as a Fulbright postdoc at the University of California-San Diego and established her group at the Universidad Autónoma de Madrid in 1990. She has been Visiting Scientist at the Salk Institute in La Jolla and Tinker Professor at the University of Wisconsin, Madison. Since 2000, she is Professor of Plant Biology at the Universidad de Castilla-la Mancha (Spain), where she teaches molecular biology, genomics, plant physiology and biotechnology. Her research group works on the regulation of gene expression and cell differentiation using molecular genetics and genomics in several biological systems, one of which is nematode feeding sites.

Part I
Introductory Chapters

Chapter 1
Introduction to Plant-Parasitic Nematodes; Modes of Parasitism

Roland N. Perry and Maurice Moens

1.1 Introduction to Nematodes

Nematodes are astonishing organisms. Despite their deceptively simple morphology and the fact that they are essentially aquatic, requiring at least a film of liquid for active life, they have been successful in colonising an enormous range of environments. Irrespective of their habitat, nematodes have a similar external morphology, with a worm shaped, bilaterally symmetrical, unsegmented body. The phylum Nematoda comprises >25,000 described species and the importance of nematodes should never be underestimated. Species parasitic on plants and animals have a massive deleterious social and economic impact on man. As will be discussed below, one major attribute that contributes to the undoubted success of nematodes is the amazing ability of some of the life cycle stages to survive adverse environmental conditions. Free-living nematodes of the species *Caenorhabditis elegans*, carried as part of the experimental payload on the Columbia spacecraft, even survived when the spacecraft broke up on re-entry in 2003 (Szewczyk and Lamb 2005).

Free-living species, which make up the bulk of the phylum Nematoda, feed primarily on bacteria and fungi, and are found in soil, marine and freshwater habitats; species have been thawed out of Antarctic ice (Cobb 1914) and others have been found in hot water springs in New Zealand (Rahm 1937). The free-living forms in the soil are beneficial as they are involved in nutrient turnover; in addition, they may be of use as indicator species for pollution monitoring (Wilson and Khakouli-Duarte 2009). The parasitic forms have devastating effects on man, his crops and his livestock as well as infecting wild plants and animals. Nematodes infecting plants are also known as ‘eelworms’ and some species infecting animals are colloquially called ‘roundworms’.

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Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* have been commercialised as environmentally acceptable control agents for several insect pests (Ehlers 2001; Gaugler and Han 2002). Like many free-living nematodes, individuals of these genera feed on bacteria but they have become specialised in using insect larval stages as a basis for culturing their food supply. The infective juvenile nematodes invade the target insects and release symbiotic bacteria in the haemocoel of the host. The bacteria multiply and kill the insect by septicaemia, usually within 48h, and the nematodes feed on the proliferating bacteria, develop and reproduce. The research information on these nematodes is extensive (Gaugler 2002).

Although the majority of nematodes are microscopic in size (less than 1 mm in length and between 15 and 20 μm in diameter; Fig. 1.1), the animal-parasitic species are often considerably larger. The largest nematode is *Placentonema gigantisma*, discovered in the placenta of a sperm whale; the adult nematode can grow to 8 m in length. In humans, nematodes are of great medical importance and it has been estimated that a quarter of the world's population suffer from a nematode infection of some sort. One of the most familiar diseases caused by nematodes is elephantiasis, caused by *Wuchereria bancrofti*. *Ascaris lumbricoides* is a major parasite of the intestine as is *Enterobius vermicularis*, which is probably familiar to many mothers as the 'pin worm' parasite of children. Dogs and cats are infected by several nematodes, among which are the microscopic *Toxocara cati* (in cats) and *T. canis* (in dogs). If the animals are not wormed the eggs voided in the faeces in parks and play areas, for example, can attach to fingers and, if ingested by humans, the juvenile nematode will hatch. The juvenile does not develop further, but not being in the proper host will wander around the body causing serious damage to organs. If the nematodes enter the cerebrospinal fluid and migrate to the brain, the victim can suffer brain damage and blindness. Deaths, usually of small children, have been reported. These are only a few examples of animal-parasitic nematodes; for further reading illustrating these pests and the horrific diseases they cause see Matthews (1998) and Lee (2001).

The subjects of this book, plant-parasitic nematodes, do not have such obvious and unpleasant effects. However, their economic and social impacts are no less severe,

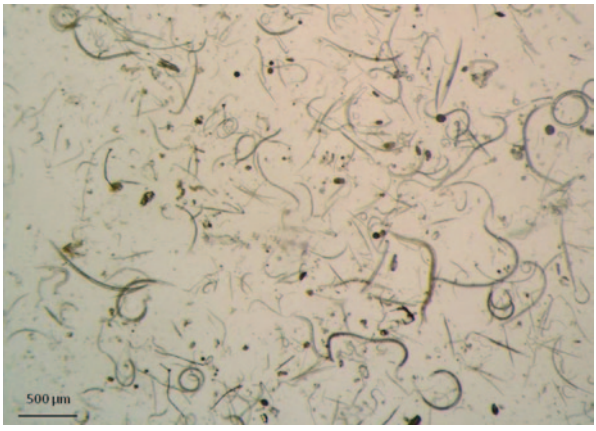

Fig. 1.1 Free-living nematodes and free-living stages of plant-parasitic nematodes obtained from a field soil extraction. (Courtesy Wim ML Wesemael, Institute for Agricultural and Fisheries Research, Belgium)

especially in developing countries where crop loss due to nematodes may be disastrous. All crop plants have one or more species of nematodes that feed on the roots as ectoparasites or invade the roots and feed internally as endoparasites. Some species are migratory endoparasitic, moving in and out of the plants and there are also species that feed on the aerial parts of plants (stems, leaves, buds and seeds). As well as the detrimental effects on the growth of the plants, causing stunting, early senescence and in severe cases total crop loss, the damage caused, especially to root crops such as carrots, can render the produce unmarketable and eliminate income. A major difficulty in controlling the plant-parasitic species is convincing farmers, growers and advisors that the crop problems are actually caused by these microscopic pests. With good reason, plant-parasitic nematodes have been called 'The Invisible Enemy'.

Among the most economically important nematodes are those endoparasitic species that form complex feeding structures in the roots of their host plants. The most damaging are the root-knot (*Meloidogyne* spp.) and cyst (*Heterodera* and *Globodera* spp.) nematodes. The root-knot nematodes cause most damage worldwide (Moens et al. 2009). In general, species of *Meloidogyne* have broad host ranges,

Fig. 1.2 Galls of the false root-knot nematode, *Nacobbus bolivianus,* on potato roots. (Courtesy Rosa H Manzanilla-López, Rothamsted Research, UK; from Manzanilla-López 2010)

and are able to infect almost all species of flowering plants. The world-wide spread of root-knot and cyst nematodes and their enormous economic impact has formed the justification for much research; information on these two groups is extensive and this bias is, of necessity, reflected in the following sections. However, there are other groups that have a major agricultural impact, especially species of the genus *Nacobbus* (Manzanilla-López et al. 2002; Manzanilla-López 2010; Fig. 1.2) and the migratory endoparasitic root lesion nematodes of the genus *Pratylenchus* (Castillo and Vovlas 2007).

With the decline in use or banning of many chemicals because of adverse environmental impacts, it is imperative that new strategies for nematode control and management are developed and implemented. In this context, understanding plant-nematode interactions will be vital, and the ability to exploit genomics will not only indicate novel control targets, but also justify re-examination of some older suggestions for control based on interrupting certain phases of the life cycle.

1.2 Evolution of Plant Parasitism

The conserved morphology of nematodes and the absence of extensive fossil records make discussion of the evolution of parasitism in nematodes problematic (Poinar 2011). Several hypotheses about the origins of plant parasitism have been put forward by nematode taxonomists (for example, Maggenti 1971; Poinar 1983; Siddiqi 1983) with little agreement. However, more recent studies using molecular phylogenies based on the small subunit ribosomal RNA (SSU RNA) demonstrate that parasitism of plants by nematodes has arisen independently on at least three separate occasions (reviewed by Baldwin et al. 2004). The rRNA phylogenies also support convergent evolution of sedentary endoparasitism and feeding site establishment by root-knot and cyst nematodes, rather than the theory that the two groups shared a common ancestor.

The co-evolution of plants and plant-parasitic nematodes has resulted in remarkable synchrony of the host and parasite life cycles that enhances the chances of the nematode infection and, thus, survival and reproduction. This integration between host and nematode has progressed furthest in cyst nematodes and the dependency of some species on stimulation from host plants to cause hatch is one aspect of this integration (see Sect. 1.3).

Comparative genomics have been used to provide insights into the evolution of parasitism in the phylum Nematoda, especially the acquisition of novel genes associated with parasitic lifestyles (Rosso et al. 2009). Several genes have been identified in the transcriptomes of plant-parasitic nematodes that are most similar to microbial genes, and these may have been acquired by horizontal gene transfer (HGT) from microbes associated with ancestral nematodes. Jones et al. (2005) argued that acquisition of such genes via HGT has played a critical role in the evolution of plant parasitism. For example, several genes coding for enzymes such as cellulase, pectate lyase and chorismate mutase have been identified in cyst and root-knot nema-

todes with bacteria as the likely origin (Jones et al. 2005). Fungi are the probable origin for the gene coding for GHF45 cellulase, which is vital for the parasitic phase of the life cycle of *Bursaphelenchus xylophilus* (Kikuchi et al. 2004).

The use of RNAi enables loss-of-function phenotypes to be analysed and will provide information on the evolution of nematode parasitism. Rosso et al. (2009) consider that RNAi will facilitate the elucidation of the molecular determinants of parasitism. Information from this approach, together with functional and behavioural data, may provide pointers to new control targets centred on perturbing aspects of the parasitic life cycle such as hatching, host location and survival.

1.3 Hatching

The embryo in each nematode egg develops through embryogenesis to a first-stage juvenile (J1), which, in some longidorids and trichodorids, hatches. However, in most species of plant-parasitic nematodes, the J1 moults within the egg to the second-stage juvenile (J2). It is this invasive J2 that hatches and then feeds on a host plant. There is little variation in the average size of nematode eggs, irrespective of the size of the adult, and the eggshell of plant-parasitic nematodes typically consists of three layers, an outer vitelline layer, a middle chitinous layer and an inner lipid layer. The lipid layer is the main permeability barrier of the eggshell and makes the egg resistant to chemicals, including non-fumigant nematicides. Physiological adaptations, such as different states of dormancy, are an essential component of the survival of nematodes in the absence of a host and are frequently associated with the unhatched juveniles (Perry 1989, 2002). In the majority of species of plant-parasitic nematodes, the juvenile hatches provided environmental conditions, including temperature and moisture content of the soil, are favourable. However, in some species, co-evolution of host and parasite has resulted in a sophisticated relationship whereby the nematode does not hatch unless stimulated by chemicals emanating from the host roots. These emanations have been termed root diffusates, root leachates or root exudates. Root diffusates is the preferred term of the present authors because ‘diffusate’ conveys the idea of volatile and non-volatile components diffusing through the soil and establishing a concentration gradient; thus, it is an especially apposite term in relation to hatching and attraction of nematodes.

The hatching process can be divided into three phases: changes in the eggshell permeability, metabolic activation of the juvenile, and eclosion (or hatch from the egg). The chronological order of the first two phases differs between genera. For example, in *Meloidogyne* spp., activation of the juvenile appears to occur first and causes eggshell changes; in others, such as *Globodera* spp., alteration of eggshell permeability characteristics appears a necessary pre-requisite for activation of the juvenile (Perry 2002). The agents for initiation of these responses vary between species and genera of nematodes but have been studied most extensively in species of root-knot and cyst nematodes. Hatching and survival attributes of these species are associated with the ‘packaging’ of eggs into ecological units (Perry and Moens

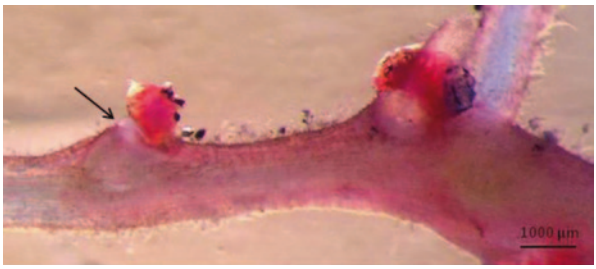

Fig. 1.3 Egg masses of *Meloidogyne chitwoodi* stained with Phloxine B; the posterior end of the adult female (*arrowed*) is visible outside the root. (Courtesy Wim ML Wesemael, Institute for Agricultural and Fisheries Research, Belgium)

2011). Females of root-knot nematodes lay eggs into a gelatinous matrix, which comprises an irregular meshwork of glycoprotein material (Sharon and Spiegel 1993). The gelatinous matrix surrounds the eggs and retains them in a package termed an egg mass (Fig. 1.3). With cyst nematodes, the death of the mature females is followed by polyphenol oxidase tanning of the cuticle resulting in a hard, brown cyst. Egg masses and cysts can each contain several hundred eggs. Egg packaging units similar to cysts and egg masses are not found in animal-parasitic or free-living nematodes.

Hatching of *Meloidogyne* spp. is, in general, temperature dependent and hatching occurs when temperatures are favourable without the need for stimulus from root diffusates. However, there are exceptions and a proportion of the unhatched juveniles of *M. hapla, M. triticoryzae* and *M. chitwoodi,* for example, have been shown to be dependent on root diffusates for hatch, especially in later generations during a host growing season (Gaur et al. 2000; Perry and Wesemael 2008). Although a few other species from other groups (e.g. *Rotylenchulus reniformis* and *Hypsoperine ottersoni*) hatch in response to host root diffusates, this phenomenon is most common among the cyst nematodes but even in this group reliance on host stimulation for hatch varies. *Globodera rostochiensis* and *G. pallida*, have a very restricted host range and are almost completely dependent on host diffusates for hatch, whereas *H. schachtii*, for example, has a very wide host range (some 218 plant species, including many weeds) and hatches well in water (Perry 2002). *Heterodera avenae* has a large hatch in water but a relatively narrow host range; however, the hosts are very common (Turner and Rowe 2006). The dependence of *G. rostochiensis* and *G. pallida* on a plant-derived hatching stimulus is an obvious control target, with the aim of inducing hatch in the absence of a host plant and thus causing the nematodes to die of starvation. However, although much research effort has been expended in elucidating the chemicals, termed hatching factors, in root diffusates, there has been no successful control strategy using analogues of the hatching factors to induce hatch in the field.

Host root diffusates induce a cascade of inter-related changes leading to eclosion, and the sequence of events has been discussed in detail by Jones et al. (1998) and Perry (2002). Unhatched J2 of *Globodera* and *Heterodera* spp. are surrounded by perivitelline fluid, which contains trehalose. Trehalose generates an osmotic pressure that reduces the water content of the J2 and inhibits movement because the turgor pressure is insufficient to antagonise the longitudinal musculature. For

hatching to occur, the pressure needs to be removed. In *G. rostochiensis* and some other species, this is achieved by a change in permeability of the inner lipoprotein membranes of the eggshell via HF binding or displacing internal Ca^{2+} (Clarke et al. 1978). In both *G. rostochiensis* and *G. pallida*, a 5 min exposure to host diffusate is sufficient to stimulate hatch (Perry and Beane 1982), suggesting the involvement of a receptor-ligand interaction between the HF and the eggshell lipoprotein membrane. The change in eggshell permeability enables trehalose to leave the egg, with a concomitant influx of water and subsequent rehydration of the J2 to a water content commensurate with movement. The eggshell of *G. rostochiensis* remains rigid during the hatching process and there is no evidence of enzyme involvement. Devine et al. (1996) demonstrated that the potato steroidal glycoalkaloids, α-solanine and α-chaconine, induce hatch of *G. rostochiensis*; glycoalkaloids are known to destabilise lipid membranes during which leakage of trehalose is possible. However, enzymes have been implicated in softening of the eggshell prior to eclosion in other species, including *Xiphinema diversicaudatum*, *Aphelenchus avenae* and *M. incognita*; in *M. incognita* lipase activity has been positively correlated with hatch (Perry et al. 1992). Rehydration of the J2 of *G. rostochiensis* is accompanied by increased metabolic activity due in part to removal of osmotic pressure and hydration and in part to direct stimulation of the J2 by root diffusate. Changes in gene expression of *G. rostochiensis* J2 appear to occur during or immediately after the hatching process (Jones et al. 1997), but more work is needed on the molecular aspects of the hatching response.

The J2 of *Globodera* spp. uses its stylet to cut a regular series of perforations in the subpolar region of the eggshell, and the J2 hatches through the resulting slit. J2 of *D. dipsaci* use a similar approach, except that the stylet thrusts are more random and the J2 uses its head to force open the slit in the eggshell. In *Pratylenchus penetrans* and *H. avenae*, a single stylet thrust penetrates the eggshell and the head extends this into a tear.

Once hatched, nematodes are vulnerable to environmental extremes and have to locate a host to start feeding. For example, under optimal conditions for movement, J2 of *G. rostochiensis* must locate a host root and set up a feeding site within 6–11 days of hatching otherwise it will exhaust its energy reserves and die (Robinson et al. 1987). Hatching in response to host root diffusates has the advantage of ensuring that the nematodes hatch and leave the protection of the egg and cyst when host roots are close by; thus, synchrony of host availability and nematode hatch is advantageous for nematode survival.

1.4 Attraction to Plants

Around actively growing roots there exist several gradients of volatile and non-volatile compounds, including amino acids, ions, pH, temperature and CO_2. It is evident that nematodes use their chemosensory sensilla, the amphids, to orientate towards the roots using at least some of these gradients. The ability to orientate

towards stimuli from plant roots enhances the chances of host location and reduces the time without food (Perry 1997). Evaluating the reality of the attractiveness or otherwise of an individual compound is difficult. Information is usually based on *in vitro* behavioural studies, often using agar plate movement bioassays, which bear little if any resemblance to the situation in the soil; care must therefore be exercised in extrapolating from such assays to the field situation (Spence et al. 2009). It will be especially important in the future for nematologists to link with plant physiologists to determine the temporal and special attributes of putative attractants in the soil. However, some generalisations can be made and certain compounds are strongly implicated in orientating nematodes to the roots.

Perry (2005) separated gradients into three types: 'long distance attractants' that enable nematodes to move to the root area, 'short distance attractants' that enable the nematode to orientate to individual roots, and 'local attractants' that are used by endoparasitic nematodes to locate the preferred invasion site. There is clear experimental evidence that CO_2 is a long distance attractant (Robinson and Perry 2006). With cyst nematodes, such as *Globodera* spp., it is apparent that the J2 responds to host root diffusate and the evidence is persuasive that diffusate contains chemicals that constitute short distance attractants (Perry 1997; Rolfe et al. 2000). Diffusates from the roots of the host plant, potato, increased the activity of the infective J2 of *G. rostochiensis* and also attracted them to the roots. As detailed in Sect. 1.3 potato root diffusate (PRD) is required to stimulate hatching of the majority of J2 of the potato cyst nematodes *G. rostochiensis* and *G. pallida* but work by Devine and Jones (2002) has shown that the chemicals in PRD responsible for hatching differ from those responsible for attracting the J2 to the root. Electrophysiological analysis of sensory responses (Perry 2001) demonstrated that spike activity of J2 of *G. rostochiensis* increased on exposure to PRD but not to root diffusate from the non-host sugar beet, thus indicating that responses to diffusates may be host specific. Pudasaini et al. (2007) found that the migration of *P. penetrans* towards a host depends on both the initial distance between the nematode and the host and the nature of the host. These authors considered that the attractiveness of the host to *P. penetrans* seems to be correlated with its efficiency as a host; the attractiveness of hosts also declines with age.

The orientation of J2 of cyst and root-knot nematodes to the preferred invasion site, the root tip, is well established but the active factors that constitute the 'local attractants' are unknown. The nematodes may orient to an electrical potential gradient at the elongation zone of the root tip but the relative importance of electrical and chemical attractants for root tip location has not been evaluated; in addition the elevated temperature at the zone of root elongation may influence nematode perception.

Blocking sensory perception so that the nematodes are unable to orientate to roots and thus exhaust their food reserves and die is an attractive control option but may be difficult to achieve. Exposure of J2 of *M. javanica* and *G. rostochiensis* to antibodies to amphidial secretions blocked the response to host root allelochemicals (Stewart et al. 1993; Perry and Maule 2004) but responses were not permanently blocked as, after a period of between 0.5 and 1.5 h, turnover of sensilla secretions

presumably 'unblocked' the amphids. A similar effect occurred with the bionematicide, DiTera®, where disruption of sensory perception was reversible (Twomey et al. 2002).

1.5 Penetration and Feeding

As mentioned in Sect. 1.1, plant-parasitic nematodes exhibit several feeding strategies. However, for all species, feeding is dependent on the use of the hollow, needle-like mouth spear, or stylet, which is inserted into a cell in order to extract its contents. A framework of definitions for biological function is rarely exact; however, some general groupings can be made that reflect the different feeding strategies.

Sedentary endoparasitic nematodes enter host roots, set up a feeding site within the root tissue and feed internally. As with other aspects of plant-parasitic nematode biology, the focus on feeding by endoparasitic species now and in the recent past has been on cyst and root-knot nematodes. However, other nematodes, such as *Nacobbus*, set up a feeding site as a nutrient sink (Manzanilla-López et al. 2002) and may become important research subjects, especially as comparative genomics progresses.

The convergent evolution of cyst and root-knot nematodes has resulted in the same outcome, feeding on nematode induced nutrient sinks, but the method of achieving the end result and the feeding sites themselves show interesting differences. The preferred invasion site for both groups is behind the root tip in the zone of elongation. J2 of cyst nematodes, with their more robust stylets, cut cortical cell walls and migrate through cells until they reach the differentiating vascular cylinder. This intracellular migration causes considerable damage to host tissue, resulting in necrosis from the invasion point to the feeding site. By contrast, root-knot nematodes migrate intercellularly. After invasion, J2 of root-knot nematodes move towards the root tip until they reach the root apex where they turn around, avoiding the barrier of the endodermis, and migrate back up the root until they reach a site near the vascular cylinder.

Cell wall-degrading enzymes from the subventral glands are secreted through the stylets of both cyst and root-knot nematodes to facilitate migration by weakening or breaking down cell walls. Among the enzymes identified in both nematode groups are cellulases and pectate lyases, and in root-knot nematodes xylanase and polygalacturonase (Davis et al. 2000; Gheysen and Jones 2006). Before the discovery of these enzymes in nematodes, they had been reported only from plants and pathogenic bacteria and fungi. They are not present in non-parasitic nematodes or other invertebrates and it is likely that they were acquired by horizontal gene transfer from bacteria to plant-parasitic nematodes (see Sect. 1.2).

Detailed information of the induction and maintenance of the feeding sites for cyst and root-knot nematodes are given in Chaps. 4 and 5 respectively, this volume. Briefly, a cyst nematode selects a cell, becomes sessile, and a multinucleate feeding site, termed a syncytium, is formed by gradual incorporation of hundreds

of adjacent cells as the intervening cell walls disintegrate. Root-knot nematodes become sessile and induce the formation of several binucleate cells followed by mitosis that does not cause cell division. These nuclei further divide and several large multinucleate giant cells result and cells surrounding them also enlarge to form a gall or root-knot. In both cyst and root-knot nematodes, secretions from the dorsal and ventral pharyngeal glands play a central role in the induction and maintenance of the feeding sites.

Only the J2 and adult males of root-knot and cyst nematodes are migratory, whereas in *Nacobbus*, for example, all juvenile stages, the male and the immature vermiform female are migratory, only the mature female being sedentary. All mobile stages of **migratory endoparasitic** nematodes, such as *Pratylenchus* and *Radopholus*, invade plant hosts but do not become sessile and have no fixed feeding site within the plant, moving around and feeding off numerous cells and causing considerable damage to plant tissue.

Semi-endoparasitic nematodes, such as *Rotylenchulus* and *Tylenchulus*, become sessile after penetrating the root but only the anterior part of the nematode penetrates and remains embedded in the root tissue, the rest of the body remains outside the root in the soil. Some genera, such as *Hoplolaimus* or *Helicotylenchus*, may be either semi-endoparasitic or migratory ecto-endoparasitic, depending on the host. **Ectoparasitic nematodes** remain outside the roots to feed externally, puncturing individual cells with their stylet, extracting food and then withdrawing the stylet to move on to another cell to repeat the process. Some ectoparasitic species, e.g. *Cacopaurus*, become permanently attached to the root by the deeply embedded stylet.

1.6 Moulting

Once feeding commences the nematode can continue development. The majority of nematodes have four juvenile stages before development to the adult; thus, there are four moults where the old cuticle is replaced by a new cuticle. Exceptions include certain species of *Xiphinema*, which have only three moults and three juvenile stages. During the moulting process the cuticle is either shed completely or, as in *Meloidogyne*, is partially absorbed. Unlike insects, the nematodes increase in size between moults and not during the moulting process.

Moulting may be a putative control target. Soriano et al. (2004) examined the effects of the ecdysteroid 20-hydroxyecdysone (20E), a major moulting hormone of insects, on *M. javanica.* Exogenous application of 20E resulted in immobility and death of J2, and invasion was partially inhibited and development was halted in spinach with induced high levels of endogenous 20E; however, of the few J2 that invaded, no abnormal moulting was observed. The biosynthesis of ecdysteroids by any nematode has yet to be demonstrated, and specific efforts to detect 20E and its precursor, ecdysone, in *M. arenaria* and *M. incognita* were unsuccessful (Chitwood et al. 1987). The complete sequences of the genomes of *M. hapla* (Opperman et al. 2008) and *M. incognita* (Abad et al. 2008) will provide information about the genes

involved in moulting in *Meloidogyne*, and this, together with data from genome projects on other species of nematodes, may provide the basis for a realistic assessment of inhibiting or disrupting moulting as a control strategy.

1.7 Reproduction

Nematodes exhibit a variety of sexual and asexual reproductive methods (reviewed by Evans 1998) and, of the plant-parasitic nematodes, most information is available on *Meloidogyne* (reviewed by Chitwood and Perry 2009). Sexual reproduction (**amphimixis**) can occur in species where there are two separate sexes, where the haploid (n) male (spermatocytes) and female (oocytes) gametes fuse to form the zygote and restore the diploid (2n) complement of chromosomes. In *A. tritici* and *D. dipsaci*, for example, female nematodes are the homogametic sex (genetically XX) and males are heterogametic (sometimes XO, usually XY), and the sex ratio is determined genetically. In genera such as *Globodera*, *Heterodera* and *Meloidogyne* sex chromosomes are absent and the sex ratio may be environmentally influenced. In many genera, including *Heterodera, Pratylenchus* and *Radopholus*, the female can be fertilised by several males, thus enhancing the genetic diversity of the offspring.

In species of nematodes that reproduce asexually, males are absent or occur only rarely. There are two main types of parthenogenesis, meiotic parthenogenesis (**automixis**) and mitotic parthenogenesis (**apomixis**). In meiotic parthenogenesis, there is a first meiotic division in the oocytes, although there are variations between species. This meiotic division allows some genetic reorganisation, even though the diploid chromosome number is restored by self-fertilization. *Meloidogyne hapla* race A exhibits facultative meiotic parthenogenesis, oogenesis and spermatogenesis proceeding as in amphimictic species to yield one haploid nucleus and two polar bodies per oocyte. Although parthogenesis predominates, amphimixis can occur if sperm are present. However, if the sperm are not present, the egg pronucleus recombines with the second (haploid) polar body to restore the diploid state. In monosexual populations of *Aphelenchus avenae*, females produce only female progeny and reproduction is by obligate meiotic parthenogenesis. Meiosis produces only one polar body, with the egg nucleus having the 2n chromosome number that then develops into the zygote.

Mitotic parthenogenesis, which occurs in several species of *Meloidogyne*, is the most common method of asexual reproduction and is always obligate; the only division is mitosis and the oocytes retain the diploid chromosome number. This would seem to prevent any genetic reorganisation, except for that resulting from mutations. Frequently, mitotic parthenogenesis is associated with polyploidy, which may increase the likelihood of mutation. Several of the most widespread and economically important species of *Meloidogyne* are obligate mitotic parthenogens. Populations of the same *Meloidogyne* species may differ in mode of reproduction; for example, 29 of 32 populations of *M. hapla* reproduced by facultative meiotic parthenogenesis, the others by mitotic parthenogenesis (Triantaphyllou 1966).

In hermaphrodites, both egg and sperm are produced in the same individual. Usually the sperm is produced first and is stored in the spermatheca, then the gonad produces oocytes, which are fertilized by the sperm until the sperm supply is exhausted. Hermaphroditism is a common method of reproduction amongst free-living nematodes but is relatively rare in plant-parasitic nematodes, being found in some members of the Criconematoidea and in *Radopholus similis* and species of *Paratrichodorus.*

1.8 Survival

For obligate parasitic species there are situations where persistence of a population requires survival of the infective stages outside the host. This may occur when the host is not available, requiring the nematode to survive in the absence of food, or environmental conditions exist that are not commensurate with life cycle progression, such as temperature extremes, osmotic stress and dehydration. Of the environmental extremes, desiccation survival has attracted most interest, partly because the ability to survive dry is implicit in the distribution of infective stages by wind and in dry plant material, such as seeds. Interest has also been generated by the spectacular abilities of some nematode to survive desiccation for periods considerably in excess of the normal life cycle. For example, *D. dipsaci* fourth-stage juveniles (J4) have been recorded as surviving for more than 20 years in the dry state, yet the whole life cycle of the nematode in favourable environmental conditions only takes about 4 weeks from egg to egg-laying adult; thus, the ability to survive in the dry state (anhydrobiosis) can prolong life by a factor exceeding 240 times!

Although some of the most astonishing survival attributes are found in species of nematodes, such as *A. tritici*, and *D. dipsaci*, that live within aerial parts of plants, the infective J2 of root-knot and, especially, cyst nematodes also survive for long periods as unhatched individuals inside the egg mass or cyst (Perry 1999). Morphological features such as the cyst, gelatinous matrix and eggshell all protect the J2 and ensure a slow rate of drying to ensure effective entry into anhydrobiosis. There is no one stage that can be termed the ‘nematode survival stage’. Where species have the ability to suspend development and withstand environmental extremes, the stage(s) involved often differ. Similarly, there are variations in the behavioural and morphological adaptations that ensure desiccation survival, but all have the role of reducing the rate at which water is lost from the nematode as it experiences drying conditions (Perry and Moens 2011).

Galls induced by *A. tritici* in the tissues of host plants contain tightly packed aggregates of J2, each of which remains uncoiled when dry, yet the galls induced by *A. amsinckia* contain hundreds of desiccated adults and juveniles of all stages, many of which are coiled. Coiling and clumping are two behavioural responses that effectively reduce the surface area exposed to drying conditions (Perry 1999; Moens and Perry 2009). Reproduction of *Aphelenchoides besseyi* stops as rice grains ripen and adults coil in clumps beneath the hulls of grains, where the nematodes can remain

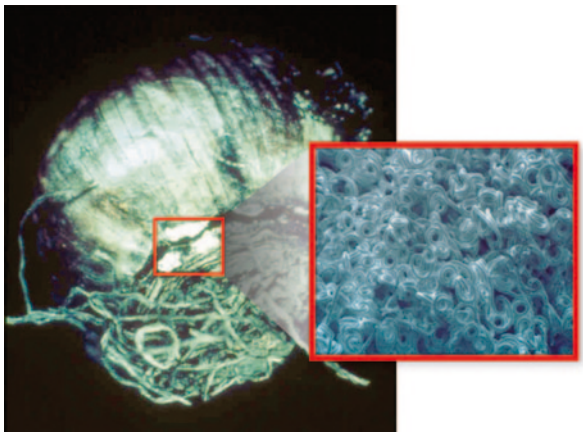

Fig. 1.4 Narcissus bulb with accumulation of *Ditylenchus dipsaci* fourth-stage juveniles (J4) as dry 'eelworm wool', and an inset showing a transmission electron microscope image of the eelworm wool with coiled, clumped, desiccated J4. (Photos courtesy of Roland N Perry, Rothamsted Research, UK; from Moens and Perry 2009)

viable for 2–3 years in dry grains. As mentioned above, in *D. dipsaci* development stops at the J4 stage and hundreds of desiccated individuals coil and clump in masses termed 'eelworm wool', often associated with infected narcissus bulbs (Fig. 1.4) or inside bean pods. The death of the peripheral J4 apparently provides a protective coat that aids survival of the enclosed nematodes by slowing their rate of drying, in a manner that Ellenby (1969) termed the 'eggshell effect'. Survival of J4 of *D. dipsaci* and J2 of *Anguina* spp. is also associated with an intrinsic property of the cuticle, involving an outer lipid layer (Preston and Bird 1987; Wharton et al. 1988), to control the rate of water loss. The cuticle dries more rapidly than other layers and slows down the rate of water loss of internal, and perhaps more vital, structures. As dry individuals in galls, as 'eelworm wool', in plant tissue, or in cysts the nematodes can survive for many years and withstand other adverse conditions, such as extremes of temperature; they are also more resistant to non-fumigant nematicides and can be dispersed effectively by wind.

Why is the rate of water loss important for survival even though the nematodes eventually lose all their body water? A slow rate of water loss appears to allow orderly packing and stabilization of structures to maintain functional integrity during desiccation. At water contents below about 20%, there is no free water in the cells. This 20%, usually referred to as 'bound water', is involved in the structural integrity of macromolecules and macromolecular structures, such as membranes. In desiccated, anhydrobiotic nematodes it is probable that the bound water has been lost and research has centred on molecules that might replace bound water and preserve structural integrity. The control of water loss enables biochemical changes to take place, including replacement of bound water, thus ensuring long-term survival of true anhydrobiotes (Barrett 1991; Perry 1999).

Ditylenchus dipsaci J4 and *A. tritici* J2 sequester trehalose, which has frequently been suggested as a desiccation protectant because of its role in preserving membrane stability, preventing protein denaturation and acting as a free-radical scavenging agent to reduce random chemical damage (Barrett 2011). However, there are contradictory reports about the importance of trehalose (Burnell and

Tunnacliffe 2011). For example, all stages of the mycophagous *D. myceliophagus* survive desiccation poorly, even at high humidities (Perry 1977). The survival of *D. myceliophagus* was unrelated to their trehalose content, and elevated levels of trehalose, generated by providing the nematodes with different food sources, did not enhance anhydrobiotic survival of this species (Womersley et al. 1998). Synthesizing trehalose during dehydration may indicate preliminary preparation for a period in the dry state, but it does not necessarily mean that survival during subsequent severe desiccation is assured. Following trehalose synthesis, it appears that other, at present unknown, adaptations are required at the cellular and subcellular levels for nematode survival, and rate of drying still has to be controlled (Higa and Womersley 1993).

Late embryogenesis abundant (LEA) proteins have been associated with survival in some nematodes, including *C. elegans* (Gal et al. 2004; Burnell and Tunnacliffe 2011), and LEA proteins may protect cellular components against the effects of desiccation (Goyal et al. 2005). Homologues of LEA genes have been identified in *B. xylophilus* (Kikuchi et al. 2007).

Aspects of the biochemical adaptations and the genes that are switched on during the induction of anhydrobiosis are likely to become evident when progress is made with comparative genomics and transcriptome analyses. In addition, the molecular information will also progress our understanding of the extent of the 'dauer' phenomenon in plant-parasitic nematodes. The dauer stage of the free-living nematode, *Caenorhabditis elegans*, represents a developmental arrest (Riddle and Albert 1997). Dauer larvae are enclosed by a dauer-specific cuticle and exhibit several characteristics associated with survival of adverse conditions, including reduced metabolism, elevated levels of several heat shock proteins and an enhanced resistance to desiccation (Kenyon 1997). Bird and Bird (1991) suggested that the survival forms of *Anguina*, may be regarded as dauers. In *Ditylenchus dipsaci*, the J4 that accumulate in response to adverse conditions are larger, have more lipid reserves and show a propensity to aggregate compared with J4 in a population feeding and developing under ideal conditions (Perry, unpublished), all properties that reflect the dauer state. In some species of the genus *Bursaphelenchus* a dauer form (J4) is present as a specialised survival and dispersal stage of the life cycle. *Bursaphelenchus xylophilus* is a migratory endoparasitic nematode that has a complex life cycle involving beetles of the genus *Monochamus* as the vector (Mota et al. 2008). *Bursaphelenchus xylophilus* has a dauer stage, which uses the insect for transport to susceptible hosts where the nematodes enter the shoots of trees through the feeding wounds caused by the vector. In an analysis of more than 13,000 ESTs from *B. xylophilus*, Kikuchi et al. (2007) looked for homologues of 37 genes involved in dauer entry and maintenance in *C. elegans*. They identified 31 homologues of 18 *C. elegans* genes, including nine homologues for *daf* (dauer formation) genes.

Meloidogyne hapla carries 14 orthologues of *C. elegans daf* genes as well as three further matches that are weak (Abad et al. 2008; Abad and Opperman 2009) but it does not carry the *daf-28* orthologue, which is key in the signal transduction pathway. Abad and Opperman (2009) conclude that basic development mechanisms are conserved, although signalling is not. Thus, there may be marked differences

between free-living and parasitic nematodes in developmental response to adverse changes in the environment.

These studies provide initial evidence that the dauer phenomenon may be more widespread than currently recognised. Certainly, the indications in some species of plant-parasitic nematodes of an alternative developmental stage similar to a dauer larva are convincing (see Sect. 1.8 above). However, there are difficulties in relating information on dauer formation in *C. elegans* to parasitic nematodes. Comparison of expression profiles of dauer genes in *C. elegans* and in survival stages of parasitic nematodes (Elling et al. 2007) reveals marked differences in expression patterns between *C. elegans* and other nematodes; as yet, there is insufficient information to be able to link individual *daf* genes to specific survival traits.

1.9 Conclusions

There are many putative control targets in the life cycle of plant-parasitic nematodes. Comparative genomics will undoubtedly increase our knowledge of the parasitic life style and the vulnerable phases. However, the challenge will be to translate this information to viable, environmentally benign control or management options. This chapter has focused on the nematode side of the plant-nematode interaction and has highlighted some putative targets. We have not discussed plant resistance or genetic engineering to interfere with nematode invasion and development. This research area of developing plant resistance to nematodes is undoubtedly important, but it is possible that future management options may be based on perturbing several different aspects of the nematode's life cycle to achieve a desired level of control.

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Chapter 2
Current Nematode Threats to World Agriculture

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2.1 Key Nematodes Threatening Major Agricultural Crops of Importance Worldwide

A major global challenge in the coming years will be to ensure food security and to feed the increasing human population. Nowhere will the need to sustainably increase agricultural productivity in line with increasing demand be more pertinent than in resource poor areas of the world, especially Africa, where populations are most rapidly expanding. Although a 35% population increase is projected by 2050 (World Bank 2008), an increase in food demand in the order of 75% is anticipated, due to economic development and changes in food preferences (Keating et al. 2010). Significant improvements are consequently necessary in terms of resource use efficiency. In moving crop yields towards an efficiency frontier, optimal pest and disease management will be essential, especially as the proportional production of some commodities steadily shifts. For example, over half the global potato production (>150 million t) now occurs in Asia, Africa and Latin America, as a result of steady increases in recent years (FAO 2010). With this in mind, it is essential that the full spectrum of crop production limitations are considered appropriately, including the often overlooked nematode constraints. For example in a recent review of intractable biotic constraints in Africa, not a single mention of nematodes was made (Gressel 2004), while for the potato crop in the UK alone, it is estimated that the cyst nematodes, *Globodera rostochiensis* and *G. pallida*, account for an estimated ~$70 million per annum or 9% of UK production (DEFRA 2010). Given the current withdrawal from use of inorganic pesticides (UNEP 2000), the primary source of pest and disease management over the past decades, the need to consider nematode pests is more acutely brought into focus.

Although over 4,100 species of plant-parasitic nematodes have been identified (Decraemer and Hunt 2006), new species are continually being described while

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others, previously viewed as benign or non-damaging, are becoming pests as cropping patterns change (Nicol 2002). However, the plant parasitic nematodes of economic importance can be grouped into relatively restricted specialized groups that either cause direct damage to their host or act as virus vectors (Table 2.1). Most affect crops through feeding on or in plant roots, whilst a minority are aerial feeders. In addition to direct feeding and migration damage, nematode feeding facilitates subsequent infestation by secondary pathogens, such as fungi and bacteria (Powell 1971).

On a global scale the distribution of nematode species varies greatly. Some are cosmopolitan, such as certain *Meloidogyne* spp. while others are particularly restricted geographically e.g. *Nacobbus* spp. or are highly host specific, such as *Heterodera carotae* which attacks only carrots. Some crops may have very few nematode pests while others have a particularly wide range of genera and species associated with them, such as sugar cane and rice, leading to difficulties for nematode control strategies. Distribution maps and host range data are available and updated regularly as a useful source for determining nematode damage potential (http://www.cabi.org/dmpd).

Although plant parasitic nematodes are among the most widespread pests, and are frequently one of the most insidious and costly (Webster 1987), data on their economic impact remain less than comprehensive, especially for crops produced in resource poor areas. In the tropical and sub-tropical climates, crop production losses attributable to nematodes were estimated at 14.6% compared with 8.8% in developed countries. Perhaps more importantly, only ~0.2% of the crop value lost to nematodes is used to fund nematological research to address these losses (Sasser and Freckman 1987). One difficulty with assessing nematode impact is that damage resulting from nematode infection is often less obvious than that caused by many other pests or diseases. Losses that result from nematode attack may not necessarily be as a consequence of direct cell death, necrosis or 'diseased' tissue but may derive from other more insidious aspects, such as interference with the root system, reducing their efficiency in terms of access and uptake of nutrients and water; to the unaware, nematode-affected plants present typical drought and nutrient stress symptoms, which are easily and often misdiagnosed. On *Musa* spp. (bananas and plantains) nematode damage affects root efficiency on the one hand, but additionally leads to root necrosis and death, undermining plant anchorage; heavily infected bunch-bearing plants can topple due to poor root anchorage leading to total loss of the unripe fruit (Gowen et al. 2005). Moreover, nematode manifestation over time leads to a gradual decline over seasons with misdiagnosis common. Plants are rarely killed outright, although impressive exceptions of full scale crop devastation can occur; *Ditylenchus angustus,* for instance, which causes Ufra disease on deepwater rice in Asia (Cox and Rahman 1980). More generally, Sikora and Fernández (2005) suggest that vegetable production in tropical and sub-tropical environments cannot be considered without some form of nematode management.

In the USA a survey of 35 States on various crops indicated nematode-derived losses of up to 25% (Koenning et al. 1999). More recently Handoo (1998) estimated global crop losses due to nematode attack in the region of $80 billion, which, given

Table 2.1 World Food Production for major food commodities and main nematode pests of importance

Crop	Total production (million metric tonnes)	Top 3 producers	Production (million metric tonnes)	Main nematode pests (Luc et al. 2005; Evans et al. 1993; McDonald and Nicol 2005; Nicol and Rivoal 2007)
Coarse Grains	**856.2**			
Maize (*Zea mays*)	681.5	United States of America China Mexico	331.2 152.4 23.4	*Meloidogyne* spp., *Pratylenchus* spp., *Heterodera* spp., *Punctodera chalcoensis*, *Paratrichodorus* spp., *Longidorus breviannulatus*
Barley (*Hordeum vulgare*)	92.3	France United States of America Australia	9.5 4.7 5.9	*Heterodera avenae, Meloidogyne* spp., *Anguina tritici, Pratylenchus* spp.
Sorghum (*Sorghum bicolor*)	49.4	United Stated of America Nigeria India	12.6 9.1 7.2	*Belonolaimus longicaudatus, Paratrichodorus* spp., *Pratylenchus* spp., *Criconemella* spp.
Oats (*Avena sativa*)	19.3	Canada Finland United Kingdom	4.7 1.2 0.7	*Heterodera avenae, Meloidogyne* spp., *Ditylenchus dipsaci, Pratylenchus* spp.
Rye (*Secale cereale*)	13.7	Russian Federation Poland Germany	3.9 3.1 2.7	*Anguina tritici, Heterodera avenae, Pratylenchus zeae*
Rice (*Oryza sativa*)	**621.6**	China India Indonesia	187.4 144.5 57.2	*Ditylenchus angustus, Aphelenchoides besseyi, Heterodera* spp., *Meloidogyne* spp., *Hirschmanniella* spp., *Pratylenchus* spp.
Roots & Tubers	**554.5**			
Potatoes (*Solanum tuberosum*)	255.8	China Russian Federation India	64.8 36.8 28.6	*Globodera* spp., *Meloidogyne* spp., *Nacobbus aberrans, Pratylenchus* spp., *Trichodorus* spp.,
Cassava (*Manihot esculenta*)	203.6	Nigeria Thailand Indonesia	43.4 26.9 20.0	*Pratylenchus brachyurus, Rotylenchus reniformis, Helicotylenchus* spp., *Meloidogyne* spp., *Scutellonema bradys*
Sweet potatoes (*Ipomoea batatas*)	95.1	China Uganda Nigeria	75.8 2.6 2.4	*Meloidogyne* spp., *Pratylenchus* spp., *Rotylenchus reniformis, Ditylenchus destructor*

Table 2.1 (continued)

Crop	Total production (million metric tonnes)	Top 3 producers	Production (million metric tonnes)	Main nematode pests (Luc et al. 2005; Evans et al. 1993; McDonald and Nicol 2005; Nicol and Rivoal 2007)
Wheat (*Triticum aestivum*)	**525.8**	China	109.3	*Heterodera spp., Pratylenchus* spp., *Meloidogyne* spp., *Anguina tritici, Ditylenchus dipsaci*
		India	75.8	
		United States of America	55.8	
Oil crops	**291.2**			
Soybeans (*Glycine max*)	217.3	United States of America	72.9	*Meloidogyne* spp., *Heterodera glycines, Rotylenchulus reniformis, Hoplolaimus columbus, Pratylenchus* spp.
		Brazil	57.9	
		Argentina	47.5	
Rapeseed (*Brassica napus*)	48.7	China	10.6	*Heterodera schachtii*
		Canada	9.6	
		India	7.4	
Sunflower seed (*Helianthus* spp.)	25.2	Russian Federation	5.7	*Meloidogyne* spp.
		Ukraine	4.2	
		Argentina	3.5	

the more subtle effects of low infestation levels is probably a vast underestimate. Globally, a wide range of crops are produced, with some grown in specific areas (Table 2.1). Others have a broader geographical plasticity, which can result in a greater range of pests, varying according to region, continent and climate. Moreover, some crops are produced in regions of varying levels of economy, leading to different levels of nematode management, often as a consequence of awareness as well as the availability of options for their management. The degree of damage a nematode causes can also be dependent upon host and age. In addition, prevailing soil, environmental and climatic conditions all influence the threshold population density, above which measurable damage occurs. For example, *Tylenchorhynchus martini* causes damage on sugarcane at populations between 600 and 6,400/plant, whilst on onions just 5 individuals per seedling of *Pratylenchus penetrans* will result in serious damage (www.enclyclopedialive.com).

Nematode attack can also predispose plants to attack by other pathogens either through mechanical damage but also on a genetic basis. For example, Sidhu and Webster (1974) determined the genetic basis of the *Meloidogyne incognita—Fusarium oxysporum lycopersici* disease complex on tomatoes, from sequential inoculations of F_2 progeny and further demonstrated the role of nematodes in disease interactions through the breakdown of resistance to *F. oxysporum lycopersici* in the presence of *M. incognita*.

In addition to the immediate concerns surrounding global food security issues, there is growing concern for pest and disease management under the predicted climate changes and the threat of the emergence of new pests, including nematodes. The Intergovernmental Panel on Climate Change (IPCC) assessments (2007) have concluded that, even if concentrations of all greenhouse gases had been kept constant at the levels present in 2000, a further overall warming of ~0.1°C per decade would be expected, due to the slow response of the oceans. About twice as much warming would be expected if emissions are within the range of scenarios used in IPCC assessments. Resulting changes would include an increase in frequency of heat extremes, heat waves and heavy precipitation; changes in wind, precipitation and temperature patterns; precipitation increases at high latitudes and decreases in most sub-tropical land regions. This would impact on species range shifts; water scarcity and drought risk in some regions of the dry tropics and sub-topics; and coastal damage from floods combined with sea-level rise. For example, *Radopholus similis* occurs only below ~1,400 m altitude in the East African Highlands where it is a principal pest of banana and plantain, a regional key starch staple for over 20 million people. A small raise in temperature would result in *R. similis*, which is cold-sensitive, infecting millions more bananas grown at higher altitudes. In an alternative example the rice root knot nematode, *Meloidogyne graminicola*, can be maintained under damaging levels through good water management. However, with reduced availability of water following climatic changes and/or competition for urban use, reduced quality of water management, or the introduction of water saving mechanisms such as direct wet seeding is favouring the development of high populations of *M. graminicola*, drastically raising its economic significance as a damaging pest (de Waele and Elsen 2007).

Nematodes are excellent bio-indicators for environmental change as once they are present in a habitat and in proximity of hosts conducive to their development, they may rapidly multiply. Indigenous species that have remained in balance may emerge to pest status on agricultural crops with small changes to their habitat, either through changes in cropping practice (crop, cultivars, rotation cycle, etc.) or climate. A good example of this is illustrated by the rapid and alarming emergence of *Meloidogyne minor* in Europe (Karssen 2004). Plant damage symptoms were first observed in The Netherlands on sports turf in 1997 and on potato in 2000 (Karssen 2004). Since then this nematode has been recovered from a range of pasture, amenity turf and potato crops in mainland Europe and the British Isles (PRA 2007). Its varying morphology on different hosts creates confusion with the quarantine root knot species *M. chitwoodi* and *M. fallax*, with both morphological and molecular characterisation essential for accurate diagnosis (C. Fleming, personal communication). Consequently, we can be sure that nematodes will continue to emerge as new or more aggressive pests of crops as farming practices adapt to fashion, as climate change occurs and as cropping systems intensify in response to an increasing global demand for food. In a world of limited means for nematode management, focus on plant parasitic nematodes as a significant affliction of crop production is highly pertinent.

2.2 Quarantine Nematodes of Global Importance

2.2.1 Potato Cyst Nematodes

Cyst forming nematodes, or cyst nematodes, are one of the most specialized and successful plant-parasitic nematode pests of agriculture. These nematodes usually have a very narrow host range. In the case of the potato, the potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida* are able to infest potato crops and some other Solanaceae such as tomatoes and woody nightshade (bitter sweet). These nematodes belong to the family Heteroderidae and originated in South America and were probably introduced to Europe along with potato breeding material around 1850 (Turner and Evans 1998).

The life cycle of cyst nematodes is well adapted towards the host and they can survive in various environments. The cyst, the dead body wall of the female, contains the eggs which hatch in the presence of the host. Root exudates from Solanaceae activate these juveniles and this can cause hatch of up to 80% of the nematodes under suitable environmental conditions. The juveniles enter the roots near the root tip and induce a feeding cell or syncytial "transfer cell" (See Chap. 4). The juveniles become sedentary and feed from the syncytia, until their development is complete after four moults. After the 4th moult the female is round and swollen and protrudes from the root. The males are slender, leave the roots, mate and fertilize the females. After mating the female forms eggs and when the female dies the cuticle tans to

form a protective cyst with 200–500 eggs within. The life cycle is complete and may take up to three months. The cyst (pinhead size) falls off the roots, waiting for the next suitable host plant.

Damage is related to the level of nematode infestation. Infested plants show retarded growth and heavily infested fields normally show badly growing patches, especially under dry conditions, and as a result yield can be decreased by up to 50%. Apart from the yield reduction, the financial benefits of growing potatoes are also reduced by the costs of control measures and reduction of marketable products.

Sampling soil on land prior to planting of seed potatoes is one way of minimizing spread of these nematodes and forms part of the statutory measures for the production of seed potatoes of all countries. However, when the cysts are present at low density they are often overlooked, or below detection level, whilst the population is spread, mainly by machinery. The difference between the status "not known to occur" and "known not to occur" is not always clear.

When PCN is present in the field there are several ways to minimize the population to such an extent that growing potatoes is feasible although complete eradication is not possible due to the persistence of the cysts in the soil. Available management tools include growing resistant potato varieties, using a lengthened crop rotation, applying chemicals, using biological methods such as solarisation or biofumigants or growing catch (trap) crops. The most reliable method in recent years has been the growing of resistant potato varieties.

Potato varieties can differ greatly in the extent to which the nematodes can multiply on them. On a fully susceptible cultivar the nematodes can multiply freely on the roots, stolons and even on the tubers. On resistant cultivars no multiplication can take place and partially resistant cultivars give intermediate multiplication, thus reducing the rate at which the nematode population builds up in the soil. The multiplication of the nematodes depends on the resistance genes present in the potato and on the virulence genes present in the nematodes. When the same resistant potato variety is grown successively, selection for virulent nematodes that can overcome the resistance source may occur. A prime example of this has occurred in the UK since the introduction of commercially viable cultivars carrying the H1 gene. This gene gives complete control against the pathotypes of *G. rostochiensis* present in the UK and wheras *G. rostochiensis* was the prevalent species present in the UK in the past, repeated use of cultivars containing H1 has led to an increase in the prevalence of *G. pallida*, which was previously rarely encountered (Minnis et al. 2002).

Outbreaks of potato cyst nematodes have now occurred in most of the potato growing areas of the world. PCN remains rare in some countries with extensive potato acreages, most notably Australia, Canada, USA, India and, probably, some parts of the former USSR. Most outbreaks involve *G. rostochiensis* or both species. The relative scarcity of commercially acceptable varieties with full resistance to *G. pallida* makes the control of this species much more difficult to achieve. Identification of the species, generally based on morphology, is now being taken over by molecular methods as these techniques are easier to learn and adapt than the more specialised knowledge of taxonomy. The EPPO Diagnostic

protocol for identification of potato cyst nematodes (OEPP/EPPO 2009a) gives an overview of the more recent identification tools and this topic is also covered in Chap. 21.

2.2.2 Endoparasitic and Free-Living Parasitic Nematodes

Endoparasitic and free-living plant-parasitic nematodes present a different challenge to plant health services. Endoparasites spend most of their life inside bulbs, corms, roots or tubers and hence can be unknowingly spread and escape detection, whilst free-living species are found in soil residues that need to be processed to confirm their presence. There is more potential for unlisted species in these groups to be assigned quarantine status because international trade is facilitating the spread of species that were not known to have potential to become economic pests when most international plant health legislation was first implemented; it usually takes several years to collect evidence with a Pest Risk Analysis (PRA) that a species should be listed as quarantine. The challenge to plant health services is to detect and identify not only listed species but also new or unusual species that might pose a threat to agriculture should they be allowed to establish and spread. Nematologists specialising in identification largely rely on morphological characters for identification, but molecular techniques offer potential for a new range of tools to facilitate this. This is especially important when frequently samples only contain a few or immature specimens that cannot be identified by morphological means.

The root-knot nematodes, *Meloidogyne* species, are endoparasitic species widespread throughout the world and usually have a wide range of host plants. *Meloidogyne chitwoodi* was first described from the Pacific Northwest of the USA in 1980 (Golden et al. 1980), but it is not clear if this is its area of origin. It was causing damage to potato tubers and a few years later was also detected in potatoes in The Netherlands, although there is evidence that it may have been present in Europe for some time (OEPP/EPPO 1991). It has a wide host range including other economically important crops such as carrots and other root crops such as salsify. *M. chitwoodi* lowers the market value of such crops as a result of internal necrosis and external galling and yields are reduced. A PRA (Tiilikkala et al. 1995; Braasch et al. 1996) led to the pest being listed as a quarantine organism in the EU in 1998. This meant that plant health service inspectors were alerted to look specifically for symptoms of this pest, particularly on potato tubers, and samples were submitted to quarantine laboratories for detection and identification.

It is important to distinguish *M. chitwoodi* and *M. fallax,* listed species in Europe and elsewhere, from other nematodes, particularly related, unlisted species, that might also be found in potato tubers and roots. In addition, survey work has demanded that large numbers of samples are dealt with quickly so prompt action on control could be taken. This has led to the development of molecular

tools to assist identification, most recently using TaqMan technology. The EPPO standard on *M. chitwoodi* and *M. fallax* gives additional information (OEPP/EPPO 2009b).

The free-living pine wood nematode, *Bursaphelenchus xylophilus,* is associated with pine wilt disease but depends on bark beetles (*Monochamus* spp.) to spread from tree to tree. It is native to North America and is thought to have been carried to Japan at the beginning of the twentieth century on timber exports. In Japan, it is causing massive mortality of native pine trees. In 1999, *B. xylophilus* was found in Europe for the first time, in Portugal. Quarantine nematologists were already researching the identity of this species in order to be able to distinguish it from the many species of *Bursaphelenchus* that inhabit wood. Unfortunately there is a variation in characters between species in the *Bursaphelenchus* group, which makes morphological identification particularly difficult, so biomolecular tools are highly recommended (OEPP/EPPO 2009c).

The majority of plant-parasitic nematodes are free-living species that feed ectoparasitically on roots; this group contains thousands of species, some well known to science but others not. Quarantine is concerned with the detection of any species that may pose an economic risk to agriculture, horticulture and, increasingly, the native biodiversity of recipient countries. This demands specialist skills in nematode identification in order to distinguish such species from native ones. Nematodes in the EU-listed group, *Xiphinema americanum sensu lato*, are listed in many countries because of the ability of some species to transmit viruses. The identification of this group is in a state of flux, but recent work in Europe has led to the development of an EPPO Diagnostic protocol (OEPP/EPPO 2009d). It is hoped that research into molecular tools will provide further tools to facilitate the harmonisation of protocols worldwide.

Control programmes may be aimed at eradication or suppression of intercepted pests, but in the long term the development of certification schemes to produce nematode-free material is important. The development of hosts with resistance to quarantine species is a prolonged and expensive process but may be justified for economically important species. Sustainable methods of control are receiving more attention as chemical controls become scarce and this stimulates research into biological control methods. Combined with knowledge of the biology and life-cycle of the species such research offers the potential to minimise the effect of plant-parasitic nematodes spread in trade.

2.3 Key Nematodes on Food Staples for Food Security in Developing Countries

Cereals constitute the world's most important source of food. Amongst cereals, rice, maize and wheat occupy the most prominent position in terms of production, acreage and source of nutrition, particularly in developing countries (Table 2.1). It has

been estimated that about 70% of the land cultivated for food crops is devoted to cereal crops. The global population is projected to increase steadily to around 9 billion by 2050 and with this demand for the staple cereals of rice, maize and wheat will increase (Dixon et al. 2009). Projections suggest that over this period the demand for maize will grow faster than that for wheat due to the use of maize as animal and poultry feed and the increasing demand for biofuel. The demand for wheat will grow faster than that for rice and is likely to follow closely the growth in global population over this period (FAO 2006; Dixon et al. 2009).

In order to meet the expected food demand, further research is required on how to produce more from less. Research will need to also focus on the less understood and appreciated nematodes which are known to be economically important on all three cereal crops. As described above, there is a void of representative information from developing countries for nematodes on many crops, which has affected recognition by the scientific and policy community with respect to agriculture research. As time advances the challenge to meet food security for developing countries is more acute. As their economic situation improves consumption of meat based products is increasing, resulting in greater demands for cereals for animal feed. It is also predicted that there will be reductions in irrigation water for agriculture as the value of this compared with other industries is challenged. This is further compounded by the climate change scenarios and extremes of drought and floods which have been forecast (IPCC 2007).

2.3.1 Maize

2.3.1.1 Introduction

Maize (*Zea mays*) has the highest production of all three cereal staples (752 Mt). It is grown largely in tropical and subtropical regions with the three largest producers found in North America Asia and Europe (Tables 2.1 and 2.3). Over 60 nematode species have been found associated with maize in different parts of the world. Most of these have been recorded from roots, or soil around maize roots, with information on the biology or pathogenicity of many of these species not readily available. The most important groups of plant parasitic nematodes demonstrated to be important limiting factors in maize production from all over the world include the root knot nematodes, *Meloidogyne* spp., the root lesion nematodes, *Pratylenchus* spp. and the cyst nematodes, *Heterodera* spp. A questionnaire survey to agricultural research institutions in South Africa put *Pratylenchus* species second overall after root knot nematodes in terms of economic importance (Keetch 1989). *Pratylenchus,* along with *Meloidogyne* and *Hoplolaimus* were the most frequently reported genera on maize in the USA. (Koenning et al. 1999). There are also reports of other plant parasites on maize (Table 2.2), but knowledge of their importance and distribution is limited.

Table 2.2 Plant Parasitic Nematodes of Economic Importance. (Adapted from Handoo 1998)

Genus	Common Name	Type	Plant tissue
Primary damage (15)			
Anguina	Seed gall	Migratory endoparasite	Seeds, stems, leaves
Bursaphelenchus	Wilt	Migratory ectoparasite	Seeds, stems, leaves
Criconemella	Ring	Sedentary ectoparasite	Roots
Ditylenchus	Stem and Bulb	Migratory ectoparasite	Stems, leaves
Globodera	Cyst	Sedentary endoparasite	Roots
Helicotylenchus	Spiral	Migratory ecto-, endoparasite	Roots
Heterodera	Cyst	Sedentary endoparasite	Roots
Hirschmanniella	Root	Migratory endoparasite	Roots, tubers
Hoplolaimus	Lance	Migratory ectoparasite	Roots
Meloidogyne	Root-knot	Sedentary endoparasite	Roots
Pratylenchus	Lesion	Migratory ecto-, endoparasite	Roots
Radopholus	Burrowing	Migratory endoparasite	Roots, tubers
Rotylenchulus	Reniform	Sedentary semi-endoparasite	Roots
Scutellonema	Spiral	Migratory ecto-, endoparasite	Roots, tubers
Tylenchulus	Citrus	Sedentary semi-endoparasite	Roots
Virus vectors (3)			
Xiphinema	Dagger	Migratory ectoparasites	Roots
Longidorus	Needle	Migratory ectoparasites	Roots
Trichodorus	Stubby	Migratory ectoparasites	Roots

Table 2.3 World and regional production of key staple cereals. (Sourced from the FAO website, www.faostat.fao.org May 2010. Figures represent five years average 2004–2008)

Continent	Total production (million metric tonnes)		
	Maize	Rice	Wheat
World	752.05	645.18	633.14
Africa	49.57	21.23	21.68
Asia	209.61	584.92	273.52
Australia and New Zealand	0.56	0.41	18.78
Central America	25.28	1.12	3.26
Europe	84.01	3.46	211.49
North America	307.54	9.55	82.77
South America	74.86	23.43	21.64

2.3.1.2 Economic Importance and Distribution

Several species of root knot nematodes including *M. incognita* and *M. javanica* have been detected at damaging levels in almost all maize growing regions of the world (McDonald and Nicol 2005). *M. africana* and *M. arenaria* have been

recorded on maize in India and in Pakistan, and *M. arenaria* has also been reported by several authors from the USA (McDonald and Nicol 2005). Above ground symptoms include stunting, leaf chlorosis and patchy growth. Root galls may be small or large, terminal or sub-terminal or in some cases totally absent. For this reason maize has often mistakenly been considered a poor host or even a non-host for root knot nematodes (McDonald and Nicol 2005). Although root knot nematodes occur frequently in maize fields, information on economic losses is lacking and requires further study. However, indirect observations when nematicides are applied in root knot nematode infected soils, suggest that these nematodes are economically important (Riekert 1996; Riekert and Henshaw 1998). It is important that growers are alert to the possibility of root knot nematode infestation of maize, particularly in low input production conditions (Table 2.2).

Many more reports exist for the lesion nematodes which have a cosmopolitan occurrence in maize fields with several of these species reported to be associated with poor growth and yield reduction (McDonald and Nicol 2005). The most commonly occuring species include *Pratylenchus brachyurus*, *P. zeae* and *P. penetrans* in subtropical and tropical regions but many other species have been noted. Lesion nematodes have wide host ranges which can affect the selection of the crop used to control nematodes in rotations. In addition, the presence of weed hosts in a field can strongly influence lesion nematode (and indeed root knot nematode) densities. The nematode species, population density and environmental conditions affect symptom expression of root lesion nematodes and hence the aboveground symptoms are not specific. Nematode damage is associated with lesions to the root as a result of the destruction of cortical parenchyma and epidermis, which may cause sloughing-off of the tissue and severe necrosis (McDonald and Nicol 2005). In addition, severe root pruning as well as proliferation of lateral roots may occur. More definitive yield loss studies have been conducted for root lesion nematodes on maize. Smolik and Evenson (1987) found direct relationships between *P. hexincisus* and *P. scribneri* and maize yield loss, with *P. hexincisus* more damaging to dry land maize than *P. scribneri* to irrigated maize. In Nigeria, *P. brachyurus* has been reported to be responsible for a 28.5% yield reduction, with this reduction being correlated with a 50% increase in nematode density (Egunjobi 1974). Indirect evidence has been obtained with nematicides where detected yield increases suggest that lesion nematodes are important limiting factors in maize cultivation with yield increases of 33–128% in South Africa, 10–54% in the USA and a two-fold increase in Brazil reported (reviewed by McDonald and Nicol 2005).

The third group of nematodes of importance for maize are the cyst nematodes. Although more than nine species of cyst nematodes have been recorded as being associated with maize in subtropical and tropical countries, only three (*Heterodera zeae, H. avenae* and *Punctodera chalcoensis*) are considered economically important (Luc 1986). *Heterodera cajani*, *H. delvii*, *H. gambiensis*, *H. graminis*, *H. oryzae* and *H. sorghi* have been recorded sporadically, but their role as parasites of maize remains uncertain (reviewed by McDonald and Nicol 2005). As with other nematodes the above ground symptoms are relatively non-specific. *H. zeae* infested plants exhibit poor growth and are stunted and pale green in colour (Koshy and

Swarup 1971). *Punctodera chalcoensis* is limited in distribution to Mexico where it has been given the local name of Mexican corn cyst nematode and is considered of extreme importance. The symptoms of *P.chalcoensis* are the same as those for *H. zeae*, with the chlorotic leaves also exhibiting pale colour stripes (McDonald and Nicol 2005).

Pathogenicity of the cyst nematode *H. zeae*, has been demonstrated on maize but data on economic damage to the crop is lacking (Koenning et al. 1999). Plant growth reductions are directly correlated with initial nematode population density and maize growth and yield are suppressed by 13–73% in the presence of *H. zeae* with this damage more profound under hot and dry conditions (reviewed by McDonald and Nicol 2005). It is also important to note the wide host range of *H. zeae* and the need to select crop rotations carefully in order to minimize population increase (Ismail 2009). There is limited published information about the importance of *H. avenae* on maize but this could be very important in wheat & maize production systems as this is a well acknowledged pathogen on wheat. Unlike the other two cyst species the host range of *P. chalcoensis* is highly restricted with only two plants, *Z. mays* and *Z. mexicana* (Teosinte), considered hosts (Stone et al. 1976). Damage by *P. chalcoensis* can be severe and is dependent on cultivar susceptibility, nematode density and adequate soil moisture levels in the later part of the growing season. Under glasshouse conditions, Sosa-Moss and Gonzales (1973) obtained a reduction of about 60% in yield in heavily infested soils. Although yield loss in the field is considered to be high, experimental data is lacking.

2.3.1.3 Major Methods of Control

Although there are limited groups working on controlling nematodes in maize production systems there have been many local reports identifying resistance against not only the 3 main groups described above but also many other genera and species (reviewed by McDonald and Nicol 2005). Some of these are in commercial varieties but most are within breeding lines and land races.

Cultural practices such as crop rotation, planting time, application of organic amendments and biological control have been tested and in many cases were demonstrated to be effective in reducing various nematode populations but these are genera and species specific (McDonald and Nicol 2005). For example in Mexico early sowing dates and adequate fertilization reduces damage caused to maize by *P. chalcoensis* (Sosa-Moss and Gonzalez 1973; Sosa-Moss 1987). In terms of rotation maize has been suggested as a good 'rotation crop' that can help reduce populations of some nematodes but little is actually known about the effects of rotation on root knot population densities in a maize crop. Crop rotations or sequences where maize was involved demonstrate the dangers of ineffective crop choices in rotations due to the susceptibility of maize to various species of nematodes (McDonald and Nicol 2005). Biological control has been investigated against several species of plant parasitic nematodes of importance to maize (McDonald and Nicol 2005), with many of these offering potential. However, none of

these biocontrol agents can be used economically at the present time in extensive cereal crops.

2.3.2 Rice

2.3.2.1 Introduction

Rice (*Oryza sativa* L) belongs to the Graminae family and as a cereal grain is the most important food resource for a large part of the world's population. As with maize, rice is grown in most tropical and subtropical regions. Rice is grown in 114 countries throughout the world in Asia, Africa, Central and South America and Northern Australia. Asia accounts for 90% of world rice production with China, India, Indonesia, Bangladesh and Vietnam the five highest rice-producing countries (FAO 2008; Tables 2.1 and 2.3).

There are different systems of cultivating rice that have evolved to suit specific environments, including irrigated, rainfed lowland, deepwater, tidal wetlands and upland. Irrigated rice is the dominant growing system in the world. Plant parasitic nematodes have adapted to each cultivation system with both foliar and root parasites being important.

2.3.2.2 Economic Importance and Distribution

Parasitic nematodes on rice can be divided into two groups: the foliar parasites and the root parasites. The foliar parasites include two well known species. *Aphelenchoides besseyi* Christie, 1942 the causal agent of 'white tip disease' in rice is widely spread throughout the rice growing areas of the world. *A. besseyi* is seed borne in rice and hence the infested seed acts as a primary source of infestation. The characteristic symptom caused by *A. besseyi* in rice is a whitening of the leaf tip that turns to necrosis, there is also distortion of the flag leaf that encloses the panicle. Infected plants are stunted, have reduced vigour and their panicles contain small and distorted grain (Ou 1985). Two other species, *A. nechaleos* and *A. paranechaleos* have been reported from rice stems in Sierra Leone and Vietnam. Both have a similar morphology and biology to *A. besseyi* (Hooper and Ibrahim 1994; Ibrahim et al. 1994). However, both species show marked differences in their pathogenicity to rice and the inconsistencies observed in the damage caused by *A. besseyi* to rice might be due to incorrect nematode identification (Ibrahim et al. 1994). The economic damage threshold density was determined to be 300 live nematodes per 100 seeds (cited by Bridge et al. 2005). Yield losses of up to 60% due to *A. besseyi* have been widely reported from various infested regions (Bridge et al. 2005). Such high losses are probably rare as the disease is now easily controlled by seed treatment and resistant cultivars (Whitehead 1998).

The other major foliar nematode pest in rice is *Ditylenchus angustus,* which is mostly limited to the south and southeast of Asia where deepwater and lowland cultivation systems are used (Table 2.3). Although it has been suggested that *D. angustus* cannot survive severe desiccation (Ibrahim and Perry 1993; Sein 1977), a recent survey indicated the recovery of live nematodes from dried seeds three months after harvest, mainly located in the germ portion (Prasad and Varaprasad 2002). This finding emphasises the importance of seed (and nematode) exchanges between different regions as a source of infection. *D. angustus* causes 'ufra' disease in rice and the most prominent symptoms of infected plants are chlorosis, twisted leaves and swollen lower nodes. Infected panicles are usually crinkled with empty, shrivelled glumes, especially at their bases, the panicle head and flag leaf are twisted and distorted (Bridge et al. 2005). The annual yield loss due to *D. angustus* has been estimated at 4% in Bangladesh (Catling et al. 1979) and 10–30% in Assam and West Bengal, India (Rao et al. 1986). However, the importance of *D. angustus* has reduced as the area sown to deep-water rice has declined (Plowright et al. 2002).

Nematode parasites of rice root systems include migratory endoparasites, sedentary endoparasites (cyst and root knot nematodes) and ectoparasites. Many species of *Hirschmanniella,* known as the rice root nematodes, have been reported from the majority of rice growing regions which are irrigated, lowland and deep water rice. Seven species are reported to damage rice, with the most commonly reported species being *H. oryzae*. The symptoms caused by *Hirschmanniella* species are not specific and include poor growth, leaf chlorosis, reduction of tillering and yield. Nematodes invade roots and migrate through the cortical tissues causing cell necrosis and cavities with infected roots, which turn brown and rot. The crop losses due to *Hirschmanniella* spp. have been estimated at 25% (Hollis and Keoboonrueng 1984).

Four cyst nematodes species are known to affect rice; *Heterodera oryzicola*, *H. elachista*, *H. oryzae* and *H. sacchari* on upland, lowland and flooded rice in Japan, India, West Africa and Iran. As with *Hirschmaniella* the infected plants show reduced growth, chlorosis, fewer tillers and a reduction in root growth. The crop losses due to cyst nematodes have been documented by Bridge et al. (2005), *H. elachista* decreases yield by 7–19% and even higher yield losses have been attributed to *H. oryzicola* in India. In Côte d'Ivoire increasing *H. sacchari* populations are expanding rapidly with intensive wet season rice cropping, leading to yield losses of up to 50% (Bridge et al. 2005).

Although several species of root knot nematodes have been reported on rice, the key species is *M. graminicola* which is mainly distributed in South and South East Asia. This species has also been reported from the USA and some parts of South America (Table 2.2). *M. graminicola* causes damage to upland, lowland, deepwater and irrigated rice. The most prominent symptoms of *M. graminicola* on the root system are swollen and hooked root tips which are characteristic for *M. graminicola* and *M. oryzae* (Bridge et al. 2005). Typical above ground symptoms include stunting and chlorosis leading to reduced tillers and yield. The effects of *M. graminicola* on grain yield in upland rice has been estimated at 2.6% for every 1,000 nematodes

present around young seedlings (Rao and Biswas 1973). The tolerance limit of seedlings has been determined as less than one second stage juvenile/cm3 of soil in flooded rice (Plowright and Bridge 1990).

2.3.2.3 Major Methods of Control

Using clean seed is the most effective means of preventing yield loss due to *A. besseyi*. Fumigating seed with methyl bromide, gamma radiation, seed dressing with effective nematicides, hot water or chemical seed treatment are the most useful methods for reducing crop losses. There are many reports on seed treatment of rice by hot water but the most effective control requires pre-soaking seed in cold water for 18–24 h followed by immersion in water at 51–53°C for 15 min (Bridge et al. 2005). Using resistant or tolerant cultivars and low seed planting densities are other control measures for reducing the crop losses due to *A. besseyi*.

There are many different methods to control 'ufra' disease in rice caused by *D. angustus*. These include destroying or removing the infested stubble or straw, burying crop residues, growing non-host crops such as jute or mustard in rotation with rice, using early maturing cultivars, removing weed hosts and wild rice to prevent the build up of nematodes for the next crop and improving water management to prevent spread of the nematodes. There are good sources of resistance to *D. angustus* and advanced generation breeding material is available for development of resistant cultivars suitable for lowland and deep-water environments (Plowright et al. 2002).

Management of *Hirschmanniella* spp. comprises various methods including soil solarisation, fallow, weed control, use of resistant cultivars and rotation with non host plants (Bridge et al. 2005). For *H. sacchari,* there are good sources of resistance in the African rice *O. glaberrima*. Flooding of soil reduces the population density of this nematode. Soil solarization and use of resistant cultivars are the main methods used for control of root knot nematodes in rice cultivation (Whitehead 1998).

2.3.3 Wheat

2.3.3.1 Introduction

Wheat (*T. aestivum* and *T. durum*) is the third largest cereal staple with production of 633Mt each year. The three largest producers are China, India and the USA (Table 2.1). It is considered the key crop of importance for food security in the regions of West Asia and North Africa. This section will focus on the primary nematodes of global economic importance on wheat: Cereal Cyst Nematode (*Heterodera*) and Root Lesion Nematode (*Pratylenchus*). Other important nematode genera including Root Knot (*Meloidogyne*), Stem (*Ditylenchus*) and Seed Gall (*Anguina*) will not be described here. However, further information on all these nematodes can

be found in the reviews of Kort (1972), Griffin (1984), Sikora (1988), Swarup and Sosa-Moss (1990), Rivoal and Cook (1993), Nicol (2002) and McDonald and Nicol (2005), Nicol and Rivoal (2007) and Riley et al. (2009).

2.3.3.2 Economic Importance and Distribution

The most globally recognized and economically important nematode on wheat is the Cereal Cyst Nematode (CCN). The CCN complex is represented by a group of twelve valid and several undescribed species. Three main species, *Heterodera avenae*, *H. filipjevi* and *H. latipons,* are thought to be the most economically important. One of the complexities of the CCN is the presence of pathotypes, making the use of genetic control challenging. The above ground symptoms caused by CCN occur early in the season as pale green patches, which may vary in size from 1 to more than 100 m^2, with the lower leaves of the plant being yellow and in which plants generally have few tillers. The symptoms can easily be confused with nitrogen deficiency or poor soils and the root damage caused by CCN exacerbates the effect of any other abiotic stress, e.g. water or nutrient stress. The below ground symptoms may vary depending on the host. Wheat attacked by *H. avenae* shows increased root production such that roots have a 'bushy-knotted' appearance, usually with several females visible at each root. The cysts are glistening white-grey initially and dark brown when mature. Root symptoms are recognisable within one to two months after sowing in Mediterranean environments and often later in more or less temperate climates (Tables 2.1 and 2.3).

As reviewed by Nicol and Rivoal (2007), *H. avenae* is the most widely distributed and damaging species on cereals cultivated in more or less temperate regions. *H. avenae* has been detected in many countries, including Australia, Canada, South Africa, Japan and most European countries, as well as India, China and several countries within North Africa and Western Asia, including Morocco, Tunisia, Libya and Pakistan, Iran, Turkey, Algeria, Saudi Arabia and Israel. *Heterodera latipons* is essentially only Mediterranean in distribution, being found in Syria, Cyprus, Turkey, Iran, Italy and Libya. However, it is also known to occur in Northern Europe, and Bulgaria. Another species with an increasingly wide distribution is *H. filipjevi*, formerly known as Gotland strain of *H. avenae*, which appears to be found in more continental climates such as Russia, Tadzhikistan, Sweden, Norway, Turkey, Iran, India, Pacific North West USA and Greece. A relatively new report also decribes this species from Himachal Pradesh in India (SP Bishnoi, pers. com.). There are also several other species of *Heterodera* reported on wheat but these are not considered to be of major economic importance (Nicol and Rivoal 2007).

In terms of economic importance the review of Nicol and Rivoal (2007) provides a long list of published yield loss studies. Interpretation of the damage threshold between specific nematological studies should be done with extreme caution, as very few studies are truly comparable, with inherent differences in sampling protocol, extraction procedure and nematode quantification. Several authors have

reported that water stress is one of the key environmental conditions that can exacerbate damage caused. Yield losses due to *H. avenae* on wheat are reported to be 15–20% in Pakistan, 40–92% in Saudi Arabia, 23–50% in Australia, 24% in the Pacific North West of the USA and 26–96% in Tunisia. It has been calculated that *H. avenae* is responsible for annual yield losses of 3 million pounds sterling in Europe and 72 million Australian dollars in Australia (Wallace 1965; Brown 1981). The losses in Australia are now greatly reduced due to their control with resistant and tolerant cultivars.

Little is known about the economic importance of the species *H. latipons*. As reviewed by Nicol and Rivoal (2007) there is one report from Cyprus on barley that indicated 50% yield loss. Recent studies in Iran in field microplots reported yield losses of up to 55% on winter wheat (Hajihasani et al. in press). Because the cysts of *H. avenae* and *H. latipons* are similar in size and shape it is possible that damage caused by this recently described nematode species has previously been attributed to *H. avenae* (Kort 1972). Similarly, *H. filipjevi* is most likely an economically important nematode on cereals due to its widespread distribution but has previously been misidentified as *H. avenae* in the former USSR and Sweden. In Turkey significant yield losses (average 42%) in several rainfed winter wheat locations have been reported. In Iran under microplot field trials yield losses of 48% were found on common winter wheat over two wheat seasons (Hajihasani et al. 2010). Natural field trials conducted over several seasons have clearly indicated greater losses under drought conditions. Given increased recognition and incidence, *H. filipjevi* and *H. latipons* are now being identified as a constraint to cereal production (Philis 1988; Oztürk et al. 2000; Scholz 2001).

The second group of nematodes considered economically important on wheat production systems is the migratory endoparasitic genus *Pratylenchus*. At least eight species infect small grains and the four most important species *P. thornei*, *P. neglectus*, *P. penetrans* and *P. crenatus* are polyphagous and have a worldwide distribution (Rivoal and Cook 1993). *P. thornei* is the most extensively studied of these species on wheat and has been found in Syria, Yugoslavia, Mexico, Australia, Canada, Israel, Iran, Morocco, Tunisia, Turkey, Pakistan, India, Algeria, Italy and the USA (Nicol and Rivoal 2007). *P. neglectus* has been reported in Australia, North America, Europe, Iran and Turkey while the other species have only been reported from local studies.

In terms of economic importance *P. thornei,* causes yield losses in wheat from 38 to 85% in Australia, 12–37% in Mexico, 70% in Israel and has also recently been reported to cause losses on wheat in the Pacific North West of the USA. *P. neglectus* and *P. penetrans* appear to be less widespread and damaging on cereals compared to *P. thornei.* In Southern Australia, losses in wheat caused by *P. neglectus* ranged from 16 to 23% while at sites infested with both *P. thornei* and *P. neglectus* yield losses of 56–74% were reported. In North America and Germany, *P. neglectus* has been shown to be a weak pathogen to cereals. Sikora (1988) identified *P. neglectus* and *P. penetrans* in addition to *P. thornei* on wheat and barley in Northern Africa, and all these as well as *P. zeae* in Western Asia. Further work is necessary to determine the significance of these species in these regions.

The life cycle of *Pratylenchus* is variable between species and environment and ranges from 45 to 65 days (Agrios 1988). Eggs are laid in the soil or inside plant roots. The nematode invades the tissues of the plant root, migrating and feeding as it moves. Feeding and migration of *Pratylenchus* causes destruction of roots, resulting in characteristic dark brown or black lesions on the root surface, hence their name 'lesion' nematodes. Secondary attack by fungi frequently occurs in these lesions. Aboveground symptoms of *Pratylenchus* on cereals, like other cereal root nematodes are non-specific, with infected plants appearing stunted and unthrifty, sometimes with reduced numbers of tillers and yellowed lower leaves.

2.3.3.3 Major Methods of Control

The major method of control for both Cereal Cyst Nematode and Root Lesion Nematode is the use of non-hosts in rotation with wheat and also genetic host resistance. Since CCN is host specific, rotation with non-cereals offers good potential to reduce nematode density. However, as *Pratylenchus* is largely polyphagous, rotational options for these nematodes are far fewer (Nicol and Rivoal 2007). Successful use of rotation requires a thorough understanding of the effectiveness of a particular rotation and, in the case of *Pratylenchus,* a clear understanding of the host status of the other plants used in the rotation.

Resistance is one of the most cost effective and straightforward methods for nematode control. Many sources have been reported and reviewed for CCN and *Pratylenchus* (Nicol and Rivoal 2007). Genetic resistance is favoured with the addition of genetic tolerance (the ability of the plant to yield despite attack by the nematode). The progress in understanding and locating resistance sources in cereals is more advanced for cyst (*H. avenae*) than lesion (*Pratylenchus* spp.) nematodes, in part due to the specific host-parasite relationship that cyst nematodes form with their hosts (Cook and Evans 1987). In contrast, the relationship of migratory lesion nematodes with their hosts is less specialized and therefore less likely to follow a gene for gene model. The identified sources of resistance to *H. avenae* have been found predominantly in wild relatives of wheat in the *Aegilops* genus and have already been introgressed into hexaploid wheat backgrounds for breeding purposes. Unlike cereal cyst nematode, no commercially available sources of cereal resistance are available to *P. thornei*, although sources of tolerance have been used by cereal farmers in Northern Australia for several years (Thompson and Haak 1997).

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Chapter 3
Phylogeny and Evolution of Nematodes

Wim Bert, Gerrit Karssen and Johannes Helder

3.1 Introduction

A phylogenetic framework is needed to underpin meaningful comparisons across taxa and to generate hypotheses on the evolutionary origins of important properties and processes. In this chapter we will outline the backbone of nematode phylogeny and focus on the phylogeny and evolution of plant-parasitic Tylenchomorpha. We will conclude with some recent insights into the relationships within and between two highly successful representatives of the Tylenchomorpha; the genera *Pratylenchus* and *Meloidogyne*.

3.2 Backbone of Nematode Phylogeny

The phylum Nematoda can be seen as a success story. Nematodes are speciose and are present in huge numbers in virtually all marine, freshwater and terrestrial environments. Analysis of large EST data sets recently reconfirmed the placement of the phylum Nematoda within the superphylum Ecdysozoa (Dunn et al. 2008), a major animal clade proposed by Aguinaldo et al. (1997) that unites all moulting animals.

Blaxter et al. (1998) (53 taxa) and Aleshin et al. (1998) (19 taxa) were among the first to exploit the potential of small subunit ribosomal DNA (SSU rDNA) sequence data to resolve phylogenetic relationships among nematodes. Holterman et al. (2006) presented a subdivision of the phylum Nematoda into 12 clades based on a series of mostly well-supported bifurcations in the backbone of the tree (339 taxa) (Fig. 3.1). The under-representation of marine nematodes in these phylogenetic overviews was, to some extent, lifted by SSU rDNA-based papers from Meldal et al. (2007) and Holterman et al. (2008a). Recently, a phylogenetic tree based on

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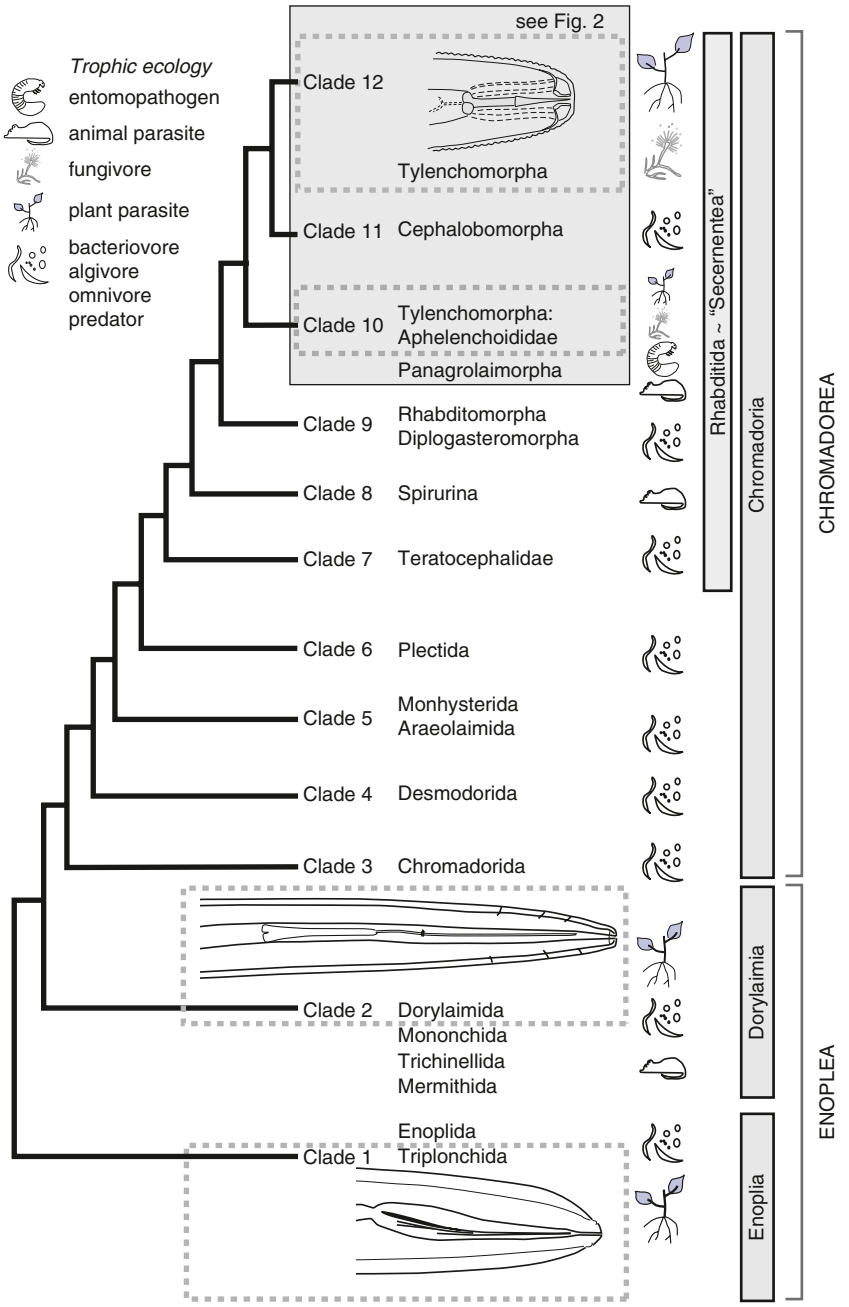

Fig. 3.1 Schematic overview of the evolution of the phylum Nematoda derived from SSU rDNA sequence data (based on Holterman et al. 2006). Major lineages of plant-parasites are indicated by *dotted boxes* (Tylenchomorpha, Dorylaimida and Triplonchida). It is noted that the infraorder Tylenchomorpha is possible a polyphyletic group; it includes the members of Clade 12 and the Aphelenchoididae, a family within Clade 10

1,215 small subunit ribosomal DNA sequences covering a wide range of nematode taxa was presented by van Megen et al. (2009). The overall topology of this phylogenetic tree resembles that of Holterman et al. (2006). However, the support values for the backbone tend to be lower. The deep subdivision of the phylum Nematoda should be regarded as a 'work in progress', and a multi loci approach will be required for a more definitive framework.

The extensiveness of convergent evolution is one of the most striking phenomena observed in the phylogenetic tree of nematodes—it is hard to find a morphological, ecological or biological characteristic that has not arisen at least twice during nematode evolution. Convergent evolution appears to be an important additional explanation for the seemingly persistent volatility of nematode systematics. One of the peculiarities of the phylum Nematoda is the multitude of times that (animal- or plant-) parasitic lifestyles have arisen. Understanding the phylogenetic history of the acquisition of particular phenotypes associated with successful parasitism permits fuller appreciation of the evolutionary constraints experienced by organisms adapting to new hosts. Plant-parasitism has evolved independently in each of three major clades in the phylum Nematoda (Fig. 3.1). The plant-parasitic Tylenchomorpha, Dorylaimida, Triplonchida have acquired their ability to parasitize plants independently. The infraorder Tylenchomorpha comprises manifestly the economically most relevant plant-parasites and within this chapter we will mainly focus on this particular group.

Apart from the scientific merits of studying of the phylogeny of the Nematoda, the underlying molecular framework can be used for DNA barcode-based nematode detection and community analysis. It is (in most cases) possible to define species-specific sequence signatures and to design simple and cheap PCR primers that allow real-time PCR-based detection and quantification of pathogenic nematodes in complex DNA backgrounds. At the same time, the SSU rDNA alignment has been used to design many family-specific PCR primers (see for example Holterman et al. 2008b) and quantitative DNA barcode-based nematode community analyses under field conditions are currently being tested.

3.3 Phylogeny of Tylenchomorpha

3.3.1 Overview

The Tylenchomorpha, the most intensively investigated infraorder within the Tylenchina, comprises the largest and most economically important group of plant-parasitic nematodes. Although there are examples of nematodes that exploit all plant organs including flowers and seeds, they mostly attack roots. The evolution of plant-parasitic Tylenchomorpha is of particular interest because associations range from transitory grazing by root-hair feeders to the highly complex host-pathogen interactions of gall-inducing nematodes and their hosts. Non plant-parasitic Tylenchomorpha feed on fungi, algae, lichens, mosses, insects, mites, leeches or frogs

(Siddiqi 2000). However, the evolution of this diversity of complex feeding traits is not yet fully understood. In recent years LSU and SSU rDNA sequences have been used to infer relationships among Tylenchomorpha (Subbotin et al. 2006; Bert et al. 2008; Holterman et al. 2009). A multiple gene approach derived from an EST mining strategy has been used to characterise the relationships between the plant-parasitic genera *Meloidogyne, Heterodera* and *Globodera* (Scholl and Bird 2005). Other phylogenetic studies within Tylenchomorpha, mainly based on rDNA sequences or the internal transcribed spacers, have been restricted to individual (super) families or genera. Recent studies include analyses of Heteroderidae (Subbotin et al. 2001), Anguinidae (Subbotin et al. 2004), Criconematoidea (Subbotin et al. 2005), Hoplolaimidae (Subbotin et al. 2007), *Meloidogyne* (Tandingan De Ley et al. 2002; Tenente et al. 2003; Tigano et al. 2005) and *Pratylenchus* (Subbotin et al. 2008).

In Fig. 3.2, a schematic phylogenetic framework of the Tylenchomorpha based on Bert et al. (2008) and Holterman et al. (2009) is shown. The families Hoplolaimididae (including Heteroderinae), Pratylenchidae (except *Pratylenchoides*) and Meloidogynidae, which comprises the economically most important plant-parasites, plus the genera *Tylenchorhynchus* and *Macrotrophurus* form a well supported clade. A robust sister relationship between *Meloidogyne* (root-knot nematodes) and representatives of the migratory endoparasitic Pratylenchidae (*Pratylenchus*, *Zygotylenchus* and *Hirschmanniella*) can be observed. The Hoplolaimidae, which include the Heteroderinae, Hoplolaiminae, Rotylenchoidinae and Rotylenchulinae according to the classification of de Ley and Blaxter (2002), appear as a monophyletic group. Remarkably, the migratory endoparasitic *Radopholus*, a notorious pest in banana and citrus, has a well-supported sister relationship with the Hoplolaimidae. Thus, the cyst-forming (Heteroderinae) and root-knot nematodes (*Meloidogyne*) are likely to have arisen independently and the migratory endoparasitic Pratylenchidae appears to be polyphyletic.

Between the earliest divergences within the Tylenchomorpha (Aphelenchoidea) and the top parts of the tree, a number of branching points remain unresolved. Although we cannot define the relationship between the suborder Criconematoidea and other Tylenchomorpha, its members clearly constitute a separate and well supported clade. Also, the tylenchid nematodes with supposedly ancestral morphological characters, including Tylenchidae and Sphaerularioidea, do not have an established phylogenetic relationship. Nevertheless, the tylenchid nematodes—those Tylenchomorpha that are characterized by a tylenchid stylet (Tylenchomorpha without Aphelenchoididae)—appear to be clearly monophyletic. The Aphelenchoidea or "aphelenchs" comprising the mainly fungal-feeding Aphelenchidae and Aphelenchoididae are appointed as polyphyletic in all molecular analyses to date. However, the morphology based hypothesis of their monophyly could not be significantly rejected based on statistical analysis of molecular data (Bert et al. 2008). Several studies have confirmed the sister relationship of the predominantly plant-parasitic Tylenchomorpha (without Aphelenchoidea) with the bacteriovorous Cephalobidae (Blaxter et al. 1998). However, this was not unequivocally supported by van Megen et al. (2009).

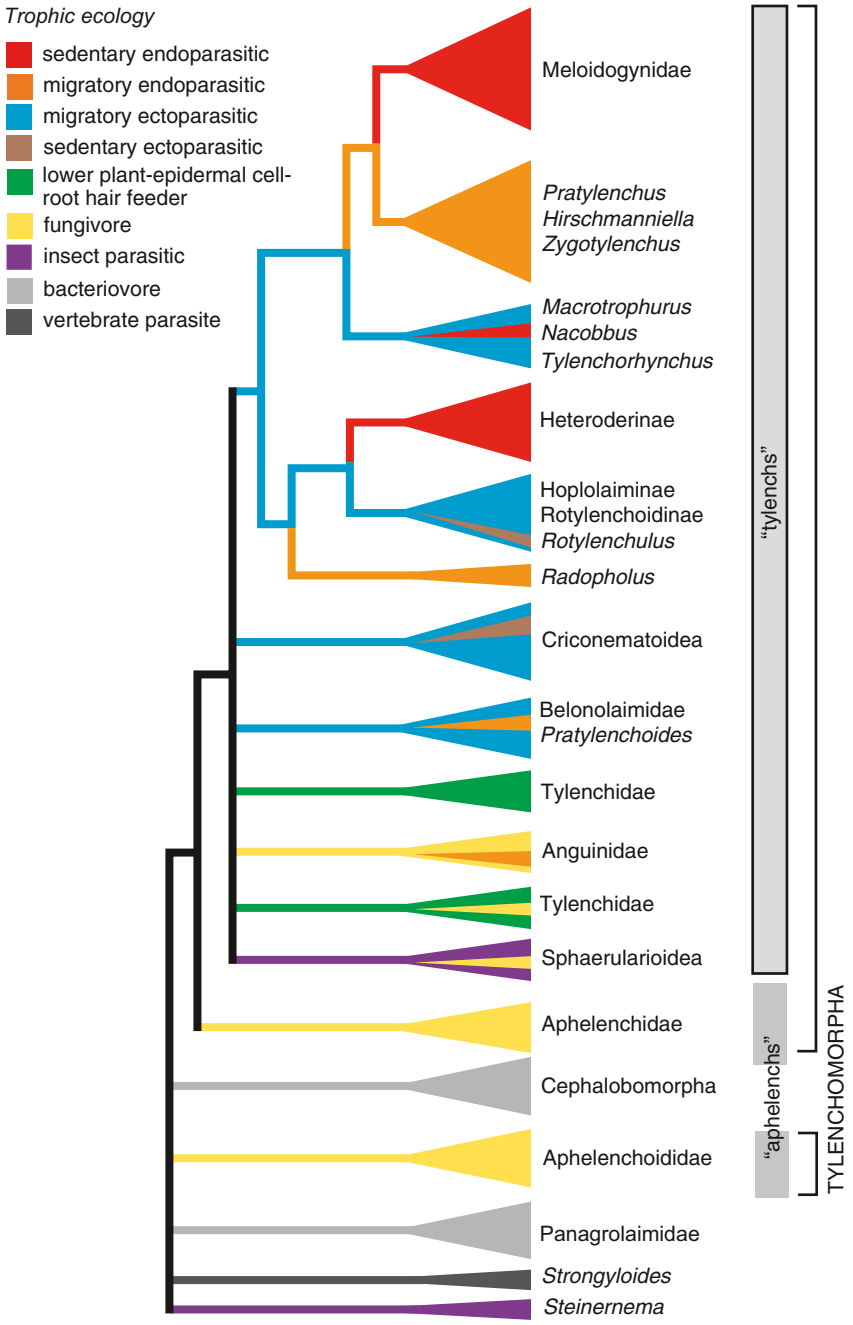

Fig. 3.2 The evolution of feeding among Tylenchomorpha super posed on a ribosomal DNA-based phylogenetic backbone. (Based on Bert et al. 2008; Holterman et al. 2009)

3.3.2 Evolution of Plant-Parasitism in Tylenchomorpha

The development of plant-parasitism in the Tylenchomorpha has traditionally been seen as a gradual evolution from fungal feeding to facultative parasitism of root hairs and epidermal cells into more complex forms of plant-parasitism, culminating in the development of sedentary endoparasitism (Luc et al. 1987). To investigate this hypothesis, ancestral feeding types were reconstructed among Tylenchomorpha using three different methods: unordered parsimony, parsimony using a stepmatrix, and likelihood (Bert et al. 2008; Holterman et al. 2009). Here we present only a schematic overview of these results (Fig. 3.2).

The feeding type analysis supported the classical hypothesis of the gradual evolution of simple forms of plant-parasitism, such as root hair and epidermal feeding and ectoparasitism towards more complex forms of endoparasitism. However, the ancestral feeding state of the Tylenchomorpha (without Aphelenchoididae) is still ambiguous; depending on the reconstruction method this is fungal-feeding, lower plant/root-hair feeding or bacteriovore feeding. Conclusions on this hypothesis await a better resolution in the basal part of the Tylenchomorpha tree and additional information on the feeding behavior of the basal Tylenchomorpha such as the Tylenchidae and Anguinidae. Within the tylenchid nematodes, migratory ectoparasitic feeding is ancestral for all major clades of nematodes that exclusively parasitize higher plants (Fig. 3.2). Migratory endoparasitism has evolved independently several times within Anguinidae and four times, always from migratory ectoparasitic ancestors, in the polyphyletic Pratylenchidae. Sedentary endoparasitism has also evolved three times independently; *Nacobbus* (false root-knot nematodes) and the cyst-forming nematodes most likely evolved from migratory ectoparasitic nematodes, while root-knot nematodes appear to have evolved from migratory endoparasitic nematodes. The number of independent developments is higher than expected mainly due to the polyphyly of the Pratylenchidae. Although the development of plant-parasitism is usually gradual, endoparasitism seems to have developed directly from several simple forms of plant-parasitism including ectoparasites (giving rise to *Heteroderinae,* Pratylenchidae, *Pratylenchoides* and *Tylenchulus*) and epidermal and root hair feeders (from which the Anguinidae evolved).

Although the parasitic biology of certain plant-parasitic Tylenchomorpha is now relatively well documented, we should achieve a broader understanding of the evolution of the mode and direction of plant-parasitism from intermediate groups, including economically less important plant-parasites. Within Clade 12 the family Aphelenchidae (fungivores) appears in a sister position to all “tylenchs” (Holterman et al. 2006; Bert et al. 2008). However, it should be noted that in a more recent, maximum likelihood analysis this positioning could not be robustly confirmed (van Megen et al. 2009). For the plant-parasitic Tylenchomorpha families positioned at the base of clade 12 such as the Tylenchidae, the Psilenchidae and the Belonolaimidae (the ‘economically less important plant-parasites’), the number of (ribosomal)

DNA sequences available is very limited. It is expected that molecular characterisation of specimens from these families and improved insight in their feeding habits will give us more insight in the transition from fungivorous lifestyles, via facultative plant-parasitism towards obligatory plant-parasites.

3.4 Tylenchomorpha—Top End Plant-Parasites

3.4.1 Root-Knot Nematodes—Towards the Origin(s) of This Highly Successful Genus

3.4.1.1 Introduction

Root-knot nematodes—members of the genus *Meloidogyne*—can be said without too much exaggeration to represent the ultimate success story among plant-parasitic nematodes. The more distal members of this genus have host ranges encompassing more than a thousand plant species, including numerous major crops, and are spread all over the temperate and (sub)tropical regions of the world. On the basis of SSU rDNA sequences Tandingan de Ley et al. (2002) defined three distal clades within the genus *Meloidogyne*. Clade I comprises a number of species often referred to as the tropical root-knot nematodes (e.g. *M. incognita, M. javanica* and *M. arenaria*), the most well known representative of Clade II is *M. hapla*, while Clade III harbors a number of species with EPPO A2 quarantine status such as *M. chitwoodi* and *M. fallax* (EPPO = European and Mediterranean Plant Protection Organisation) (Fig. 3.3). Analysis of a larger molecular data set and more *Meloidogyne* species resulted in a confirmation of these distal clades. Ribosomal DNA sequence data point at *Meloidogyne artiellia*—a polyphagous root-knot nematode typically inducing very small galls (host plant range includes both mono and dicotyledons)—as being sister to the distal *Meloidogyne* clades I, II and III (III being basal to Clades I and II) (Holterman et al. 2009).

One of the elements that could explain the success of this genus is the ability of these endoparasites to migrate between plant cells. Cell wall-degrading enzymes and expansins are likely to be widespread throughout *Meloidogyne,* although they have so far only been characterized for some of the more derived members of this genus. These enzymes and proteins make it possible for these plant-parasites to move towards the most nutritional part of the plant root, namely the vascular cylinder (stele). Migrating between cells, rather than through cells as is the case for cyst nematodes, may help the pre-parasitic second-stage juveniles to outpace and/or avoid the host plant defense response. The question why lesion (*Pratylenchus* spp.) and cyst nematodes (*Globodera* spp. and *Heterodera* spp.) do not migrate intercellularly while infective life stages are producing and secreting various cell wall degrading enzymes and proteins remains to be answered.

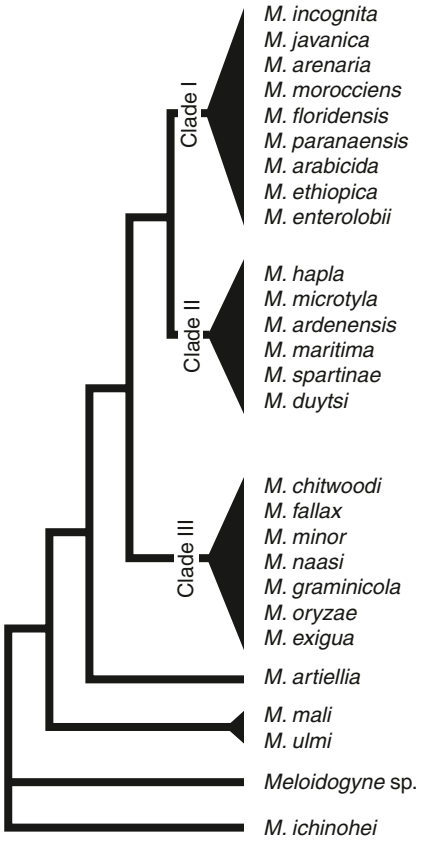

Fig. 3.3 Schematic overview of the phylogeny of the Meloidogynidae derived from SSU rDNA sequence data. (Based on Holterman et al. 2009)

3.4.1.2 Modes of Reproduction Among Root-Knot Nematodes

Another fascinating aspect of root-knot nematode biology is the wide range of modes of reproduction present with the group. *Meloidogyne incognita* multiplies by mitotic parthenogenesis. Mothers that produce genetically identical daughters could be considered as a dead end road in evolutionary terms—such a genetic constitution would make it impossible for an individual to cope with changing environmental conditions. Nevertheless, in terms of distribution and host plant range this is probably the most successful member of the genus *Meloidogyne*. *M. incognita* is one of the members of Clade I (as defined above). It is notable that Clade I is not homogenous in its mode of replication. Although mitotic parthenogenesis dominates this clade, one of its members (*M. floridensis*) multiplies by meiotic parthenogenesis. The same holds true for Clades II and III; although meiotic parthenogenesis is most frequently found there are a number of exceptions. Most remarkable is the case of *Meloidogyne hapla* for which two races are defined. Race A reproduces by facultative meiotic parthenogenesis, whereas the polyploid Race B multiplies by mitotic

parthenogenesis. It would be useful to examine more basal root-knot nematode species when seeking further insight into the extreme diversity in modes of reproduction of root-knot nematodes.

According to McCarter (2008), most root-knot species are "sexually reproducing diploids, a status thought to reflect the root-knot nematode ancestral state". A careful examination of *M. artiellia,* which is sister to the distal *Meloidogyne* Clades I—III, suggests this could be correct; the frequent occurrence of males suggests this species could multiply sexually. However, based on the analysis of rDNA sequence data at least four *Meloidogyne* species are placed in more basal positions, *viz. M. ulmi, M. mali,* an undescribed *Meloidogyne* species found on Sansevieria, and *M. ichinohei* (Holterman et al. 2009).

Meloidogyne ichinohei is an unusual root-knot nematode species both in its morphology and in its extreme host specificity. Unlike most RKN females, adult females of *M. ichinohei* show a prominent posterior protuberance, a laterally located neck and a perineal pattern showing remarkably faint and broken striae (Araki 1992). Previously the first two characters were considered to be rather atypical, and they had been the characteristics defining the genus *Hypsoperine*, a genus synonymized with *Meloidogyne* by Jepson (1987). *M. ichinohei* males are very rare according to the original description by Araki (1992). This observation has been confirmed by the Dutch National Plant Protection Organisation (NPPO); not a single male was found in *M. ichinohei* population C2312 (G. Karssen, unpublished data).

3.4.1.3 On the Former Genus *Hypsoperine*

Araki remarked that *M. ichinohei* would have been a typical member of the genus *Hypsoperine* if it still existed (Araki 1992). Sledge and Golden (1964) suggested that the genus *Hypsoperine* actually occupied a position in between *Heterodera* and *Meloidogyne* (though showing closer resemblance to the root-knot nematodes). This information prompted us to check a number of other *Hypsoperine*-like root-knot nematodes: (1) *Hypsoperine acronea* (now *Meloidogyne acronea*); (2) *H. graminis* (*M. graminis*); (3) *H. mersa* (*M. mersa*); (4) *H. ottersoni* (*M. ottersoni*); (5) *H. propora* (*M. propora*); (6) *H. spartinae* (*M. spartinae*) and (7) *H. megriensis* (*M. megriensis*). As very little is published about the members of this genus, information about host range and mode of reproduction (if known) is summarized here.

1. *Meloidogyne acronea* is a root-knot nematode-like parasite found on roots of sorghum (*Sorghum vulgare*) by Coetzee in 1956. The author reported that this particular isolate could also parasitize and multiply on beans and tomatoes. In the original description morphological characteristics of both males and females are given. *M. acronea* seems to have a very restricted geographical distribution as it has been reported from southern Africa only.
2. *Meloidogyne graminis* was found in 1964 by Sledge and Golden as a parasite on St. Augustine grass (*Stenotaphrum secundatum*). When few males were present, *M. graminis* reproduces by meiotic parthenogenesis. However, in the presence of

males reproduction was by amphimixis (Triantaphyllou 1973). Based on cytological data, Triantaphyllou (1973) suggested a close phylogenetic relationship of *M. graminis, M. ottersoni, M. graminicola* (Clade III) and *M. naasi* (Clade III).

3. *Meloidogyne mersa* is an unusual root-knot nematode found in a marine habitat (mangrove swamps in Brunei) parasitizing roots of the mangrove apple (*Sonneratia alba*). The original paper describes both males (100 paratypes) and females (50 paratypes) and males are not rare (Siddiqi and Booth 1991). The morphology of *M. mersa* resembles that of *M. spartinae,* another root-knot nematode species living in marine or brackish habitats.
4. Thorne (1969) described *Meloidogyne ottersoni* as a parasite of canary grass (*Phalaris arundinacea*). As for *M. graminis, M. ottersoni* was reproduced by meiotic parthenogenesis when males are absent but in the presence of males reproduction was by amphimixis (Triantaphyllou 1973). Based on cytological data, Triantaphyllou suggested for a close phylogenetic relationship of *M. ottersoni, M. graminis, M. graminicola* (Clade III) and *M. naasi* (Clade III).
5. *Meloidogyne propora* was first described by Spaull (1977) as a parasite on the roots of *Cyperus obtusiflorus* and *Solanum nigrum* from an atoll in the Indian Ocean (Aldabra). Males were reported to be common in soil around infested roots and near ovigerous females.
6. *Meloidogyne spartinae* is a root root-knot nematode producing galls on cordgrass (*Spartina alterniflora*) which is found in intertidal wetlands, especially estuarine salt marshes. On the basis of SSU rDNA sequences Plantard et al. (2007) clearly showed this species to be related to *M. maritima*, and as such residing in *Meloidogyne* Clade II.
7. *Meloidogyne megriensis*—rather incomplete description published in Russian by Poghossian (1971)—was collected from roots of *Mentha longifolia*. Only known from the type locality, an orchard in Megri, and a nearby village named Vagravar, Armenia Karssen and van Hoenselaar 1998.

This overview, based on various data, indicates that members of the former genus *Hypsoperine* are scattered all over the *Meloidogyne* phylogenetic tree, and—other than the observation that their morphology is different from what is considered to be typical for root-knot nematodes—they have little in common.

Another *Meloidogyne* species sharing a number of morphological characteristics with *M. ichinohei* (but never classified as member of the genus *Hypsoperine*) is *Meloidogyne kralli. M. kralli* was first found in 1968 by Dr. E. Krall on the roots of sedge (*Carex acuta*) (Jepson 1983). Adult females share a number of characteristics with *M. ichinohei*; they have a distinct neck (contrary to *M. ichinohei*, the neck was not set off) and the vulva was positioned on a posterior protuberance. Although males have been described for *M. kralli* (Jepson 1983), they are seldom found under natural conditions (Karssen 2002). *Meloidogyne kralli* seems to have a relatively small host range as it is found only on four *Carex* species, namely *C. acuta, C. vesicaria*, *C. riparia* and *C. pseudocyperus,* and on *Scirpus sylvaticus*. Under laboratory conditions *M. kralli* populations were reported to reproduce well on barley (Jepson 1983). It is noted that both *M. ichinohei* and *M. kralli* exclusively parasitize a small number of monocotyledons. However, SSU rDNA sequence analysis revealed that

M. ichinohei and *M. kralli* are rather distinct, with *M. kralli* robustly positioned in *Meloidogyne* Clade III close to *M. oryzae* (Helder and Karssen, unpublished results).

Based on the information discussed above, *M. acronea* would be worthwhile investigating in more detail, as it resembles *M. ichinohei*. Cytological and scattered molecular data place most of the former members of the genus *Hypsoperine* in the distal *Meloidogyne* Clades II or III, except for *M. acronea* and *M. propora*. It is concluded that molecular data from particularly these two species could possibly contribute to a better understanding of the origin of the genus *Meloidogyne*. However, both species seem to have a restricted distribution (southern Africa, and an atoll in the Indian Ocean) and this complicates the collection of DNA from these highly interesting root-knot nematode species. Currently, *M. ichinohei*—together with a so far non-described *Meloidogyne* species from Kenya found on Sansevieria (Holterman et al. 2009)—seem to reside genuinely at the very base of the *Meloidogyne* tree of life. Both species have a very limited host range, and for both species males are very rare. In our eyes, this justifies a hypothesis stating that (at least facultative) asexual reproduction is the root-knot nematode ancestral state.

3.4.2 Lesion Nematodes—A Stenomorphic Genus Closely Related to Root-Knot Nematodes

Lesion nematodes—members of the genus *Pratylenchus*—can be recognized easily. Lesion nematodes are relatively small (usually around 500 µm), have a short and stout stylet (11–22 µm) with strong basal knobs, a low lip region with a well-developed sclerotized framework, and glands forming a rather short lobe which ventrally overlaps the intestine (see e.g. Castillo and Vovlas 2007). However, recognizing individual species is difficult as the number of diagnostic characters at this particular taxonomic level is small. The identification of lesion nematodes is further complicated by intra-specific variation in some of these characters. *Pratylenchus* identification to species level is usually done on the basis of the morphology of adult females. They have more informative characters than males, and—at least equally important—for a number of species males are extremely rare or even non-existent. Despite these difficulties (or possibly as a consequence of them), this genus comprises approximately 70 nominal species (Castillo and Vovlas 2007).

Using the labial region as a distinguishing character, Corbett and Clark (1983) distinguished three groups of *Pratylenchus* species. Group I includes (among others) *P. brachyurus*, *P. coffeae, P. crenatus, P. loofi,* and *P. zeae*; group 2 includes *P. neglectus* and *P. thornei*; and *P. penetrans*, *P. pratensis, P. scribneri* and *P. vulnus* belong to group 3. Combined analysis of D2–D3 of 28S (= LSU rDNA) and partial 18S (= SSU rDNA) alignments revealed a subdivision of the genus *Pratylenchus* into six groups (Subbotin et al. 2008). Members of group 1 *sensu* Corbett and Clarke (1983) are scattered over major clades a, b and d. Group 2 (Corbett and Clarke 1983) corresponds to group c (Subbotin et al. 2008), and Group 3 (Corbett and Clarke 1983) members all reside in group a according to Subbotin et al.

(2008) except for *P. scribneri* which resides in clade b. No representatives of the genus *Meloidogyne* were included in this analysis. Holterman et al. (2009) presented a phylogenetic tree based on a full length SSU rDNA data set including nine *Pratylenchus* species. However, poor backbone support in this particular part of the tree did not allow for the identification of a *Pratylenchus* (or at least Pratylenchidae) candidate that could be a likely living representative close to the common ancestor of all root-knot nematodes.

3.4.3 Phylogenetic Relationship Between the Genera Meloidogyne and Pratylenchus

On the basis of shared morphological characteristics of the labial region and pharyngeal structures Ryss (1988) and Geraert (1997) proposed a common ancestry between the genera *Pratylenchus* and *Meloidogyne.* Analysis of SSU and LSU rDNA sequences from a considerable range of lesion and root-knot nematodes supports a close phylogenetic relationship between the two genera. Subbotin et al. (2006) found "evidence for a *Pratylenchus*, *Hirschmanniella* and *Meloidogyne* clade" (based on D2-D3 sequence data). Two years later Bert et al. (2008) concluded "… root-knot nematodes are most closely related to *Pratylenchus spp.* and appear to have evolved from *migratory endoparasitic* nematodes" (based on SSU rDNA data). In 2009 Holterman et al. concluded "our data suggest that root-knot nematodes have evolved from an ancestral member of the genus *Pratylenchus*, but it remains unclear which species is closest to this branching point" (based on more extensive SSU rDNA data). All in all the following can be concluded:

1. Ribosomal DNA sequences suggest that the genus *Pratylenchus* is paraphyletic as all *Meloidogyne* species are nested in it.
2. If this (1.) is correct, the ultimate consequence would be the abolishment of the genus *Pratylenchus* (*Meloidogyne* has priority following the rules of the International Commission on Zoological Nomenclature). This could be undesirable for numerous practical reasons, but in scientific research and especially in our thinking about root-knot nematode evolution this could be useful.
3. The discussion about ancient (a)sexuality as the ancestral state of root-knot nematodes should be replaced by a discussion about the ancestral mode of reproduction of a clade encompassing (at least) all members of the genera *Pratylenchus* and *Meloidogyne*.

3.5 Concluding Remarks

The steep increase in the amount of molecular data over the last decade has allowed for the establishment of more and more robust and versatile phylogenetic frameworks for the phylum Nematoda (Blaxter et al. 1998; Holterman et al. 2006, Van

Megen et al. 2009). It is noted that current frameworks are based on a single cistron (SSU and/or LSU ribosomal DNA sequences), and marine taxa (including numerous plant-parasites) are strongly underrepresented. Current data suggest at least three independent lineages of plant-parasites. This number will probably increase over time; analysis of a 5′ region of LSU rDNA pointed at multiple plant-parasite lineages among the Dorylaimida (Holterman et al. 2008b).

The infraorder Tylenchomorpha (equivalent to Clade 12 (Fig. 3.1) with the addition of the family Aphelenchoididae) comprises the largest group of plant-parasitic nematodes. Analysis of rDNA sequences resulted in a good resolution in the more distal parts of this clade only. Basal Tylenchomorpha—mostly plant-parasites with virtually no economic importance—are currently underrepresented, and this might co-explain why the relationships within this group are poorly resolved (Subbotin et al. 2006; Bert et al. 2008; Holterman et al. 2009). Additional sequencing of basal Tylenchomorpha probably will improve our understanding of the evolution of the more advanced (and successful) members of this infraorder. The relationships between and within distal taxa in Clade 12 including cyst, lesion and root-knot nematodes are far better resolved. Within this chapter the relatedness of the two latter genera—*Pratylenchus* and *Meloidogyne*—was discussed in more detail, and current data suggest that root-knot nematode are in fact nested in (and derived from) the *Pratylenchus* branch.

At this moment, several, so-called next generation DNA sequencing technologies are becoming widely available. This will make it possible to generate sequence data from hundreds if not thousands of genes from individual nematodes at a reasonable cost price. Given that we still have nematode taxonomists around to ensure the link between classical systematics and multigene phylogenetics, we foresee that within a few years from now the notoriously volatile nematode systematics will be turned into a single, more or less robust and widely accepted framework.

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Chapter 4
Cyst Nematodes and Syncytia

Miroslaw Sobczak and Wladyslaw Golinowski

Abbreviations

DAI	day after inoculation
ISC	initial syncytial cell
J2	second stage juvenile
J3	third stage juvenile
J4	fourth stage juvenile
TEM	transmission electron microscopy

4.1 Introduction

Sedentary root parasitic nematodes from the genera *Heterodera* and *Globodera* (collectively called 'cyst nematodes') are obligatory biotrophs. They withdraw nutrients only from a feeding site (syncytium) induced in plant roots and composed of specifically modified plant cells. They are amphimictic species and thus development of a new generation of the parasite begins only after mating and egg fertilisation. The first stage juvenile develops inside the egg-shell and moults to a second stage juvenile (J2) before hatching (See Chap. 1) (Raski 1950). In some species, the unhatched J2 may enter a period of diapause that can last for several years before it becomes stimulated by internal or external triggers to hatch (Jones et al. 1998). Eggs of cyst nematodes are contained within a protective cyst formed by the body of the female, which dies after completing oviposition (Turner and Stone 1984). Females of *Heterodera* deposit some of their eggs outside the cyst in a gelatinous egg-sac (Raski 1950). When the diapause of J2s is terminated, invasive vermiform J2s emerge from the eggs and seek host roots. The nature of the hatching signal and

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mechanisms of its perception, as well as the mechanisms and signals attracting J2s to the host root are not fully resolved (Bird et al. 2008; see also Chap. 1). Inside the plant root the J2 induces development of a syncytial feeding site (Golinowski et al. 1996; Jones and Northcote 1972). The syncytium is the only source of nutrients for the nematode throughout its life. The infective J2 is sexually undifferentiated, with sex determined by epigenetic factors such as environmental conditions and the amount and quality of food (Betka et al. 1991; Grundler et al. 1991; Mugniéry and Bossis 1985; Mugniéry and Fayet 1981, 1984). The first differences in the organisation of the genital primordium indicating the sex of the juvenile appear at the end of the J2 stage shortly before the moult to the J3 (Wyss 1992). When the development of the sedentary J2 is finished, it retracts its stylet and moults. During this moult no food is withdrawn from the syncytium and the nematode stylet is substantially rebuilt (Wyss 1992; Wyss and Zunke 1986). After the moult the stylet is inserted again and the J3 begins to withdraw food from the syncytium. It was calculated that a J3 female consumes three times more food than a male J3 (Müller et al. 1982). This food uptake period lasts for several days and the J3 becomes swollen and oblong. The J3 developmental stage ends with a moult, after which the developmental pathways of male and female juveniles diverge. The male J3 undergoes two subsequent moults (into J4 and then adult male) inside the cuticle of the J3 (Raski 1950; Wyss 1992). The J4 male and adult male revert to the vermiform body shape. These stages do not take up food from the syncytium and use energy reserves stored as lipid droplets in their bodies. Adult males leave the root and seek females. In contrast, the female remains sedentary after the moult to J4, continues to withdraw food and the shape of her body becomes spherical. After another moult the adult female is produced and she continues to feed from the syncytium. After mating the female starts to produce fertilised eggs (Raski 1950). The syncytium remains functional for as long as the attached nematode continues to feed on it (Golinowski et al. 1996; Müller et al. 1982; Sobczak et al. 1997). Müller et al. (1982) calculated that during development the total food consumption of a female was 29 times greater than that of a male. Kerstan (1969) calculated that the mean volume of a syncytium associated with a male is approximately 0.002 mm^3 while the volume of that associated with a female is about 0.026 mm^3. Degradation of the syncytium after feeding has stopped provides an indication of the intimacy of the relationship between sedentary parasitic nematodes and their host. On the one hand the size and efficiency of the syncytium determines the sex of the attached juvenile, while on the other hand the syncytium degrades when the stimulation from the parasite is missing.

4.2 Root Invasion and Selection of the Initial Syncytial Cell

After locating a suitable host, the J2 uses a combination of physical and chemical means to penetrate the root. Thrusts of the stylet are used along with a set of endogenous cell wall degrading and modifying proteins to weaken cell walls as the

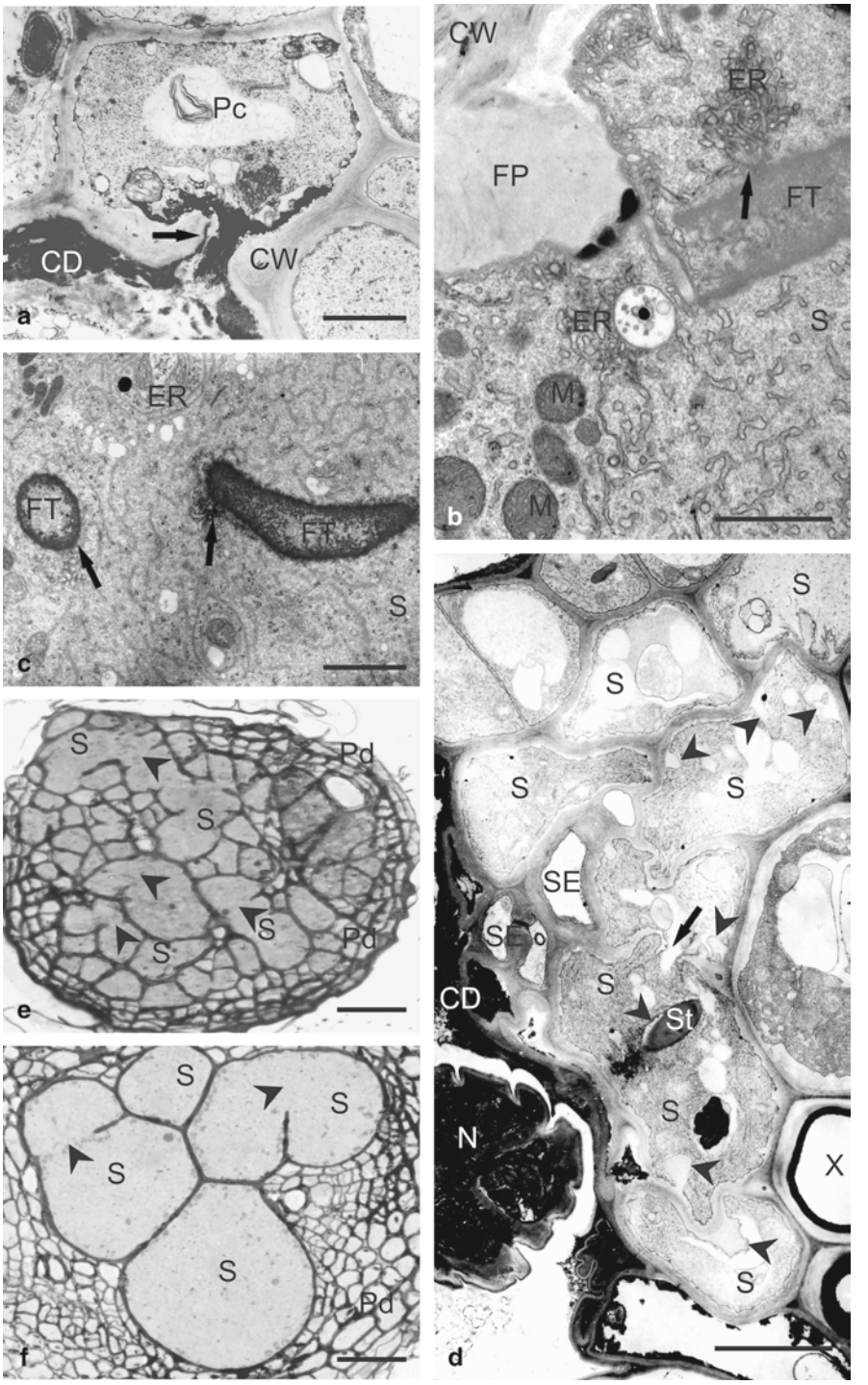
nematode enters the host cells and migrates intracellularly (see Chap. 12). Once migration is complete, the nematode chooses a cell that will become the initial syncytial cell (ISC) (Sobczak et al. 1999; Wyss 1992; Wyss and Zunke 1986).

During migration in the outer root tissues the juvenile behaves very destructively. Stylet thrusts are quick and numerous and head movements are very rapid and vigorous. This behaviour changes and becomes more delicate and exploratory when the J2 approaches the inner cortex layers (in the case of *Globodera* sp.) or the vascular cylinder (in the case of *Heterodera* sp.). Here, stylet thrusts are less numerous and are more gentle (Wyss 1992). The J2 now gives the impression of tasting and probing the cells along its migration pathway (Fig. 4.1a). As the cell is encountered the J2 inserts its stylet very carefully and waits for the cell response. If the preliminary response or cell reaction is unsatisfactory for the juvenile, *e.g.* if the protoplast collapses (Wyss 1992) or if the stylet becomes covered with a thick layer of callose-like material (Golinowski et al. 1997; Sobczak et al. 1999), the stylet is retracted. The J2 then probes another cell next to that previously examined (Golinowski et al. 1997) or the probed cell is destroyed with several stylet thrusts and the juvenile migrates through this cell to another cell (Wyss 1992). This behaviour is repeated until a suitable cell that does not respond adversely to J2 probing is found. This cell becomes the ISC. At this time the J2 stops moving and it enters the preparation phase with the stylet inserted into the cell wall of the ISC. The J2 now becomes sedentary and is never again able to leave the root even if the selected ISC is destroyed (Wyss 1992; Wyss and Zunke 1986).

Observations of *H. schachtii* infecting *A. thaliana* roots suggested that juveniles which become females usually cause little damage during migration and selection of the ISC. These juveniles also tended to select procambial cells as their ISC (Golinowski et al. 1996). When the J2 fails to induce a syncytium in the procambium the extent of destruction is more pronounced and a syncytium is induced in pericyclic cells (Sobczak and Golinowski 2008; Sobczak et al. 1997).

The process of syncytium induction and the subsequent feeding process can be subdivided into several phases. After selection of an ISC, the juvenile of *H. schachtii* enters a preparation phase which lasts for approximately 7 h. During this time no secretions emanate from the stylet orifice and the metacorpal bulb is motionless. The activity of the oesophageal glands also changes: the number of granules in the ampullae and extensions of both subventral glands decreases whereas that in the dorsal gland increases (Wyss 1992; Wyss and Zunke 1986).

When the preparation phase is complete the stylet is withdrawn and reinserted. This time secretions from the nematode oesophageal gland cells are injected into the cytoplasm of the ISC and the metacorpal bulb begins to pump (Wyss 1992). The tip of the re-inserted stylet is covered by a layer of callose-like material except for its orifice where a small puncture in the plasmalemma is formed (Hussey et al. 1992; Sobczak et al. 1999). Nematode secretions are released directly into the cytoplasm of the ISC through this puncture. They appear as a strongly osmiophilic region attached to the stylet tip and orifice when viewed using transmission electron microscopy (TEM) (Golinowski et al. 1997; Sobczak et al. 1999). It is not known whether this osmiophilic region is composed solely of nematode secretions or if it is ISC cytoplasm that is changed as a result of contact with nematode secretions.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It is also not known whether this osmiophilic region represents all the nematode secretions introduced into the plant or whether it is only a subset of them, with other components dispersed in the plant cytoplasm leaving no visible traces. A feeding tube (see below) is not formed at this early stage (Sobczak et al. 1999). After this injection of secretions the juvenile begins to withdraw food from the selected cell. Cyst nematodes withdraw food in cycles lasting for few hours and consisting of three distinctive stages. During the first stage, lasting for 1–2 h, the stylet is inserted into the syncytium and the nematode withdraws food. In the second stage the stylet is withdrawn and reinserted and in the third stage nematode gland secretions are injected into the syncytium where they form a feeding tube (Wyss 1992; Wyss and Zunke 1986). Feeding tubes have been identified in a wide range of plant-parasitic nematodes including migratory ectoparasites such as *Trichodorus similis* (Wyss et al. 1979) and sedentary endoparasites including *Meloidogyne* sp. (Berg et al. 2008; Hussey and Mims 1991), *Rotylenchulus reniformis* (Rebois 1980) and cyst nematodes (Rumpenhorst 1984; Sobczak et al. 1999). Their nature and origin are not fully understood. It was suggested that they are formed from solidified nematode secretions because of structural similarities between their core to proteinaceous deposits present in the nematode oesophageal glands (Hussey and Mims 1991; Rebois 1980). However, no definitive studies linking a nematode secreted protein to the structures observed in plants have been published. It is also possible that feeding tubes are formed as a result of an interaction between nematode secretions and the syncytial cytoplasm (Berg et al. 2008; Rumpenhorst 1984; Sobczak and Golinowski 2008; Sobczak et al. 1999). Whatever their origin, feeding tubes have one sealed end within the cytoplasm of the host with the second end attached to the nematode stylet tip (Fig. 4.1b, c). They are uniformly thick and straight or slightly curved with a diameter of between 1.3 and 1.8 µm (Rumpenhorst 1984) and a length of about 10–20 µm (Fig. 4.1b, c). The feeding tubes formed by *R. reniformis* are an exception as they are spirally coiled (Rebois 1980). When observed in TEM, feeding tubes are composed of an electron dense core (Fig. 4.1b, c) which has a more or less organised structure and an electron translucent lumen that opens directly to the stylet orifice (Rebois 1980). The core of the feeding tube has been shown to be rich in sulphur and nitrogen, suggesting that it may be composed of sulphur-rich proteins (Berg et al. 2008). The outside surface of the feeding tube core is connected to the

Fig. 4.1 Development of syncytium (S) induced by *H. schachtii* in *A. thaliana* root. **a** Perforation (*arrow*) made with the stylet of migrating J2 in the cell wall (CW) of procambial cell (Pc) (migration stage). **b** Feeding tube (FT) present next to the feeding plug (FP) formed in syncytial cell wall (CW) (14 DAI). *Arrow* points to junction of feeding tube with endoplasmic reticulum (ER) (Courtesy of S Siddique). **c** Feeding tubes (FT) in syncytial cytoplasm (10 DAI). *Arrows* point to junctions of feeding tube with endoplasmic reticulum (ER). **d** Ultrastructure of young syncytium (2 DAI). *Arrow* points to cell wall opening and *arrowheads* indicate depositions of callose-like material at cell wall and around nematode stylet (St). **e** Anatomy of syncytium associated with J3 male (10 DAI). *Arrowheads* indicate cell wall openings (Courtesy of S Siddique). **f** Anatomy of syncytium associated with J3 female (10 DAI). *Arrowheads* indicate cell wall openings (Courtesy of S Siddique). *Abbreviations*: CD, cell debris; M, mitochondrion; Pd, peridermis-like tissue; SE, sieve tube; X, xylem vessel. *Scale bars*: (**a**), (**b**) and (**c**) 1 µm; (**d**) 2 µm; (**e**) and (**f**) 20 µm

cisternae of the endoplasmic reticulum (Berg et al. 2008; Hussey and Mims 1991; Sobczak et al. 1999; Fig. 4.1b, c).

It is not known exactly what parasitic nematodes 'eat'. It is generally accepted that they ingest fluid food and that feeding tubes function as molecular sieves protecting the stylet orifice and channel from occlusion caused by syncytial membranes and small organelles. It has been calculated that the feeding tube of *H. schachtii* juveniles permits withdrawal of dextrans of diameter smaller than 8.8 nm (Böckenhoff and Grundler 1994). Interestingly, GFP of a molecular weight of 32 kDa was ingested from syncytia by juveniles of *G. rostochiensis* (Goverse et al. 1998), but another form of GFP with a molecular weight of 28 kDa was not withdrawn by juveniles of *H. schachtii* (Urwin et al. 1997). The feeding tube is detached from the stylet tip during the stylet withdrawal that occurs during the second stage of nematode feeding (Wyss 1992; Wyss and Zunke 1986; Fig. 4.1b). The detached feeding tube becomes filled with syncytial cytoplasm and is degraded (Sobczak et al. 1999). In addition to the feeding tubes, a feeding plug is formed during the feeding process of cyst nematodes. This is described in more detail in Chap. 19.

4.3 Syncytium Development

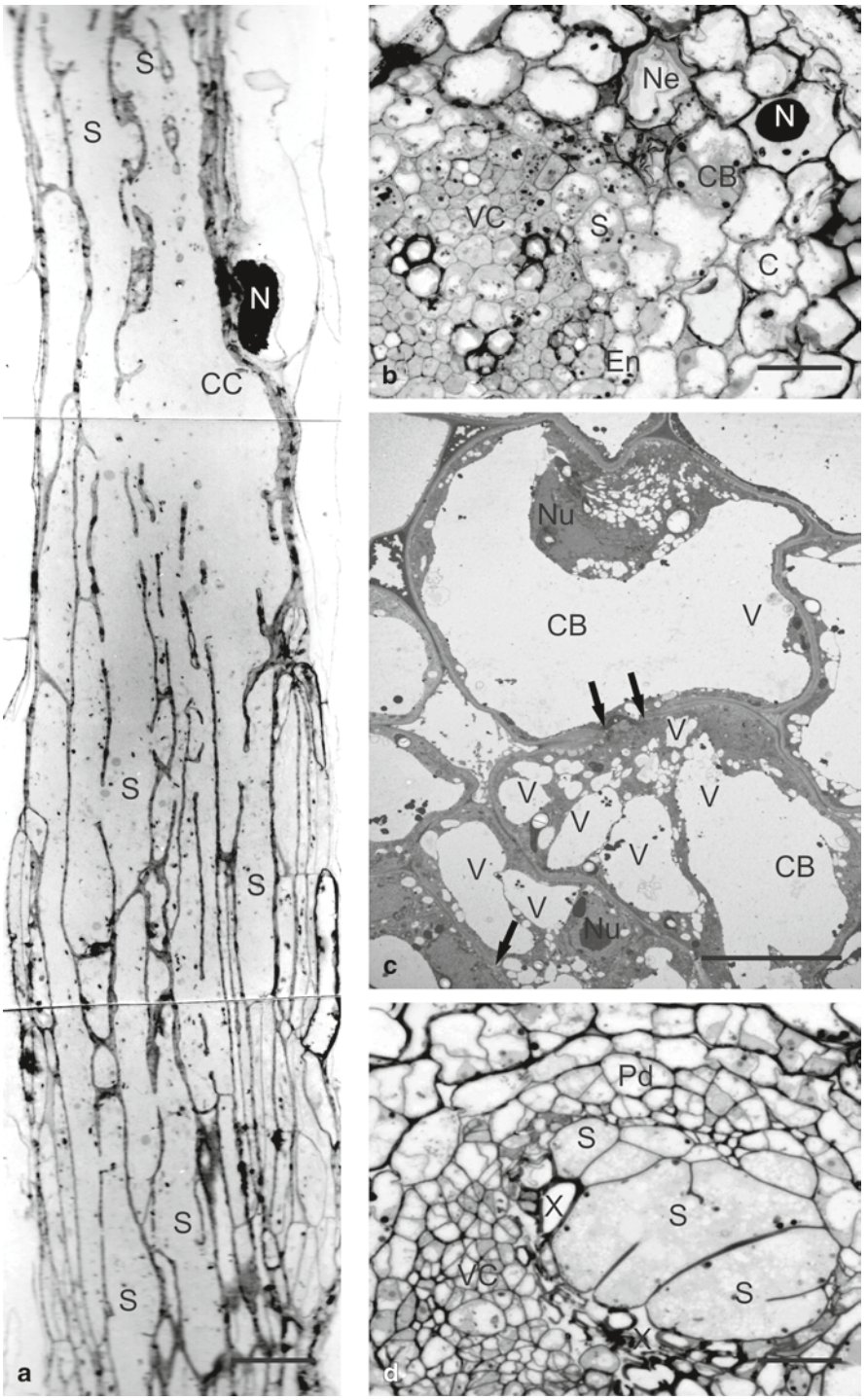
J2s of *H. schachtii* induce syncytia among procambial or pericyclic cells (Golinowski et al. 1996; Sobczak et al. 1997; Fig. 4.1d). The ISC and its nucleus are slightly enlarged and cytoplasmic streaming increases a few hours after completion of the preparation phase (Golinowski et al. 1997; Wyss 1992). Its cell wall and the walls of neighbouring cells are covered locally by patchy depositions of callose-like material. The same material also covers the tip of the inserted nematode stylet (Sobczak and Golinowski 2008; Sobczak et al. 1999; Fig. 4.1d). The cytoplasm of the ISC proliferates while the volume of the vacuole decreases (Sobczak et al. 1999). Similar changes occur also in neighbouring cells that undergo the same ultrastructural changes before they fuse with the ISC via cell wall openings (Magnusson and Golinowski 1991).

Cell wall openings are formed about 6 h after ISC induction via widening of pre-existing plasmodesmata (Grundler et al. 1998). A syncytium induced by *H. schachtii* in the procambium preferentially incorporates procambial cells located next to xylem vessels and sieve tubes. When metaxylem precursor cells are present that are not yet differentiated into vessels they are preferentially incorporated into syncytia (Golinowski et al. 1996). Thus a syncytium may expand into the opposite side of the xylem bundles and occupy a central position inside the vascular cylinder. Additionally, the syncytium may develop an extensive interface with conductive elements which makes it more effective in the uptake of nutrients and water from these tissues. Expanding syncytia induced in the pericycle incorporate primarily other pericyclic cells and they are usually collar- or C-shaped structures when observed in cross sections. Such syncytia have a less well developed interface with conductive tissues. These developmental differences seem to be caused by patterns of plasmodesmata distribution. Plasmodesmata are abundant between cells of the

same tissue type but few are present between the procambium and pericycle. These initial differences also have an impact on the anatomy of older syncytia. After establishing direct contact with conductive elements, the syncytium spreads along the vascular elements in acro- and basipetal directions via incorporation of new cells derived from the procambium. The distal parts of the syncytium (most remote from the nematode head) are always procambial cells abutting xylem vessels. Secondary xylem and phloem elements differentiate around the syncytium associated with J4s or adult females (Golinowski et al. 1996). Pericyclic cells usually divide abundantly and form tissue similar to the peridermis-secondary cover tissue (Golinowski et al. 1996; Sobczak and Golinowski 2008; Sobczak et al. 1997; Fig. 4.1e, f). At later developmental stages the syncytium induced in the pericycle associated with male juveniles may show some abnormalities (Fig. 4.1e). It is composed of numerous, but weakly hypertrophied elements. The syncytial elements are connected by few narrow cell wall openings. Procambial cells adjacent to xylem vessels or sieve tubes often appear to have resisted incorporation into the syncytium and degenerate. Thus, compressed degenerated cells separating the syncytium from conductive elements are often present close to male-associated syncytia. It is possible that the development of a syncytium in the pericycle is a form of 'emergency exit' for the juvenile. Syncytia formed in this tissue face a range of obstacles to their development. They are less effective and provide fewer nutrients, sufficient to support the development of males only. Distal parts of syncytia induced in the procambium are composed of extremely strongly hypertrophied elements derived from procambium (Fig. 4.1f). Cell wall openings are most pronounced next to the nematode head where the region of confluent cytoplasm almost devoid of cell walls is formed (Fig. 4.2a). The cell wall openings are less numerous and narrower in distal parts of the syncytium.

In *A. thaliana* the syncytium reaches its maximal size at about ten DAI (Urwin et al. 1997) and is composed of about 200 cells (Hussey and Grundler 1998). When the feeding of the attached juvenile is complete the syncytial protoplast degenerates very rapidly and syncytial elements are compressed by dividing and developing root cells (Sobczak et al. 1997).

The detailed observations above have been derived from studies on *H. schachtii* in roots of Brassicaceous plants and studies on other cyst nematodes have shown some differences in the details of syncytium induction. The J2 of *Globodera* sp. usually selects its ISC among inner cortical parenchyma cells or in the endodermis (Fig. 4.2b). It does not usually enter the vascular cylinder (Goverse et al. 1998; Sembdner 1963; Sobczak et al. 2005; Figs. 4.2b and 4.4b). The syncytium develops from the ISC towards the vascular cylinder via formation of a so called "cortex bridge", composed of cortical cells connected by cell wall openings (Sembdner 1963). These cells are only slightly enlarged (Fig. 4.2b, c) and their cytoplasm proliferates (Fig. 4.2c). Vacuoles decrease in size and divide into smaller ones but in general are preserved during all stages of syncytium development. Many small vacuoles are formed *de novo* in syncytial elements. The syncytium reaches the vascular cylinder about 48 h after selection of the ISC. As in syncytia induced by J2 of *Heterodera* sp., procambial cells abutting xylem vessels are preferentially incorporated into the syncytium, which spreads along vascular tissues in both directions. Once the developing syncytium reaches the vascular cylinder only pericyclic and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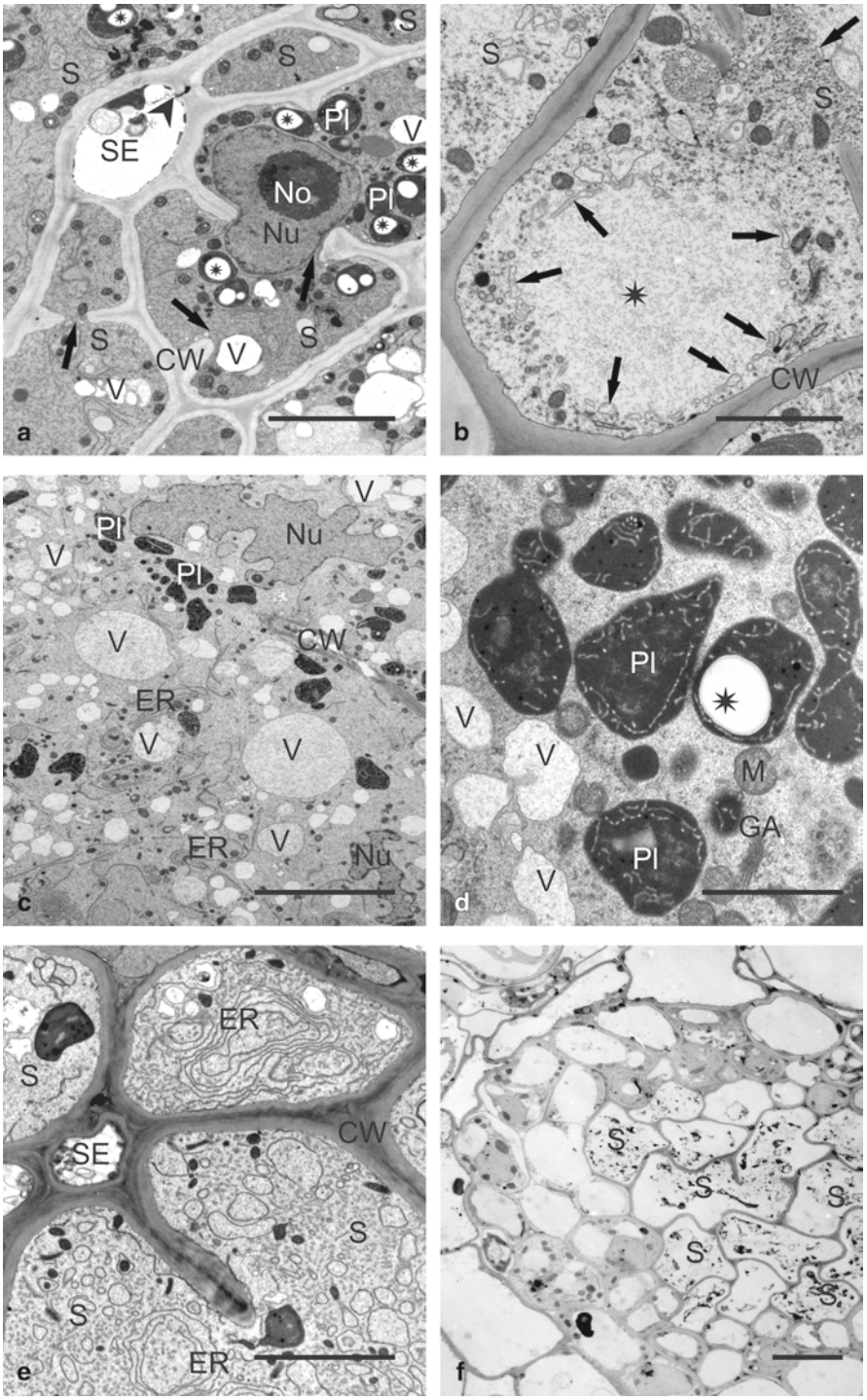
procambial cells are incorporated and distal syncytial elements are always derived from procambium (Fig. 4.2d). The 'cortex bridge' hypertrophies during syncytium development but new cells are not incorporated. Procambial cells included in the syncytium are strongly hypertrophied. The cytoplasm of syncytial elements derived from the procambium proliferates and the central vacuoles dedifferentiate and are substituted by smaller ones (Fig. 4.3a). If procambial cells resist incorporation into syncytia and degenerate, the syncytium is separated from conductive elements by a layer of compressed cells. Male juveniles are usually associated with such syncytia indicating that they are not sufficiently effective to provide the nutrients required to induce development of females. The part of the syncytium derived from the procambium is surrounded by dividing pericycle cells forming a structure similar to secondary cover tissue (Fig. 4.2d).

4.4 Syncytium Ultrastructure

Syncytia induced by different species of cyst nematodes have numerous ultrastructural features in common during their entire development. The most pronounced features are hypertrophy of syncytial elements and proliferation of their cytoplasm. The cytoplasm is electron dense and its streaming is strongly increased as observed using *in vivo* microscopy (Golinowski et al. 1996; Wyss 1992; Wyss and Zunke 1986; Figs. 4.1d and 4.3a, c, e). Turgor pressure inside syncytia induced in *A. thaliana* roots by *H. schachtii* reaches 9,000–10,000 hPa (Böckenhoff and Grundler 1994). Microtubules and microfilaments are present in abundance in syncytial cytoplasm, but they are organised in short and randomly arranged bundles (de Almeida Engler et al. 2004). No cortical microtubule network, mitotic spindles or phragmoplasts are created during syncytium development. The reorganisation of the cytoskeleton in syncytia, and genes involved in these processes are discussed in Chap. 18.

As the cytoplasm of the syncytium proliferates, the volume of the central vacuoles in syncytial elements decreases (Figs. 4.1d and 4.3a, c, e). In syncytia induced by *Heterodera* sp. and in elements derived from the vascular cylinder cells in syncytia induced by *Globodera* sp., central vacuoles are re-differentiated before or soon

Fig. 4.2 Sections of syncytia (S) induced in *A. thaliana* (**a**) or *S. tuberosum* root (**b–d**). **a** A portion (about ½) of longitudinal section through syncytium associated with J3 female (N) of *H. schachtii* (10 DAI). **b** Anatomy of syncytium with 'cortex bridge' (CB) induced by J2 (N) of *G. pallida* in transgenic potato root (3 DAI) (Courtesy of K Koropacka). **c** 'Cortex bridge' (CB) elements connected by cell wall openings (*arrows*) contain proliferating cytoplasm and enlarged nuclei (Nu) in syncytium induced by *G. pallida* in transgenic potato root (3 DAI). Central vacuoles (V) dedifferentiate into smaller ones and new numerous small vacuoles are formed in cytoplasm of 'cortex bridge' elements (Courtesy of K Koropacka). **d** Anatomy of distal part of syncytium associated with J4 female of *G. pallida* induced in transgenic potato root (14 DAI) (Courtesy of K Koropacka). *Abbreviations*: C, cortical parenchyma; CC, confluent cytoplasm; En, endodermis; Ne, necrosis; Pd, peridermis-like cover tissue; VC, vascular cylinder; X, xylem vessel. *Scale bars*: (**a**), (**b**), (**c**) and (**d**) 20 μm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after the cell becomes incorporated into the syncytium (Magnusson and Golinowski 1991). Organelle-free regions delineated by swollen endoplasmic reticulum (ER) cisternae are observed in functional syncytial elements (Fig. 4.3b). They resemble the provacuolar regions described during vacuole biogenesis (Amelunxen and Heinze 1984; Hilling and Amelunxen 1985). Numerous small vacuoles formed *de novo* by widening of the ER cisternae are present in functional syncytia (Sobczak and Golinowski 2008; Fig. 4.3a, c, d). In syncytia induced by *Globodera* sp. the central vacuoles are present in cells forming the 'cortex bridge' (Fig. 4.2c) while syncytial elements derived from the vascular cylinder contain only small vacuoles (Fig. 4.3a, c).

Syncytial nuclei start to become enlarged soon after syncytium induction (Wyss 1992). They are highly enlarged and acquire lobed or amoeboid shapes in fully developed syncytia (Bleve-Zacheo and Zacheo 1987; Gipson et al. 1971; Golinowski et al. 1996; Fig. 4.3a, c). The amount of heterochromatin in syncytial nuclei decreases during syncytium development. Syncytia associated with adult females contain almost only uniformly electron translucent chromatin (Fig. 4.3c). Tritiated thymidine is abundantly incorporated into nuclei at distal parts of syncytia implying that DNA synthesis is an important step in syncytium development and incorporation of new cells into syncytium (Endo 1971). Syncytial nuclei undergo endoreduplication without cytokineses (Goverse et al. 2000; de Almeida Engler 1999) and further details on this process can be found in Chap. 17. Although syncytial nucleoli become enlarged their number does not change (Fig. 4.3a). However, they contain numerous small nucleolar vacuoles indicating that they are highly metabolically active.

Syncytial plastids increase in number and size (Fig. 4.3c, d). Although they are most similar in appearance to leucoplasts they also have some features typical of amyloplasts and chloroplasts. At all stages of syncytium development plastids with constriction are present, indicating that they divide throughout the lifetime of the syncytium (Fig. 4.3d). The shape of plastids observed in TEM sections changes

Fig. 4.3 Ultrastructural details of syncytia (S) induced in *S. tuberosum* (**a**, **c** and **d**), *A. thaliana* (**b** and **e**) and *Sinapis alba* (**f**) roots. **a** Ultrastructure of syncytial elements derived from the vascular cylinder cells of transgenic potato root infected with *G. rostochiensis* (3 DAI). Syncytial cytoplasm contains small vacuoles (V), plastids (Pl) with starch grains (*asterisks*) and enlarged nucleus (Nu) with large nucleolus (No). Inside sieve tube member (SE) wall a plasmodesma-like structure (*arrowhead*) is present (*arrow*-cell wall opening) (Courtesy of A Karczmarek). **b** Electron translucent and organelle-free region (*asterisk*) delineated by dilated cisternae of endoplasmic reticulum (*arrows*) in syncytium induced by *H. schachtii* (5 DAI) (Courtesy of S Siddique). **c** Enlarged amoeboid nuclei (Nu), plastids (Pl) without starch grains, endoplasmic reticulum (ER) and many vacuoles (V) of different sizes in syncytium induced by *G. pallida* in transgenic potato root (28 DAI) (Courtesy of K Koropacka). **d** Ultrastructure of plastids (Pl) in syncytium induced by *G. pallida* in transgenic potato root (10 DAI) (*asterisk*-starch grain) (Courtesy of K Koropacka). **e** Ultrastructure of endoplasmic reticulum (ER) structures in syncytium induced by *H. schachtii* (14 DAI) (Courtesy of S Siddique). **f** Degenerated syncytium induced by *H. schachtii* in procambium of resistant *S. alba* cv. 'Maxi' root (10 DAI) (Courtesy of AH Soliman). *Abbreviations*: CW, cell wall; GA, Golgi apparatus; M, mitochondrion. *Scale bars*: (**a**) 5 μm; (**b**), (**d**) and (**e**) 2 μm; (**c**) 10 μm; (**f**) 5 μm

during syncytium development. Plastids have oblong or round outlines in uninfected cells and young syncytial elements (Fig. 4.3a). The first plastids with cup- or C-like shapes appear in syncytia associated with the J3s (Fig. 4.3d). These cup-like plastids become abundant in syncytia associated with J4 and adult females (Fig. 4.3c). Plastids are surrounded by a double membrane and contain strongly electron dense stroma. They usually contain several thylakoids and few plastoglobuli (Fig. 4.3d). Starch grains are not observed in syncytial plastids during periods of active food withdrawal (Hofmann et al. 2008). However, in young syncytia (Fig. 4.3a) and in syncytia associated with moulting juveniles plastids turn into amyloplasts and contain large starch grains. Starch also appears in necrotising syncytia (Hofmann et al. 2008). Chlorophyll synthesis takes place in syncytia exposed to light as indicated by detection of fluorescence patterns typical for chlorophyll (Urwin et al. 1997; Szakasits unpublished). However, the number of thylakoid cisternae does not substantially increase and they are not arranged into granae.

The number of mitochondria in syncytial elements increases (Figs. 4.1b and 4.3d) but are not structurally changed in syncytia. Most have round or rod-like shapes as seen on sections, but many are constricted, indicating a process of division.

The amount of ER is generally high throughout syncytium development (Bleve-Zacheo and Zacheo 1987; Gipson et al. 1971; Golinowski and Magnusson 1991; Golinowski et al. 1996; Grymaszewska and Golinowski 1998; Holtmann et al. 2000; Soliman et al. 2005; Wyss et al. 1984). The ER is composed of two morphologically distinct parts. Rough ER consisting of flattened cisternae covered with ribosomes is well developed and generally predominates in syncytia (Fig. 4.3e). It is often arranged in parallel lines or forms circular swirls. Its abundance indicates high levels of protein synthesis. It is present in the entire syncytium except for the region of confluent cytoplasm close to the nematode's head where smooth ER predominates (Grymaszewska and Golinowski 1998; Wyss et al. 1984; Fig. 4.1b, c). Smooth ER forms tubular structures free of ribosomes. The ER structures are often found to be connected with the osmiophilic core of feeding tubes (Fig. 4.1b, c). Plant-parasitic nematodes are unable to produce some sterols and are therefore completely dependent on the syncytium for the supply of these essential molecules (Chitwood and Lusby 1992). Sterols are synthesised by smooth ER and thus a direct connection to the feeding tube may permit delivery of sterols directly to parasitic nematodes.

Dictyosomes are not changed structurally in syncytia, but their number seems to increase (Fig. 4.3d). They are usually located along syncytial walls. Peroxisomes are present in syncytia induced in potato roots by *Globodera* sp. They have a typical structure, but they are relatively small compared to peroxisomes present in mesophyll cells. They are surrounded by a single membrane and contain large catalase crystals inside the amorphous content (Gruber et al. 1970). The cell wall of the syncytium undergoes dramatic changes including formation of cell wall openings, development of cell wall ingrowths and changes in plasmodesmata. These are described in detail in Chap. 19.

Unless it is destroyed by external triggers the syncytium produced in a susceptible interaction degenerates only when the associated nematode ceases feeding. During this process the syncytial cytoplasm rapidly becomes electron translucent

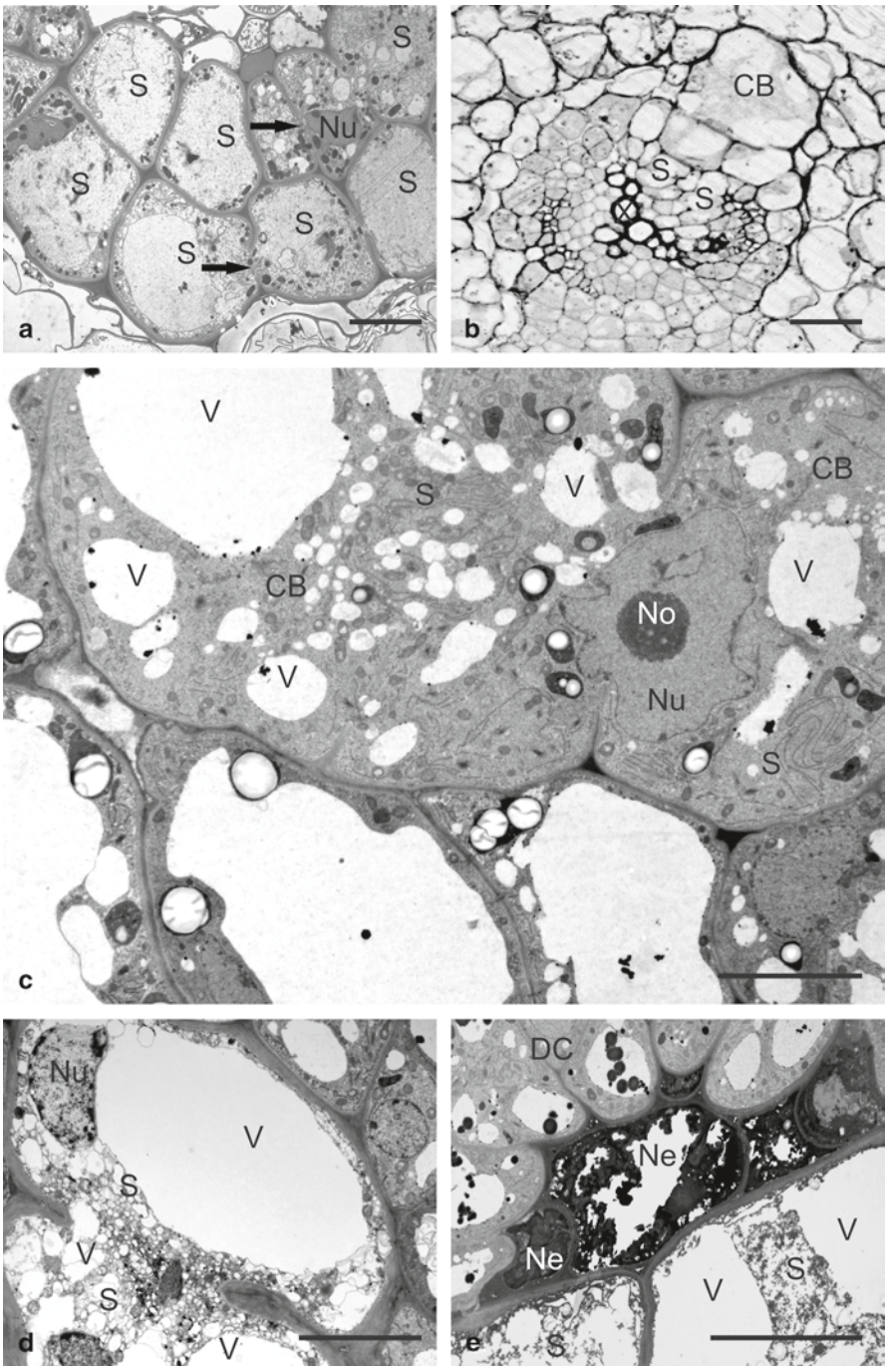
(Fig. 4.4d, e). Small vacuoles enlarge and coalesce to form larger vacuoles. The nucleoplasm becomes electron dense and flocculent. No nucleoplasm condensation similar to that seen during programmed cell death is observed. Membranes of the ER collapse forming lipid grains or myelin bodies. Plastids with starch grains and structurally well-preserved mitochondria are the last recognisable organelles in a degenerating syncytium (Sobczak et al. 1997). Finally the plastidial stroma and the mitochondrial matrix become electron translucent and both organelles deteriorate. The necrotised syncytium is compressed by neighbouring cells that enlarge and differentiate into regular root tissues.

4.5 Defence Responses

Most plant nematode resistance genes do not protect plants against infection and plants carrying them do not target cyst nematodes directly. Instead, resistance against cyst nematodes tends to decrease nematode population density (Müller 1998). Plant nematode resistance genes are usually derived from wild relatives of crop species (Tomczak et al. 2008). They are specific for nematode species, pathotypes and sometimes even races or genotypes and thus their use against a wide range of economically important plant-parasitic nematodes is restricted.

Resistance against cyst nematodes is manifested in different ways. Resistant genotypes of host plants are usually located and infected by plant-parasitic nematodes in the same way and to a similar extent as susceptible genotypes (Turner and Stone 1984). The J2 selects an ISC and induces a syncytium after a migration through the root that is not usually accompanied by any more abundant necrosis than migration through susceptible roots. The initial defence response of *Sinapis alba* cv. 'Maxi' to infection with *H. schachtii* is one of a few interactions based on a hypersensitive response to cyst nematodes (Soliman et al. 2005). In this interaction the infective J2 is surrounded by numerous necrotised cells during invasion and it is usually unable to induce a syncytium. However, if this initial response is overcome the J2 can induce a syncytium. A syncytium induced in procambial cells usually degenerates during the J2 developmental stage (Fig. 4.3f) but a syncytium induced in the pericycle develops further (Fig. 4.4a). However, although it allows the attached juveniles to develop into J3, almost all nematodes develop into males (Golinowski and Magnusson 1991; Soliman et al. 2005).

During the more typical response of resistant plants to infection with cyst nematodes syncytia develop for several days. Initially, these syncytia do not differ from the syncytia induced in susceptible plants. After this initial stage of development, syncytial elements degenerate or their collapse in some cases follows degeneration of surrounding cells. Differences in spatial and temporal patterns of defence reactions are sometimes very obvious and they depend on the type of resistance gene as well as on the plant and nematode genotype involved in the interaction. This typical resistant response against cyst nematodes is often called a 'delayed (late) hypersensitive response' or 'male-based resistance' because syncytia usually remain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functional long enough to allow development of adult males only. If an initially efficient syncytium allows J2 to enter the female development pathway, degeneration of the syncytium stops development of the female at one of the juvenile stages and the nematode does not reach maturity. This mechanism reduces nematode propagation and decreases nematode population density (Müller 1998).

Resistant responses against *Heterodera* sp. are best described for *H. schachtii* infecting resistant beet and Brassicaceous plants and for *H. glycines* infecting resistant soybean cultivars. In a resistant sugar beet line carrying the $Hs1^{pro\text{-}1}$ resistance gene *H. schachtii* induces development of small syncytia composed of weakly hypertrophied elements derived from procambial and pericyclic cells (Holtmann et al. 2000). Syncytia have well established interfaces with vessels and sieve tubes and degradation of surrounding cells occurs only rarely. The syncytial cytoplasm proliferates and becomes electron dense and the number of plastids, mitochondria and ER structures increase. Syncytial nuclei enlarge while the amount of heterochromatin decreases. Vacuoles are less numerous and larger, and rough ER predominates in syncytia induced in resistant plants as compared to syncytia developed in susceptible plants. Membranes of the ER aggregate and form large clumps leaving syncytial cytoplasm almost free of ER structures in older syncytia. Syncytia induced in resistant plants degenerate before the juveniles reach maturity. Juveniles are rarely able to develop into adult females. However, syncytia induced by these nematodes structurally resemble syncytia induced in susceptible plants and no membrane aggregations were found (Holtmann et al. 2000).

In the case of Brassicaceous plants the resistant response of radish (*Raphanus sativus*) cv. 'Pegletta' to infection with *H. schachtii* is the best described. In this interaction syncytia follow the same developmental pattern in susceptible and resistant plants during the first two DAI. Their cytoplasm becomes electron dense and proliferates. Similarly the numbers of ribosomes, plastids and mitochondria increase. Nuclei and nucleoli hypertrophy and central vacuoles are substituted by numerous small vacuoles (Grymaszewska and Golinowski 1998; Wyss et al. 1984). Then the number of small vacuoles increases and cytoplasm becomes more electron dense in syncytia induced in resistant plants at four DAI, but most syncytia contain only degenerated protoplasts at seven DAI. Few, if any, cells abutting the syncytia

Fig. 4.4 Sections of syncytia (S) induced in *Sinapis alba* (**a**) and *S. tuberosum* (**b–e**) roots. **a** Syncytium with electron translucent cytoplasm and enlarged nuclei (Nu) induced by *H. schachtii* in pericycle of resistant *S. alba* cv. 'Maxi' root (10 DAI) (*arrows*-cell wall openings) (Courtesy of AH Soliman). **b** Anatomy of syncytium with 'cortex bridge' induced by *G. pallida* in resistant transgenic potato root (7 DAI). Syncytium has well established interface to xylem elements (X) (Courtesy of K Koropacka). **c** Ultrastructure of 'cortex bridge' elements (CB) in syncytium induced by *G. pallida* in resistant transgenic potato root (3 DAI). Central vacuoles (V) are decreased in size and new vacuoles are formed. Nucleus (Nu) and nucleolus (No) are enlarged (Courtesy of K Koropacka). **d** Degenerating syncytium induced by *G. pallida* in resistant transgenic potato root (7 DAI) (Courtesy of K Koropacka). **e** Degenerating cells (Ne) abutting necrotised syncytium induced by *G. pallida* in resistant transgenic potato root (14 DAI). Cells next to the layer of degenerated cells surrounding the syncytium divide neoplastically (DC) (Courtesy of K Koropacka). *Scale bars*: (**a**) and (**d**) 5 μm; (**b**) 20 μm; (**c**) and (**e**) 10 μm

degenerate and the collapsed syncytia are never surrounded by a layer of degraded cells (Grymaszewska and Golinowski 1998; Wyss et al. 1984). However, some syncytia composed of slightly enlarged elements derived from procambial cells still remain functional and they are able to support the development of adult males and occasionally even females (Grymaszewska and Golinowski 1998).

A range of different defence responses against *H. glycines* has been described in resistant soybean (*Glycine max*) cultivars. In cv. 'Bedford' syncytia composed of several, slightly enlarged pericyclic cells are established 18 h after inoculation (Endo 1991). Their elements are connected by narrow cell wall openings and contain remnants of central vacuoles and numerous small vacuoles. The syncytial cytoplasm and nucleoplasm are osmiophilic and deteriorating, but many mitochondria and dilated structures of ER are still recognisable. At five DAI the syncytial cytoplasm is still deteriorating and apparently plasmolysed as it is retracted from strongly thickened syncytial cell walls. Some syncytial nuclei contain large patches of chromatin, electron translucent nucleoplasm and broken nuclear envelopes (Kim et al. 1987). Syncytial cytoplasm and nuclei are completely disintegrated and only their debris is present at 10–15 DAI. The necrotic reaction occurs only in the syncytium while cells around it remain alive (Kim et al. 1987). A similar defence mechanism occurs also in another resistant soybean cv. 'Peking' (Endo 1965; Riggs et al. 1973). After initial syncytium development its cytoplasm becomes extremely electron dense and clumped at 5–7 DAI. Syncytial walls and walls of abutting degenerated cells are strongly thickened. This deposition of cell wall material may form a barrier sealing off the syncytium from nutrient influx (Riggs et al. 1973). Degraded syncytia are associated with dead J2s (Endo 1965). In the resistant cv. 'Pickett71' early degradation of the syncytium is also the main mechanism of defence. Syncytia are induced in the vascular cylinder and they are usually composed of pericyclic cells (Endo 1991). Membranous aggregations and many small vacuoles are present in the electron translucent syncytial cytoplasm at two DAI. Only protoplast debris is present in slightly enlarged syncytial elements connected by wide cell wall openings at four DAI. In contrast, in cv. 'Forrest' resistance is based on degradation of cells surrounding the syncytium (Kim et al. 1987). Necrosis begins in the oldest region of the syncytium next to the nematode's head at five DAI. Thereafter it expands and surrounds the entire syncytium which becomes isolated from neighbouring cells. Concomitantly, the syncytial wall thickens more than would be seen in a susceptible interaction, thus further isolating the syncytium from nutrient influx. Completely degenerated syncytia containing osmiophilic remnants of the protoplast can be observed at 15 DAI (Kim et al. 1987).

In the case of *Globodera* sp. the anatomy and cytology of defence responses are best studied for *G. rostochiensis* and *G. pallida* infecting resistant potato and tomato genotypes. J2s of *G. rostochiensis* invade potato roots with the *H1* resistance gene normally. Syncytia are induced, usually among cortical cells, as in the susceptible interaction. The 'cortex bridge' is formed and the syncytium expands toward the vascular cylinder by incorporation of cortical, endodermal, pericyclic and procambial cells (Bleve-Zacheo et al. 1990; Rice et al. 1985). However, the incorporation of procambial cells into the syncytium usually fails and the syncytium is located at the edge of the vascular cylinder giving it very limited interface with the xylem and

phloem elements. Proliferation of the syncytial cytoplasm is weak or not observed. The syncytial cytoplasm is osmiophilic and located primarily around central vacuoles that are preserved. Additionally, many small vacuoles appear within the syncytial cytoplasm. Syncytial nuclei and nucleoli enlarge but they show features of karyolysis such as a loss of osmophility and dispersion of chromatin to the periphery of the nuclear envelope (Bleve-Zacheo et al. 1990). Plastids usually contain starch grains. Vacuolated protoplasts and mitochondria are degenerated at seven DAI. Syncytial cell walls are extremely thickened at some places and no cell wall ingrowth formations occur. Necrosis appears in cells located around the juvenile and then in cells surrounding the syncytium, isolating it from neighbouring alive cells (Bleve-Zacheo et al. 1990; Rice et al. 1985).

A similar response occurs in resistant tomato carrying the *Hero* gene cluster infected with *G. rostochiensis*. The syncytium is induced and develops forming a 'cortex bridge' that expands toward the vascular cylinder. In most cases degraded vascular cylinder cells appear around the syncytium at two DAI and it becomes surrounded by a continuous layer of degenerated cells at four DAI. In addition to the central vacuoles numerous small vacuoles are present in the syncytium. The syncytial cytoplasm turns granular and electron translucent and finally the syncytium degenerates (Sobczak et al. 2005). However, in this interaction some syncytia resist this initial defence response and develop further. Most juveniles associated with these syncytia develop into males but development of a few females may also occur (Sobczak et al. 2005). Syncytia associated with males are smaller and composed of fewer cells than syncytia associated with females. They expand along the vascular cylinder and incorporate some procambial cells but are always separated from the vessels by a layer of necrotised cells. In contrast, syncytia associated with females develop a direct interface to conductive elements (Sobczak and Golinowski 2008; Sobczak and Kumar unpublished). Transgenic tomato plants with the *Hero* gene (Ernst et al. 2002) reveal, in general, the same defence reaction as plants containing the *Hero* gene cluster. Syncytia are induced in the cortex and spread via the formation of a 'cortex bridge' toward the vascular cylinder, but therein they incorporate only pericyclic cells. Four DAI they are surrounded by a layer of degenerated vascular cylinder cells. However, the defence reaction in the transgenic plants seems to be more rapid and abrupt than in plants carrying the *Hero* gene cluster. In these plants the syncytial cytoplasm is granular, osmiophilic and plasmolysed. Organelles become destroyed and callose is abundantly deposited at syncytial walls (Sobczak and Golinowski 2008; Sobczak and Kumar unpublished).

The resistant response against *G. pallida* has been described at ultrastructural and anatomical levels in some clones of *Solanum vernei* (Rice et al. 1987) and *S. canasense* (Castelli et al. 2006). In *S. vernei* clones the syncytium is induced in the cortex and spreads centripetally to the vascular cylinder via formation of a 'cortex bridge'. It incorporates pericyclic cells only (Rice et al. 1987). It enlarges by hypertrophy and fusion of incorporated elements until seven DAI. The syncytial cytoplasm proliferates weakly and it becomes electron dense and osmiophilic in four DAI syncytia. The remnants of the central vacuoles are present and many new small vacuoles are formed. The syncytial nuclei enlarge and plastids often accumulate next to them. The abnormality of the syncytium development is variable.

Some syncytia resemble those induced in susceptible plants while others show different features of degeneration. The syncytium is usually strongly vacuolated and contains degraded cytoplasm at seven DAI. No necrotic layer is formed around the syncytium. Only a few J3s were able to develop at about ten DAI (Rice et al. 1987).

A similar response also occurs in resistant *S. andigena* clones infected with *G. pallida* (Blok et al. unpublished; Sobczak and Golinowski 2008). A small syncytium composed of cortical, endodermal and pericyclic cells only is usually separated from the vascular elements by degenerated procambial cells. The syncytial cytoplasm has a deteriorating appearance and its proliferation is weak. Central and many small vacuoles are present. The protoplast of most syncytia is totally degenerated at seven DAI.

A different response occurs in transgenic potato lines carrying the *Gpa2* resistance gene (Koropacka et al. unpublished). In this interaction the syncytium is also, typically for *Globodera*, induced in the cortex and spreads via a 'cortex bridge' toward the vascular cylinder. Reaching the vascular cylinder it incorporates pericyclic and procambial cells and its interface to the conductive tissues is therefore well developed (Fig. 4.4b). The syncytial elements are connected by wide and numerous cell wall openings. The syncytial cytoplasm proliferates and becomes electron dense. The central vacuoles decrease in size and small vacuoles are formed (Fig. 4.4c). Syncytial nuclei enlarge and become amoeboid. Syncytia with a degraded strongly osmiophilic cytoplasm, degraded nuclei and numerous small vacuoles first appear at seven DAI (Fig. 4.4d). Degenerated surrounding cells are abundant at 14 DAI, by which time most of the syncytia is degraded (Fig. 4.4e). Parenchymatous vascular cylinder cells next to necrotised cells surrounding the degenerated syncytium divide abundantly (Fig. 4.4e).

A further type of defence response was described for a resistant *S. canasense* clone (Castelli et al. 2006). J2s of *G. pallida* infect its roots at very low frequency and few of those that do invade are able to induce syncytia. The processes of root penetration, migration and ISC selection take far longer compared to these processes in susceptible potato. Many J2s leave the roots after invasion. Finally, if successful, the J2 induces a syncytium in the cortex. The syncytium consists of a 'cortex bridge' and a few parenchymatous vascular cylinder cells. The syncytial cytoplasm is granular and plasmolysed at seven DAI. It contains many small vacuoles, structurally unchanged plastids and mitochondria, and hypertrophied and amoeboid nuclei. No necrotic cell layer surrounding this degenerating syncytium has been observed (Castelli et al. 2006).

4.6 Concluding Remarks

Millions of years of co-evolution have allowed evolution of mechanisms that permit cyst forming plant-parasitic nematodes to influence and control plant morphogenetic pathways to create specific plant structures that function for the nematode's benefit only. It is one of very few situations in the plant kingdom where the plant

develops a multicellular structure resembling a specialised organ that is used only by the foreign invader. Although the structural composition and function of syncytia have been studied for almost a century we still have only rough indications about the mechanisms of the morphogenetic development of syncytia. Our understanding of syncytial development and function is still fragmentary and restricted to some basic structural features. We do not know what mechanisms are used by the nematode to induce the formation of this specific structure and which pathways are necessary for its proper development. Recently our knowledge concerning the molecular plant-nematode dialogue has been substantially increased as detailed reports concerning changes in plant gene expression profiles have been published (*e.g* Swiecicka et al. 2009; Szakasits et al. 2009; Tucker et al. 2007). However, one shortcoming of these studies is that they rarely link studies of gene function to ultrastructural studies, making it difficult to relate the role of specific genes in terms of syncytium development. Plants defend themselves against their parasites through resistance genes. Unfortunately, nematode resistance is not absolutely effective and occasionally even females develop on resistant genotypes. Their progeny may have gene combinations that allow breaking or overcoming of the resistance mechanisms provided by known resistance genes and this may lead to emergence of new virulent nematode isolates (Müller 1998). Additionally, there are only a few effective and easy to introduce cyst nematode resistance genes (Tomczak et al. 2008), thus 'artificial' resistance against nematodes has been introduced (McCarter 2008) that is based on specific expression of antifeedants or toxins in syncytia (Atkinson et al. 2003) or on specific silencing of plant susceptibility genes (de Almeida Engler et al. 2005). However, the influence of these genes on the ultrastructure and anatomy of nematode induced syncytia has rarely been studied. Such studies could help develop our understanding of the mechanisms underlying syncytium development.

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Chapter 5
Root-knot Nematodes and Giant Cells

Michael G. K. Jones and Derek B. Goto

Abbreviations

DPI	Days Post Infection
GFP	Green Fluorescent Protein
RKN	Root-Knot Nematode
J1	first stage Juvenile
J2	second stage Juvenile
NF	Nodulation Factor; other abbreviations defined when first used

5.1 Introduction

Root-knot nematodes (*Meloidogyne* species, Family Tylenchidae, genus Tylenchus), together with cyst nematodes (*Heterodera* and *Gobodera* species), are economically the most important nematode pests of broadscale and vegetable crops worldwide. Root knot nematodes have a very wide host range that includes almost all vascular plants, and although their infection is normally confined to host plant roots, tubers or tap roots in the soil, they can also reproduce in plant leaves if infiltrated into the leaf air spaces. Root-knot nematodes are classified as obligate biotrophic sedentary endoparasites, and they are possibly the easiest nematode pests to identify and study, because in most cases infection of roots or other organs is accompanied by the formation of a characteristic gall.

Although there are in the order of 60 species of root-knot nematodes, a smaller subset of these are very widespread—probably the best known species are *M. incognita, M. javanica, M. arenaria* and *M. hapla.* The nature and function of the

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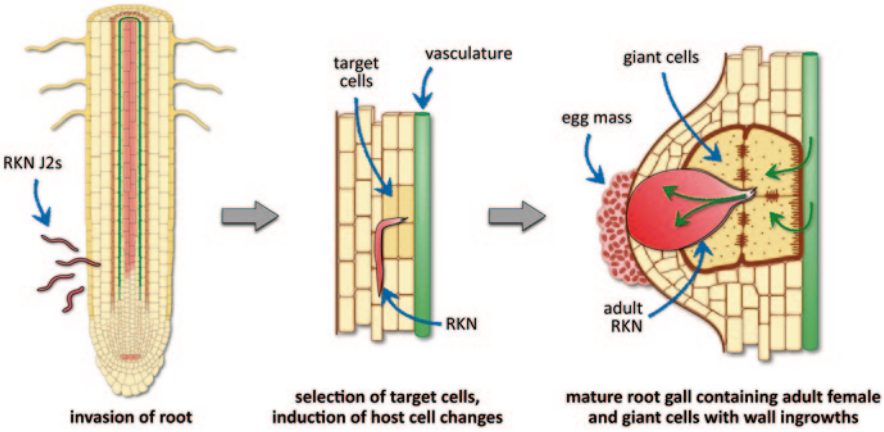

Fig. 5.1 Schematic representation of the life cycle of root-knot nematodes. J2 root-knot nematodes (RKN) migrate to and enter a host root in the zone of elongation. An individual nematode then migrates to pro-vascular 'target cells' where it re-programs them by stimulating mitosis and cell expansion to form giant cells from which it feeds and becomes surrounded by a gall. After three moults within the root (not shown), the adult female becomes spherical and lays eggs in a gelatinous egg mass. The giant cells are multinucleate 'transfer cells' with wall ingrowths that amplify their plasma membranes to enhance the flow of solutes to the nematode from vascular tissues

feeding cells induced by all of these species are very similar. However differences do exist between the species in gall structure, number of lateral roots originating from galls and tolerance to cold. For example, *M. hapla* has the unfortunate common name 'Northern root-knot nematode' because it is more cold-tolerant, but this is inappropriate for the Southern hemisphere, where north equates to a hotter climate! An overview of the life cycle of root-knot nematodes is provided in Fig. 5.1 and described further below.

Most root-knot nematode species reproduce by mitotic parthenogenesis, an asexual or clonal form of reproduction in which mitosis occurs and the oocytes keep the original diploid or polyploid chromosome number. Some species of root-knot nematodes, such as *M. hapla*, reproduce by facultative meiotic parthenogenesis. In this case, there is a first meiotic division in oocytes, and when males and sperm are present, usually under conditions of crowding or stress, sexual reproduction can occur to produce hybrid progeny. In the absence of males, diploidy is restored by fusion of sister haploid nuclei to generate diploid, inbred, progeny. As for cyst nematodes, after embryogenesis occurs in root-knot nematode adult females, the first stage juvenile (J1) develops within the protective eggshell and then moults to produce the second stage (J2) juvenile. Eggs are normally laid in a gelatinous egg mass, and in some temperate species (e.g. *M. naasi*), or under conditions of stress, there can be a period of diapause before the J2s hatch, but for most root-knot nematode species J2s hatch rapidly under favourable conditions (Fig. 5.2a).

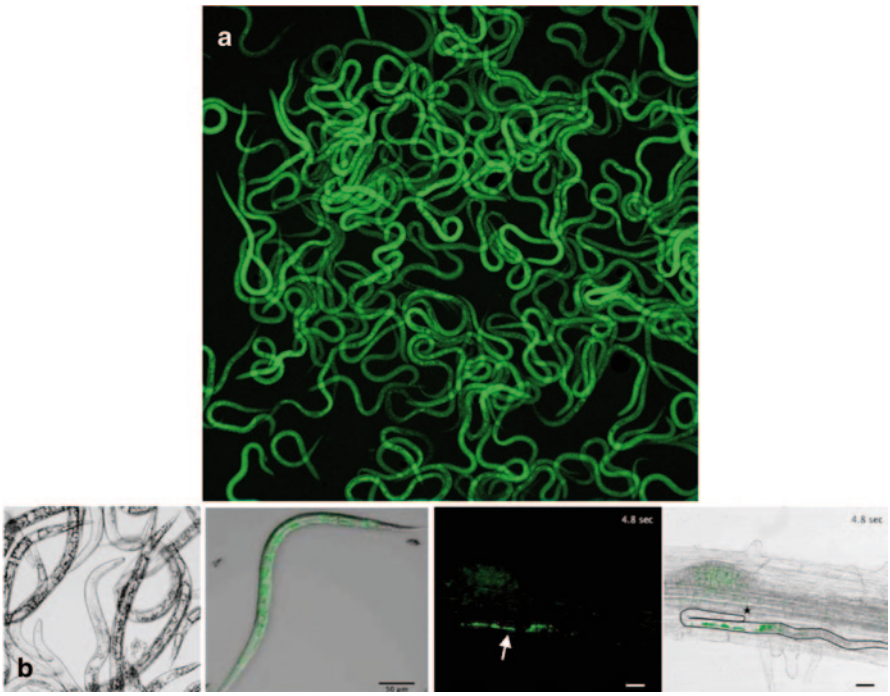

Fig. 5.2 **a** Freshly hatched *M. hapla* J2s labelled with fluorescein diacetate (FDA). **b** (*left to right*) J2 *M. javanica*, an individual J2 labelled with FDA, visible (*arrow*) in *Arabidopsis* roots *in vitro* 3 DPI, also outlined (*right*). (From Goto et al. 2010)

5.2 Root Invasion and Migration

Once J2 have hatched, they move through the soil in moisture films that surround soil particles towards roots of host plants. Growing plant roots influence the surrounding soil to generate a 'rhizosphere', as a result of secreting polysaccharide 'slime', which helps root tips penetrate the soil, and the sloughing off of root cap cells and degeneration of root hair cells. In some cases C4 acids are actively secreted by roots, and these C4 acids act as chelating agents, and aid release and uptake of mineral nutrients such as phosphorus. Mycorrihzal fungi may also be associated with roots and these also help extend the zone surrounding the root from which it can sequester nutrients. Without any clear gradients the movement of J2s is random or sometimes they are attracted to each other. However, normally the rhizosphere provides gradients of compounds that the nematodes can detect, and so enables them to move to and locate host plant roots. Although J2s are normally attracted to growing root tips, they are also attracted to emerging lateral roots and sites where other J2s have entered roots.

It also appears that signalling may occur in both directions before the J2 reaches a host root. Weerasinghe et al. (2005) provided evidence that root-knot nematode juveniles (*M. incognita*) may send out signals in advance that can influence root development and responsiveness to the nematode. Rhizobia bacteria, which form nitrogen fixing nodules with legume hosts, secrete lipochitooligosaccharide signalling molecules, generally known as 'Nod' (NF) factors that affect root hairs 2–4 h in advance of the arrival of the bacteria and root entry. Even in the absence of the bacteria, Nod factors alone can induce a series of changes in root hair cells that involve cytoskeleton, ion flux changes, root hair deformation and the formation of pseudonodules. Weerasinghe et al. (2005) demonstrated that water or culture medium that had been in contact with washed *M. incognita* J2s appeared to contain a signalling molecule(s) functionally equivalent to Nod factors, which they termed 'NemF'. *Lotus japonicus* hairs from roots grown in solution are normally straight and smooth. At 1–4 h after treatment of such *L. japonicus* roots with water exposed to J2 *M. incognita*, root hairs became wavy and branched, with accompanying changes to the cytoskeleton and nuclear movements, similar to changes seen in root hairs treated with NF. This phenomenon also occurred when tomato roots, a good host for root-knot nematodes, were treated in the same way, indicating that the plant response is not confined just to legume hosts, but did not occur in treated *Arabidopsis thaliana* (Columbia), which is a poorer host for root-knot nematodes. The suggestion from these results, which also included studies on mutants of NF perception is that, at least in part, root responses to root-knot nematodes and rhizobial nodule formation may have some signalling pathways in common, and that root-knot nematodes may have conscripted some of those mechanisms from rhizobia, perhaps by horizontal gene transfer. However, flavonoid-deficient *Medicago truncatula* roots which did not support rhizobial nodulation did support root-knot nematode giant cell formation (Wasson et al. 2009), so clearly there are also major differences in pathways of host responses between root-knot nematodes and rhizobium nodules.

Once the J2 reaches a host root, it enters the root using a combination of mechanical penetration with its hollow mouth stylet, together with enzymatic secretions (Fig. 5.3) that can degrade polysaccharide components of plant cell walls. Unlike migration of J2 cyst-nematodes, root-knot nematode J2s migrate intercellularly, that is between cells rather then through the cells themselves. Migration of J2s can be followed using differential interference contrast optics and time-lapse photography. J2s can also be pre-stained with fluorescent dyes and monitored with fluorescence optics and confocal laser microscopy (Fig. 5.2b) (Goto et al. 2010). The effects of nematode migration can also be studied using plant lines in which specific cell types or genes have been tagged with green fluorescent protein (GFP) from *Aequorea victoria* or other similar fluorescent tags, and subsequent changes in gene expression with nematode migration and feeding site development can be monitored (see below).

Although *A. thaliana* is an atypical host, the small size and transparency of its roots nevertheless make it a good subject for studies using time time-lapse photography. Wyss and Grundler (1992) used video-enhanced contrast microscopy to show that J2s of *M. incognita* usually invade roots in the zone of elongation

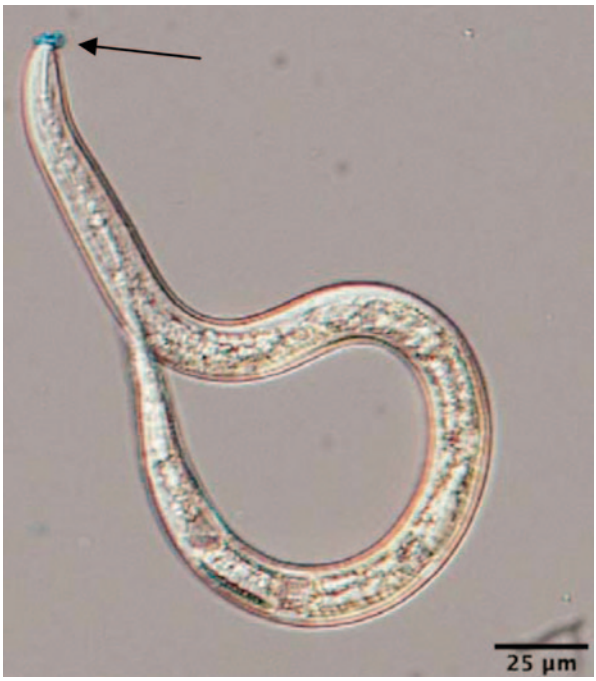

Fig. 5.3 *M. hapla* J2 producing stylet secretions indicated by the *arrow* (stained blue with Coomassie Blue). (Photo: C. Nakano, D. Goto)

behind the tip. Local exploration of the root surface involves head rubbing and stylet movements at epidermal cells and entry is usually achieved by destroying the thin-walled epidermal and sub-epidermal cells, followed by intercellular migration. Surprisingly, on entry into the root behind the tip, their migration is first in the direction of the root-tip, where they turn round, and move away from the tip towards the differentiating vascular cylinder. This migration of root-knot J2s through cell walls also requires secretion of wall degrading enzymes, and it appears that a cocktail of such enzymes is secreted and enable separation of the middle lamella of cells just ahead of the migrating nematode—this aspect is described in more detail in Chap. 12. Despite this enzymatic breakdown of some cell wall components during migration, which is accompanied by mechanical pressure and the rupture of plasmodesmata that normally interconnect the symplasts of adjacent plant cells (Jones and Payne 1978a), in susceptible interactions the host plant is not known to respond to these disturbances, or to the oligosaccharides or other wall fragments generated, with any typical defence reaction. Either elicitors of host defence are not produced, or the nematode can prevent the activation of host defences (see Chap. 13 for more details on this aspect). It is not clear that the behaviour of J2 migration described for *A. thaliana* is typical of migration in other species, although similar behaviour has been observed in early stage infection of tomato roots (Goto and Jones unpublished). In other situations, when there are multiple secondary infections away from the growing root tips, infection and migration behaviour is likely to be different.

Once within the root, as the nematode migrates, it must respond to a completely new set of signals and gradients that enable it to detect its location and to migrate towards a site suitable for the induction of feeding cells, normally termed 'giant cells' where it will become sedentary.

5.3 Giant Cell Formation and Function

Once the responses from stylet thrusting by the J2 indicate that potentially responsive feeding cells, usually pro-vascular cells, have been reached, the nematode stops migrating and induces the formation of about six giant cells. This involves secretion of proteins and/or other components probably from the subventral oesophageal gland cells. The genes whose products enable the nematode to invade host plants and induce giant cells are usually referred to as 'parasitism' genes, and knowledge about them is described in detail in Chaps. 12–14 of this book. Root-knot nematodes have a characteristic pattern of head movement and feeding behaviour, which consists of head and stylet movements, interspersed with stylet-tip thrusting and pumping of the metacorpal bulb (Wyss and Grundler 1992). Pre-parasitic J2s store their food reserves mainly as lipids, but two to three days after root invasion, once they sense that the feeding cells are responding, they convert their lipid reserves to glycogen. Lipid reserves do not accumulate again until the adult stage is reached (Dropkin and Acedo 1974). One of the first external signs that a root-knot nematode J2 has become sedentary is the swelling of the root and the formation of a gall, which can be seen from about 24 h after infection.

5.3.1 Giant Cell Characteristics

The process of giant cell formation follows a typical developmental pathway, which differs of course from the cellular differentiation that would normally take place. The nematode can therefore re-program plant differentiation, and different endoparasitic nematodes re-program host cell development in different ways. Infection of the same host genotype of soybean by root-knot, cyst and reniform nematodes shows that each nematode induces their typical, distinctly different feeding sites (Jones and Dropkin 1975; Jones 1981). Early after a root-knot nematode has identified giant cell initials, such as in tissues examined 24 h after infection, there is no cell wall breakdown, but there is frequent evidence of recent mitotic events in cells immediately adjacent to the nematode or one or two cell layers away. The first sign of giant cell formation is stimulation of cell division and the formation of binucleate cells, in which the vesicles that would normally fuse to form the new cell plate between daughter nuclei after mitosis align apparently as in a normal cell plate, but subsequently fail to fuse and then disperse. This results in formation of a binucleate cell, but at this stage the cells are not obviously different in size

from neighbouring cells. This nuclear division cycle—nuclear mitosis without cytokinesis—is stimulated and repeated to generate four, eight, sixteen, thirty-two or more nuclei in developing giant cells. Apart from the first nuclear division, in which spindle alignment appears normal, its alignment is abnormal in later mitotic cycles. In later cycles, metaphase plates with different number of chromosomes have been documented, such that polyploid nuclei may form, and it is probable that endoreduplication without mitosis occurs during later stages. In some instances, partial cell plate vesicle fusion occurs, usually when nuclear division has occurred near the cell wall, and cell wall stubs can be found (Fig. 5.4c) (Jones and Payne 1978a). A similar alignment followed by dispersion of cell plate vesicles occurs when dividing plant cells are treated with caffeine (Jones and Payne 1978b). The process of giant cell development from the initial cells has been followed by electron microscopy over a period of 72 h, and composite tracings of electron micrographs of this process were provided by Jones and Payne (1978a). The J2 nematode retains the ability to move its head and insert its stylet into different giant cell initials during early and later stages of the interaction (Fig. 5.4d).

5.3.2 The Cytoskeleton

Giant cell initials start out as vacuolated cells, but they expand rapidly over a period of about 2 weeks; central vacuoles are replaced with many smaller vacuoles, and ground cytoplasm (ribosomes, polysomes etc.) and organelles (Golgi bodies, mitochondria, endoplasmic reticulum, etc.) rapidly increase in number and density. Nuclei and nucleoli also become enlarged, with amoeboid profiles and strongly contrasting heterochromatic regions (Jones 1981). Given the stimulation of mitosis in giant cell development, coupled with the rapid expansion and other cytological features, it is not surprising that alterations of cytoskeletal components occur in developing giant cells (de Almeida Engler et al. 2004 and Chap. 18). As the giant cells elongate and expand, surrounding cells of the gall also divide and expand to accommodate giant cell expansion, and gall cells outside giant cells do not become crushed. When giant cells development starts near the zone of elongation they are usually longer than when formed in other tissues, and intrusive growth of giant cells (that is, the extension of giant cells by separation and growth between normal cells, particularly at either end of the complex), may also occur (Jones and Northcote 1972; Jones and Payne 1978a).

5.3.3 Giant Cells as Multinucleate Transfer Cells

About 3 days after induction, wall ingrowths typical of transfer cells start to form where giant cell initials contact vascular tissues—xylem or phloem sieve elements, (Fig. 5.4). Transfer cells are normally found in plants in a number of anatomical locations where a substantial flux of solutes is thought to cross from the apoplast

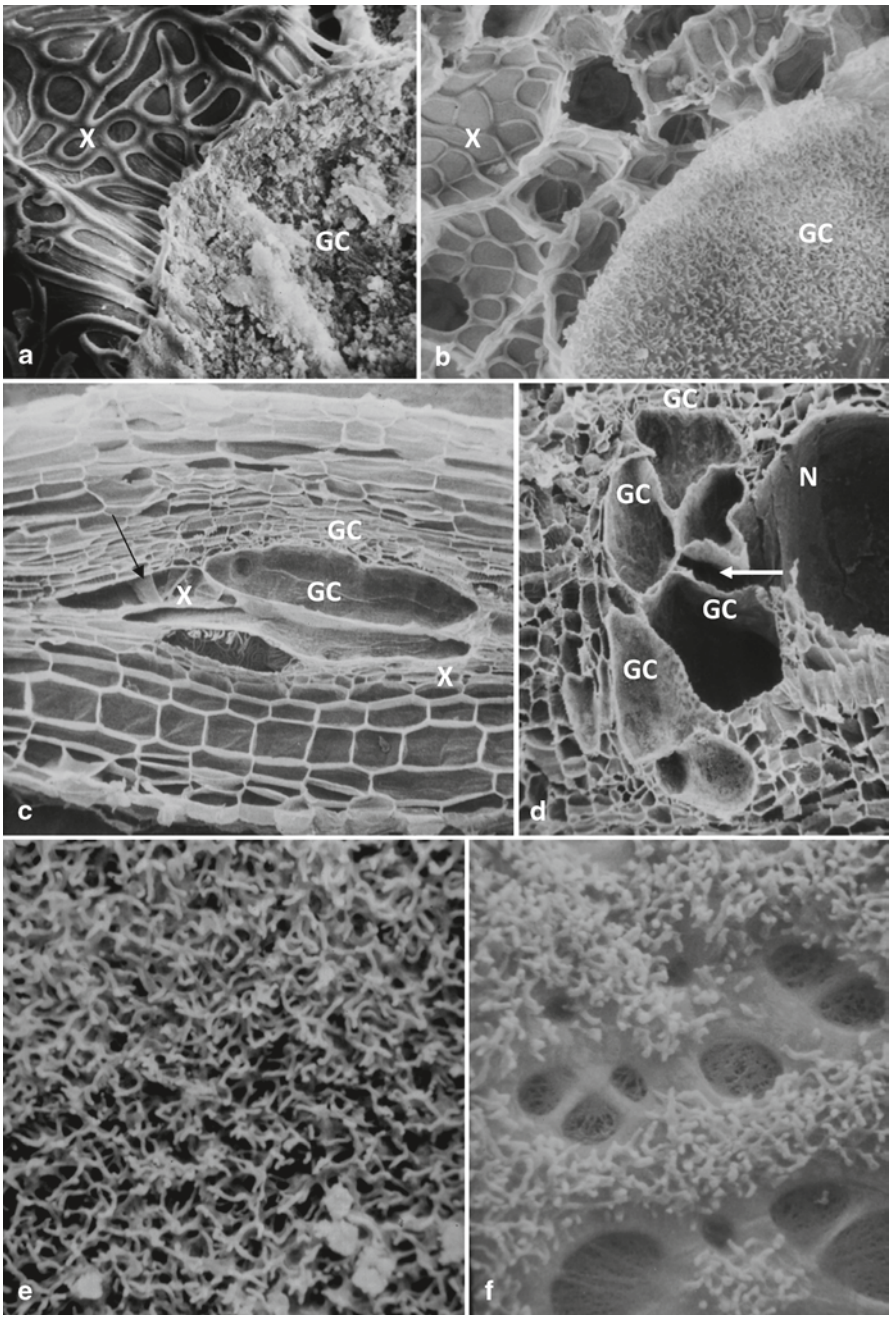

Fig. 5.4 Scanning electron micrographs of roots of *Impatiens balsamina* infected with *M. incognita*, cut through feeding sites: A—cytoplasmic contents undigested, B-F cytoplasmic contents removed. **a** 10 DPI, the giant cell is filled with granular contents, wound xylem has differentiated outside the giant cell (x595). **b** A similar giant cell with cytoplasm removed to show wall ingrowths next to xylem elements. **c** 10 DPI giant cells and surrounding gall cells, note (*arrow*) partial cross

(cell wall) compartment into the symplast (cytoplasmic compartment in which protoplasts are linked via plasmodesmata). These are typically in minor leaf veins, departing leaf traces, in root xylem parenchyma, at vascular discontinuities (e.g. the base of a developing cereal grain), in rhizobium nodules or the epidermis of water plants (Gunning and Pate 1974). The form of ingrowth is typical of each plant species, varying from branching finger-like structures to plates and flanges, and they are non-lignified secondary wall deposits. Wall ingrowths are typically polarised in the direction of solute flow. As giant cells enlarge, the extent of wall ingrowths, enveloped by the plasma membrane, increases dramatically opposite vascular cells (particularly xylem vessels), such that there is a 15–20 fold amplification in the surface area of the plasma membrane (Fig. 5.4b, e). In rare cases (e.g. *Helianthemum*) ingrowths of xylem and phloem transfer cells are morphologically different, and in root-knot nematode infected *Helianthemum*, when one giant cell abuts both xylem elements and phloem sieve tubes, the morphology of the wall ingrowths reflects the xylem type, not the phloem type, even next to sieve elements (Jones and Gunning 1976). When the walls between neighbouring giant cells are examined, very well developed wall ingrowths also occur in both giant cells, separated by thinner regions of walls where there are large pit fields that contain numerous plasmodesmata (Fig. 5.4f). This gives such walls a very irregular appearance in sections (sometimes mis-interpreted as wall breakdown) and the number of plasmodesmata suggests that secondary (*de novo*) formation of plasmodesmata may have occurred. This is in stark contrast to walls between giant cells and non-giant cells, where pit fields with plasmodesmata are not evident (Fig. 5.4b), and individual plasmodesmata, if present, are rare or perhaps non-functional (Jones and Dropkin 1976).

Because root-knot nematode giant cells form from pro-vascular cells, vascular continuity in the root is compromised, and adjacent cells often differentiate to form shorter ‘wound-type’ vascular elements with typical xylem thickenings (Fig. 5.4a, b, c)—in a symmetrical infection these wound xylem elements form an irregular cage-like structure around the giant cells. In some cases forming giant cells can revert to differentiate into tracheids with typical xylem wall thickenings, perhaps if the associated nematode stops feeding from it (Fig. 5.4c) (Jones and Dropkin 1976). Although not so obvious, phloem sieve elements also differentiate around giant cells to maintain phloem continuity.

5.3.4 Changes in Gene Expression in Giant Cells

Giant cells pass through a series of distinct stages during the life-cycle of the associated root knot nematode. These include the initial stages of induction, gall for-

walls in one giant cell, and apparent reversion of one developing giant cell to a xylem element with lignified thickenings (x85). **d** The space formerly occupied by and adult nematode (N) in relation to associated giant cells (GC), the arrow indicates the position of the head of the nematode (x115). **e** Extensive ingrowth development on an outer giant cell wall where it contacts xylem elements (x2,292). **f** Wall between two giant cells (from micrograph D). Note the pit fields, which contain numerous plasmodesmata, and wall ingrowths (x2,500). (From Jones and Dropkin, 1976)

mation, stimulation of nuclear division, mitosis without cytokinesis, wall ingrowth formation, cell elongation and expansion, increase in cytoplasmic contents, DNA endoreduplication and maximum metabolic activity and eventually senescence of the cell contents as the nematode ceases to feed and completes its life cycle. Some changes in gene expression can be inferred, for example, by studying the responses of transgenic plants with different promoter-reporter gene constructs, or by using mutant lines of *Arabidopsis*, on infection with root-knot nematodes. Many of the results from these approaches have been reviewed comprehensively for example by Gheysen and Mitchum (2009), to which the reader is referred. Some reporter genes linked to specific promoters are strongly up-regulated in giant cells at different times of their development. Most research has concentrated on identifying genes that are up-regulated early in giant cell formation (e.g. see Gheysen and Mitchum 2009), but there are also promoter/reporter gene combinations in which expression only occurs three weeks or more after giant cell induction. Alcohol dehydrogenase (*adh*) and plant haemoglobin promoters linked to a *gus* reporter gene are such examples, and since both of these promoters normally express under conditions of low oxygen tension, this suggests that the oxygen tension in giant cells drops at the time of peak nutrient demand by the egg-laying female (Hutangura 1999). Similarly there are many genes that are down-regulated in giant cells.

Using a sterile culture system with glass inserts to observe nematode feeding sites during the entire nematode life cycle by confocal laser-scanning microscopy (Blinco and Jones unpublished), transgenic *A. thaliana* plants with an enhancer trap based on the yeast transcriptional activator GAL4 and an upstream activation sequence (UAS) linked to a modified GFP marker were used to monitor changes in gene expression in and around giant cells. For example, in a line where GFP was under the control of elements conferring specific expression in sieve elements, strong up-regulation of GFP was also observed within the giant cells (Fig. 5.5a, b). Strong down-regulation of endodermal cell specific GFP expression was observed around the giant cells in a second line (Fig. 5.5c, d), suggesting that a functional endodermal cell layer may not be present around giant cells.

5.3.5 Changes in the Cell Cycle in Giant Cells

As indicated above, there is no doubt that the mitotic cycle is stimulated in giant cells, although it is not followed by cytokinesis. In plant cells, there are five groups of cyclin genes: A, B, C, D and H, which encode the regulatory subunits of specific protein kinases. These genes are responsible for controlling the stages of cell cycle progression, particularly at the principal control points late in the G1 phase and at the G2/M boundary (Francis and Sorrell 2001). A- and B- type cyclins accumulate periodically through G2 and early M phases, with A-type cyclins also essential during the S phase (Joubès et al. 2001). D-type cyclins are important in regulating the progression of the G1 phase, and in the transition from G1 to S. Cell cycle re-entry is one of the first events to occur in GC initials. Expression of A- and B-type cyclins

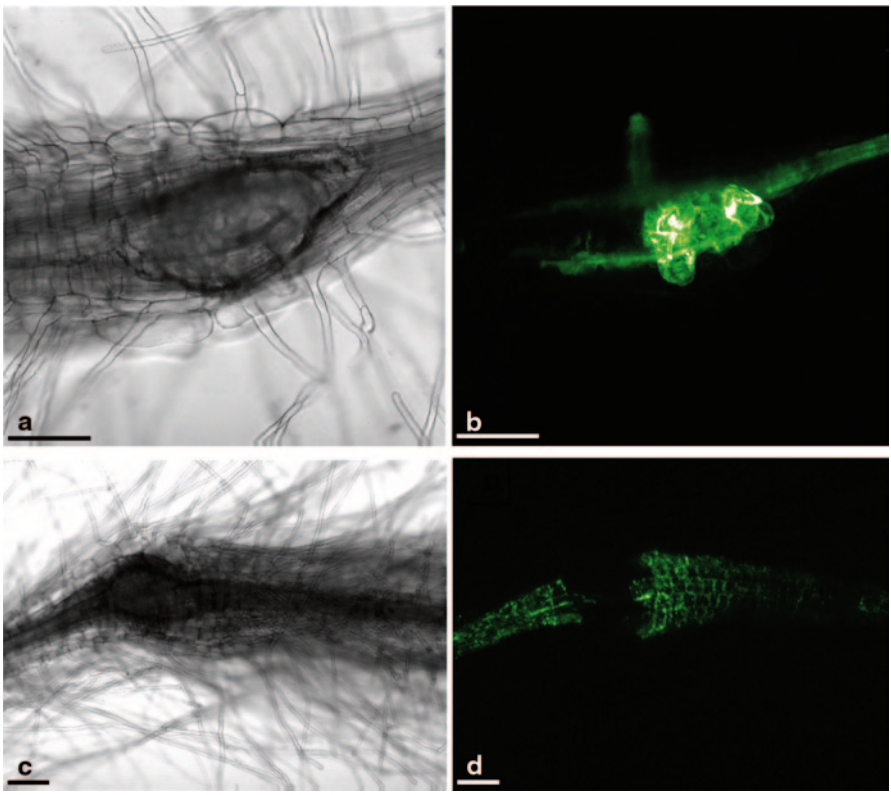

Fig. 5.5 Transgenic *Arabidopsis thaliana* plants with an enhancer trap (yeast transcriptional activator GAL4 and an upstream activation sequence UAS linked to a modified GFP marker) infected with *M. javanica* (line numbers refer to Nottingham Arabidopsis Stock Centre accessions). In line N9156, GFP expression is localised to phloem cells and giant cells. **a** The transmitted light image shows the giant cells in the central cylinder of the root at 6 DPI. **b** Confocal image shows strong up-regulation of GFP which co-localises with the giant cells seen in **a**. **c** and **d** GFP is expressed in the endodermis and root cap cells of line N9124. C Transmission and D confocal images of this line at 5 DPI. Panel D shows complete down-regulation of GFP in endodermis cells surrounding the giant cells (this pattern was retained to at least 20 DPI). Scale bars: 100 μm

has previously been studied in root-knot nematode-induced GCs and cyst nematode induced syncytia in *Arabidopsis* (de Almeida Engler et al. 1999; Goverse et al. 2000). Using a GUS reporter gene, strong promoter activity of both the CycA2;1 and the CycB1;1 genes was observed in young GCs and neighbouring cells within the gall tissue (de Almeida Engler et al. 1999). This is evidence for activation of cell cycle progression through S to G2 and G2 to M phases. However, it is possible that the trigger for the initiation of GC formation occurs before the S phase. Using laser capture microdissection (LCM) to collect cytoplasmic contents from 4 day post-inoculation giant cells in tomato roots followed by RT-PCR two D-type cyclin genes, LeCycD3;2 and LeCycD3;3, were expressed at higher levels in giant cells

compared to other cell-cycle-related cyclin genes, suggesting that the induction of the G1 phase of the cell cycle may be triggered in response to stimulation by the infecting nematode (Ramsay et al. 2004). A series of experiments aimed at understanding changes in cell cycle events in developing giant cells, using, for example, inhibitors of the cell cycle, cell cycle mutants and silencing specific cell cycle genes has been summarised by Gheysen and Mitchum (2009). The overall picture is quite complex, and the current information on this aspect is described in more detail in Chap. 17.

5.3.6 Plant Growth Regulators

A number of studies on relations of root-knot nematodes with host plants suggest that plant hormones, including auxins and cytokinins, are involved in giant cell and gall formation (e.g. Jones 1981; Hutangura et al. 1999; Karczmarek et al. 2004). Auxin responsive promoters are up-regulated early in giant cell formation (18–24 h post infection), but by 96–120 h post infection the response is much lower, suggesting that auxin accumulation is needed as a trigger for giant cell formation, but not for later enlargement. The local increase in auxin is probably a result of inhibition of local auxin transport, which correlates with gall and lateral root formation, and reduction of root tip growth, and may be preceded and regulated by changes in flavonoid metabolism (Hutangura et al. 1999). The involvement of cytokinins is less clear—similar experiments suggest that cytokinin levels are raised before giant cell development starts, but are not raised in giant cells (Lohar et al. 2004), nevertheless, cytokinin treatment can convert resistant plants to be susceptible to root-knot nematodes (Bird and Loveys 1980).

5.3.7 Giant Cells and Feeding Tubes

Although not present in the initial stages of giant cell formation, the presence of ‘feeding tubes’ is a typical feature of the root-knot nematode giant cell interaction. They were first noticed by light microscopy as straight rods in giant cells, and the first electron micrographs of their structure were described by Paulson and Webster (1970) and Jones and Northcote (1972). Feeding tubes in giant cells appear to form rapidly when feeding starts, within about 15 min of stylet insertion (Wyss and Zunke 1986) by self-assembly of nematode secretions, probably originating from the dorsal oesophageal gland cell. The stylet aperture is located just to one side of the tip of the mouth stylet, and the consensus view is that the stylet is only just inserted through the giant cell wall, such that at the stylet aperture the plasma membrane of the giant cell is breached but a seal is maintained. Nematode secretions are released that then self-assemble into the feeding tube (rather like the self assembly of rod-like virus coat proteins), which is sealed at the distal end and grows from the basal

end, the diameter being relatively constant (about 1 μm) and the length varying up to 100 μm. The aperture of the nematode stylet is continuous within the lumen of the feeding tube, and the tube itself is semi-crystalline (Jones and Northcote 1972) and proteinaceous. When formed, the feeding tube interacts with host components, and is surrounded by a complex mass of host membranes. Because root-knot nematodes can feed from different giant cells, the stylet must be withdrawn after feeding from one giant cell and the associated feeding tube is released such that a number of feeding tubes can be found in the cytoplasm of one giant cell. The latter are no longer surrounded with the complex membrane system.

The role of the feeding tube is generally thought to be twofold—it acts as an ultrafilter and as a pressure regulator. The size exclusion value for root-knot feeding tubes has been studied by expression of fluorescent proteins in transgenic plants, and appears to be between 32–40 k Da (e.g. Urwin et al. 1997; McCarter et al. pers. comm): this is somewhat larger than the size exclusion for feeding tubes in cyst-nematode syncytia (see Chap. 4). Because of the raised solute levels in giant cells, their hydrostatic pressure appears to be very high, and so the feeding tube also serves to ensure that the giant cell does not burst or leak when a nematode feeds from it. The absence of a feeding tube as giant cells are initiated may be significant in that it may enable uptake of siRNAs by the nematode that triggers RNAi, such that early concerns that feeding tubes might prevent this mechanism of transgenic resistance appear not to be a problem (Bakhetia et al. 2005).

5.3.8 Electrophysiology of Giant Cells

The ability of giant cell plasma membranes to seal around inserted objects, such as an electrophysiological micropipette, is also remarkable. In a series of electrophysiological experiments by Jones et al. (1975), in contrast to other cells, they were able to make continuous recordings of transmembrane potentials from giant cells over many hours whilst carrying out electrophysiological experiments. These were the first results to indicate H^+ ion co-transport was involved in uptake of sugars in higher plants (Jones et al. 1975), and a model for how this might occur at wall ingrowths is provided in Jones (1981). Subsequent research supports this proposal that H^+ ATPase symporter proteins (e.g. for sucrose uptake and amino acid permeases) are co-localised at high density in the plasma membranes of transfer cell ingrowths (Offler et al. 2002). Jones et al. (1974) also recorded a series of action potentials from giant cells, that were regular in nature, reproducible, and not associated with nematode feeding. Similar trains of action potentials, apparently reflecting the same phenomenon have been recoded as rhythmic Ca^{2+} oscillations—termed ‘calcium spiking’. In plants, Ca^{2+} oscillations are involved in abscisic acid signaling in guard cells, in pollen tube elongation, and more significantly in the nodulation factor (NF) signal transduction pathway of legumes (Capoen et al. 2009). The underlying mechanism is a rhythmic sequestration and release of Ca^{2+} ions from the endomembrane system.

5.4 Giant Cell Induction—A Deliberate Controlled Event

With information on the molecular process involved in giant cell formation remaining elusive, it is possible to question whether they are simply induced by the plant itself as an endogenous response to nutrient withdrawal by the nematode, rather than by input of specific nematode molecules. In terms of the general processes involved in development and function of endoparasitic nematode feeding sites—giant cells, syncytia or nurse cells—there are two general phases, initial establishment of the feeding cells followed by formation of the mature transfer cell-like structures. The answer to the above question can be found by comparison of the first phase of initial establishment. Although different species of endoparasitic nematodes always induce a similar final form of feeding structure, clear differences exist in the nature of cell expansion, wall degradation, and stimulation of mitosis (Jones and Dropkin 1975; Jones 1981), indicating that initial feeding cell establishment is a deliberate process differentially controlled by secretions from the different nematode species.

Once feeding cells have been established, the second phase could in a large part relate to the nematode acting as a nutrient sink, such that withdrawal of solutes induces a programmed response to the source- sink situation in which the plant cells attempt to maintain homeostasis. This process includes increased transcriptional, metabolic and transport properties common to different feeding structures after their establishment (Jones 1981). Development of transfer cells in plants can be thought of as forming in this manner, with the typical formation of wall ingrowths, increase in cytoplasm and mitochondria being induced by the source-to-sink flow of solutes. If this is correct, it remains to be determined for how long initial nematode secretions are required, or if their nature changes during the nematode-host cell interaction in a way that reflects differences in patterns of gene expression in different feeding structures.

5.4.1 Nematode Secretions

Induction of feeding cells involves a delicate interaction between host and parasite with secretion of specific components, mainly from gland cells, into the giant cell initials. The subventral gland cells appear to be more active during the J2 migration phase, whereas the dorsal gland cell is more active during the prolonged sedentary phase (Hussey 1989). Some of the components, encoded by nematode parasitism genes have been identified as proteins and peptides, and have been termed collectively the ‘parasitome’—that is, those genes/products that are required by the nematode for successful parasitism. Various approaches have been used to identify parasitism genes and new knowledge has been generated rapidly as molecular techniques have advanced. A major advance was to isolate mRNA from glands of dissected parasitic J2s and to identify genes expressed in gland cells with the potential to be secreted into host cells (Huang et al. 2003). Proteomics approaches have also

been used to identify proteins present in secretions of parasitic J2s that may be involved in modifying host cells (Jaubert et al. 2002; Bellafiore et al. 2008). Of the 50 or more parasitism genes expressed in root-knot and cyst nematode gland cells, few of the secreted products are common between these species, and more than 70% are pioneer genes with no significant homology in databases (Davis et al. 2009). The availability of full genome sequences of two species of root-knot nematodes (*M. hapla* and *M. incognita*) undoubtedly with more to come, application of comparative genomics, and new approaches for functional analysis of parasitism genes, such as over-expression in plant cells or silencing by RNA interference in the nematode, will provide new information that will lead to a much better understanding of how giant cells and other nematode feeding cells are induced and maintained. These aspects are described in more detail in Parts II and III of this volume.

5.5 Host Resistance to Root-Knot Nematodes

Although the focus of this chapter is on the susceptible plant response, and the formation of giant cells by root-knot nematodes, there are plants with natural resistance to root-knot nematodes, either as a result of being a non-host or through deployment of specific resistance genes. A series of genes that confer specific resistance to nematode infection have now been identified and characterised. These are typically of the 'R' gene type in which an avirulence (*Avr*) component from the nematode is recognised by the R gene, followed by a signalling cascade in plant cells in which usually a hypersensitive resistance response prevents successful nematode root colonisation. Probably the best characterised of such resistance genes is the *Mi-1.2* gene, which was introduced into tomato in a cross with *Solanum peruvianum*. This gene encodes a typical nucleotide binding leucine rich repeat (NB/LRR) cytoplasmic protein, which confers resistance to *M. incognita*, *M. javanica* and *M. arenaria*, and is remarkable in that it also confers resistance to the potato aphid (Rossi et al. 1998) and the white fly *Bemesia tabaci* (Nombela et al. 2003). Natural resistance genes may be transferred by conventional plant breeding or by introduction into transgenic plants, although compatibility in the signalling cascade is required for them to function in different host plants. Transgenic resistance may also be achieved using RNA interference (RNAi) targeted to nematode genes (Yadav et al. 2006), or by prevention of feeding cell formation (Jones et al. unpublished).

5.6 Concluding Remarks

Root-knot nematodes are ubiquitous, polyphagous and highly successful plant pathogens, which have probably evolved from free-living nematodes by a process that probably included horizontal gene transfer of bacterial genes and they have subsequently evolved the capacity to control the development of giant cells at

their feeding sites. They live in a benign environment in the root protected by surrounding gall cells, with a ready made food source available to them while they complete their life cycle. From being a relatively neglected group of pathogens, plant parasitic nematodes, and particularly root-knot nematodes, are now at the forefront of the latest research. Some of this new emphasis can be attributed to the availability of significant new resources derived from the choice of *C. elegans* as the model nematode. Certainly, the pace of advancements in understanding this host-pathogen interaction is accelerating, although often each new piece of information requires further testing and raises new questions. However, even without the *C. elegans* model, the host of advances in technology would still have been applied to study of nematode-plant interactions. In particular, advances in microtechniques have enabled changes in gene expression and metabolism in giant cells to be greatly expanded. Combined with new DNA and transcriptome sequencing technologies for both host plant and nematode pathogens, improvements in bioinformatics and comparative genomics that enable large new datasets to be analysed and compared, and an ability in functional genomics (e.g. RNAi and overexpression) to study gene function, we are now entering a new era in which we can expect to develop fundamental understanding of how a root-knot nematode finds the correct cells and re-programs them to form giant cells for their benefit. Much new information is provided in later chapters of this book, and we can predict confidently that this will result in new effective methods to control plant-parasitic nematodes.

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Part II
Resources for Functional Analysis of Plant-Nematode Interactions

Chapter 6
Genome Analysis of Plant Parasitic Nematodes

Pierre Abad and James P. McCarter

Abbreviations

7TM	Seven transmembrane
CAZyme	Carbohydrate active enzyme
daf	dauer formation
dsRNA	double stranded RNA
GHF	glycosyl hydrolase family
HGT	horizontal gene transfer
PPN	plant parasitic nematode
RdRP	RNA-dependent RNA Polymerase
RISC	RNA-induced silencing complex
RKN	Root Knot Nematode
RNAi	RNA interference
siRNA	small interfering RNA
SL	Splice Leader

6.1 Introduction

Nematodes are simple roundworms consisting of an elongated stomach and a reproductive system inside a resistant outer cuticle. Their small size and their ability to adapt to severe and changing environmental conditions have made nematodes

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the most abundant of all animals, accounting for an estimated four of every five individual animals on earth. There are about 10,000 described species classified in the phylum Nematoda. However, there may be as many as half a million species yet to be discovered. If this estimation is anywhere close to correct, it would mean that roundworms are the second most diverse group of animals on earth, trailing only the arthropods. Nematodes may be free-living, predaceous, or parasitic. The last group represents a major challenge to human health and agriculture. Presently, parasitic nematodes, including whipworm, *Ascaris,* hookworm, and filarial worms, infect more than one billion people whereas plant parasitic nematodes cause annually an estimated 100 billion euros in crop damage. Among them, root-knot nematodes (RKN) are the most damaging species infecting almost all cultivated plant species.

Nematodes have been a focus of genomics since large-scale sequencing projects began in the early 1990s: *Caenorhabditis elegans* was the first genome of a multicellular organism that was completely sequenced, providing a platform for further nematode genomics (*C. elegans* Sequencing Consortium 1998). Comparison of the *C. elegans* and *C. briggsae* genomes has allowed a powerful means of understanding the genetic basis for the development of these relatively simple metazoans. While these two *Caenorhabditis* species are nearly indistinguishable even at the microscopic level, their most recent common ancestor existed 80–110 million years ago, and thus they are more evolutionarily distant than, for example, human and mouse, a degree of divergence reflected in their genomes (Stein et al. 2003). Subsequently, genome sequence of additional *Caenorhabditis* species (including *C. remanei, C. brenneri, C. japonica*) greatly improved the annotation of the *C. elegans* genome, helping us to understand the evolutionary forces that shape nematode genomes. Recently, the genome of *Pristionchus pacificus* has provided a unique perspective on its nematode lifestyle (Dieterich et al. 2008). Despite the wealth of information available for this group of nematodes very little is known about most members of this important phylum.

Parasitic nematodes, which constitute about half of nematode species and which have huge impacts on human health and nutrition, remain largely unexplored. Analysis of the genome of the human filarial nematode parasite *Brugia malayi* revealed significant differences with *Caenorhabditis* spp. Notably, the predicted proteome provided evidence for adaptations of *B. malayi* to niches in its human and vector hosts and insights into the molecular basis of a mutualistic relationship with its *Wolbachia* endosymbiont (Ghedin et al. 2007). The differences in genome content and organization between *Caenorhabditis* and *B. malayi* underscored the importance of obtaining additional genome data from representative species from across the diversity of the Nematoda. Plant nematology has also entered an era of genomics. At this writing, two complete plant parasitic nematode genomes, the root knot nematode (RKN) species *Meloidogyne incognita* and *M. hapla*, are available. In addition, a draft genome assembly from the soybean nematode *Heterodera glycines* is available and the genome of the potato cyst nematode *Globodera pallida* is in progress. These sequences will be a platform for analysis of traits related to plant parasitic abilities. The two independent RKN projects led to identification of genes reflecting the contrasting biology of these two nematode species, and constitute the first step in comparative and functional genomics for plant parasitic nematodes (PPN). This

provides new insights into these pests and should aid the development of environmentally sustainable strategies to control these important agricultural pests.

In this review we will focus only on the outstanding features of these two RKN genomes. In the primary genome publications (Abad et al. 2008; Opperman et al. 2008), a complete description of the two genomes are available for interested readers. In addition, assemblies of both genomes are available in the public databases, including GenBank, and at project web sites (www.hapla.org and http://meloidogyne.inra.fr).

6.2 Sequencing Strategies

The RKN genus, *Meloidogyne* spp, belongs to the order Tylenchida, a very large and diverse group of nematodes, which contains the majority of the known plant parasitic species. Representatives of this order have a worldwide distribution and are encountered more frequently than any other group of nematodes. More than 80 RKN species have been described differing in morphology, host-range, geographic distribution, cytology and mode of reproduction (Eisenback and Triantaphyllou 1991; Karssen 2002). Although belonging to the same genus, *M. incognita* and *M. hapla* are very different in their biology. The first is an asexual and polyphagous species whereas the second can reproduce sexually and has a narrower host range. *M. incognita* reproduces by mitotic parthenogenesis and more than 95% of the isolates studied have 2n = 36–42 chromosomes with a haploid genome (n = 18). Therefore, it is generally assumed that these *M. incognita* isolates have extensive polyploidy and/or aneuploidy, a common feature in species that have lost meiosis. In contrast *M. hapla* has a meiotic reproduction lifestyle and controlled genetic crosses are possible, a feature that has been exploited to produce a genetic map.

The *M. incognita* genome has been sequenced in France under the initiative of the Nematology group at INRA Sophia Antipolis in close collaboration with the Génoscope at Evry (the French center for sequencing), and the Bioinformatic platform at INRA Toulouse. The ensemble of predicted and automatically annotated protein coding genes was manually annotated by an international consortium of 17 laboratories. Each laboratory focused on a particular process or gene family relevant to the different aspects of *M. incognita* biology. The *M. hapla* genome has been sequenced under the initiative of the Department of Plant Pathology at North Carolina State University. The inbred strain VW9 was chosen for sequencing due to its genetic resources including a linkage map (Liu et al. 2007). These two genome sequencing projects were carried out using the whole-genome shotgun approach using a combination of genomic libraries with small insert sizes (1–4 kb and 5–10 kb). 30–40 kb large insert fosmid libraries were also constructed. The shotgun sequencing strategy was applied using classic Sanger dideoxy chain-terminator on automated platforms. In shotgun sequencing, DNA is broken up randomly into numerous small segments, which are sequenced using the classical chain termination method to obtain reads. Typically, the read length of these sequences is 600–800 bp. Multiple overlapping reads for the target DNA are obtained by performing several rounds of this frag-

mentation and sequencing. Computer programs then use the overlapping ends of different reads to assemble them into a continuous sequence. For genome annotation, the protein-encoding genes were predicted by using Glimmer and FgenesH in case of *M. hapla* and the integrative gene-prediction EuGene for the *M. incognita* genome assembly (Foissac and Schiex 2005).

6.3 Genome Organisation

Comparative analysis of these two RKN genomes reveals striking differences in their genome organizations and sheds light on the mechanisms and evolution of parasitism. One very interesting feature of the genome of *M. hapla* is its compactness and its very small size. The assembled scaffolds of *M. hapla* VW9 provided 10.4-fold genome coverage and an estimated haploid genome size of 54 Mbp (million base pairs) with sequence spanning >99% of the genome (Opperman et al. 2008). The genome possesses a relatively small percentage of moderately repetitive DNA (~12%) with low complexity sequences and a high A+T content. Therefore, this genome represents not only the smallest nematode genome yet completed, but also the smallest metazoan genome characterized so far. This size has to be compared for example, with another plant parasitic nematode, the soybean cyst nematode *H. glycines* with an estimated genome size of 92.5 Mb (Opperman and Bird 1998) or to other nematode genome such as the free-living nematode *C. elegans* or the necromenic beetle-associated *P. pacificus*, which are 100 Mb and 142 Mb respectively (*C. elegans* Sequencing Consortium 1998; Dieterich et al. 2008). By contrast the organization of the *M. incognita* genome is much more unusual and sheds light on the genetic consequences of reproduction by asexual mitosis. The assembled sequence reads gave a total coverage of 86 Mb which is almost twice the estimated size previously obtained by flow cytometry and C*ot* analysis, with values ranging from 47 to 51 Mbp (Pableo and Triantaphyllou 1989; Leroy et al. 2003). The average sequence divergence between the aligning regions is 7 and 8%, which is among the highest observed for a sequenced heterozygous organism. We also found an additional 3.35 Mb of the assembly composed of supercontigs that align with two of the previously identified supercontig pairs, thus corresponding to triplicated genomic regions (Fig. 6.1). The observation that most of the assembly is present as pairs of homologous but divergent segments suggests that despite being a parthenogenetic aneuploid species, *M. incognita* is evolving, in the absence of sex, towards effective haploidy as proposed for bdelloid rotifers (Mark Welch et al. 2008). These so far poorly characterised mechanisms of genetic plasticity could account for the rapid adaptation to environmental changes and the extremely wide host range and geographic distribution of this species. As the first obligate plant pathogenic animal to be sequenced, *M. incognita* could constitute a model for evaluating the impact of these mechanisms at the genomic scale.

Computational predictions from genomic sequence indicate a proteome of 14,454 protein coding genes in *M. hapla* and 19,212 protein coding genes in *M. incognita*. Gene number differences between these nematodes could be explained

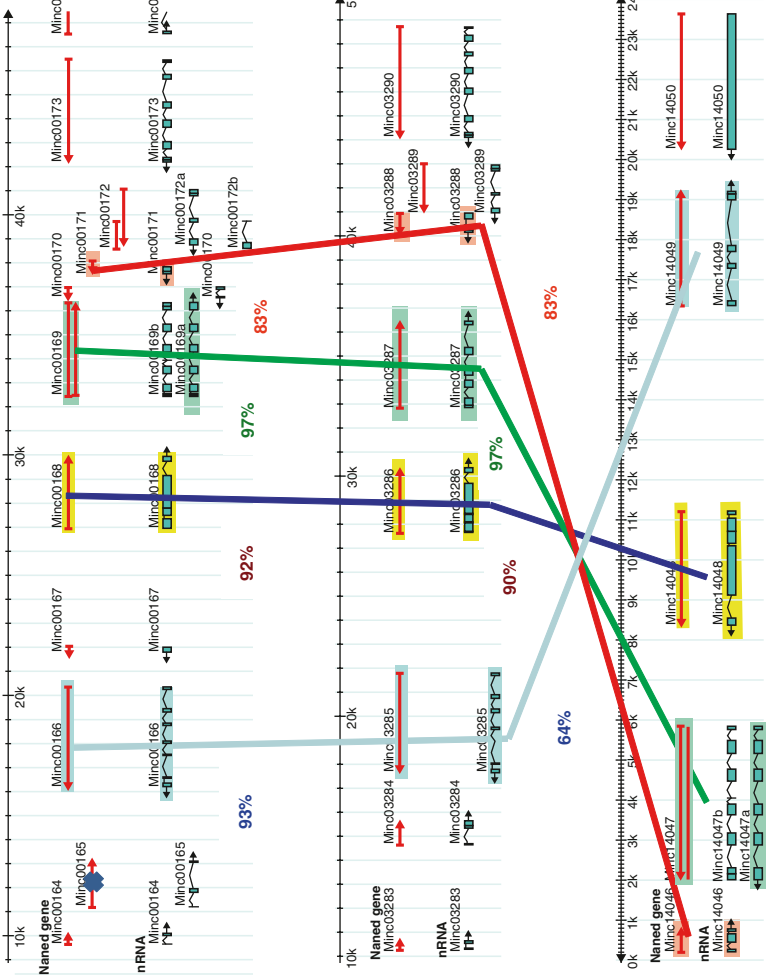

Fig. 6.1 Example of three allelic regions in the *M. incognita* assembly. (Three aligning portions of *M. incognita* contigs are represented, MiV1ctg2, MiV1ctg67 and MiV1ctg757, the first two being in sense orientation and the last one in antisense (as illustrated by *black arrows*). Percent identities between proteins encoded by pseudo-allelic gene models on different contigs are indicated in color boxes. Exons are represented by boxes and are linked together to form genes. Arrows indicate the direction of transcription. Gene order is not well conserved between these three allelic regions)

by the *M. incognita* genome organization. Interestingly, 69% of protein sequences in this species are less than 95% identical to any other protein. The high divergence between pseudo-alleles is probably due to the absence of meiotic recombination. For comparison, 20,621 and 29,201 genes were predicted in non parasitic nematodes such as *C. elegans* and *P. pacificus* respectively, and 11,515 were predicted in the human parasite *B. malayi*. Gene loss in parasitic nematodes compared to free-living species is a clear attribute of a parasitic life style where selective pressure on anabolic processes is reduced by the presence of host-supplied nutrients and more generally, a host-controlled environment.

A major source of conserved local synteny in nematodes is the existence of operons (Spieth et al. 1993). In *C. elegans*, operons are defined as sets of adjacent cotranscribed genes, which are characterized by a small intergenic distance and an alternation between splice leaders (SL), such as SL1 splicing of the first gene and SL2 (or related splice leaders) splicing of downstream genes (Blumenthal et al. 2002). The vast majority of the ~1,000 *C. elegans* operons have a conserved counterpart in *C. briggsae* (Stein et al. 2003). This number drops dramatically looking at more distantly related nematodes such as *Meloidogyne* species and the comparison is complicated by the evolution of SL sequences in the nematode phylum. Based on gene spacing, 1,585 putative operons were identified in the *M. incognita* genome (Abad et al. 2008). 516 operonic genes have a *C. elegans* ortholog, yet only nine operons, each containing two genes, are shared between *C. elegans* and *M. incognita*. In *M. hapla*, operons could not be predicted based on gene spacing but 140 candidate operons were indicated by the close proximity of gene pairs each having a *C. elegans* ortholog in the same operon. Taken as a whole, these results indicate that most operons and synteny in general are not ancient conserved features in nematodes. Comparisons of *C. elegans* to *B. malayi* are consistent with the rarity of conserved operons and synteny, though linkage is conserved (Ghedin et al. 2007).

6.4 Plant Parasitism

Compared to its free-living relatives, one specific requirement of plant root parasites is their ability to invade plant roots. The RKN have developed specific capabilities and complex biotrophic interactions adapted to its hosts by navigating between cells to reach its feeding sites and eluding host defense responses for the several weeks during which the feeding site is required to support female development up to egg hatching.

One of the most remarkable findings that has emerged from these RKN genome studies is the identification of an extensive set of plant cell wall-degrading, carbohydrate-active enzymes (CAZymes), which has no counterpart in any other animal genome. This “suite” of enzymes likely modifies and subverts the host to support nematode growth. First evidence for the presence of cellulase genes and activity in plant parasites was provided by Smant et al. (1998). The complete RKN genome sequences now provide a very broad set with the presence of 61 CAZymes genes

Table 6.1 *Meloidogyne* enzymes with predicted plant cell wall-degrading activities, compared with those from others nematodes and *D. melanogaster*

Species Family	Cellulose/Xylan		Pectin/Pectate		Arabinose	Total
	GH5_2	GH5_8	GH28	PL3	GH43	
M. incognita	*21*	*6*	*2*	*30*	*2*	*61*
M. hapla	*6*	*1*	*2*	*22*	*2–3*	*33–34*
P. pacificus	0(7)	0	0	0	0	0(7)
C. elegans	0	0	0	0	0	0
B. malayi	0	0	0	0	0	0
D. melanogaster	0	0	0	0	0	0

Number of genes encoding enzymes with candidates activity on different substrate is listed in the different species
GH glycoside hydrolases, *PL* Polysaccharide lyases

from five different families in *M. incognita* and 33 corresponding genes in *M. hapla* (Abad et al. 2008) (Table 6.1). This difference in gene number could be attributed to the particular genome structure of *M. incognita*. This set includes cellulases, xylanases and other glycosylhydrolase family members (GHFs). Several of these enzymes are present as multigene families. For example, more than 20 candidate cellulases and 30 candidate pectate lyases are found in the *M. incognita* genome. Interestingly, *M. incognita* and *M. hapla* differ in the exact copy numbers of these enzymes with cellulases being expanded in *M. incognita* and pectate lyases being expanded in *M. hapla* (Abad et al. 2008; Opperman et al. 2008). Additional CAZymes such as enzymes from family GH43 (candidate arabinases) and family GH32 (invertases) have been identified in these RKN genomes. Invertases catalyze the conversion of sucrose (an abundant disaccharide in plants) into glucose and fructose, which can be used by *M. incognita* as a carbon source.

All family members were possibly acquired by horizontal gene transfer (HGT) because similar genes are absent from free-living nematodes and the genes of PPN are most similar to those of unicellular organisms and bacteria (Smant et al. 1998; Abad et al. 2008; Opperman et al. 2008). One of the key adaptations towards plant parasitism therefore appears to have been achieved by the acquisition of cell-wall degrading enzymes by HGT. As a supplement to their arsenal of plant cell wall degrading CAZymes, large number of candidate expansin genes (20 in *M. incognita*) were found in RKN genomes (Table 6.1). While the precise biochemical function of these proteins remains unknown, it has been shown that PPNs produce expansin-like proteins (Qin et al. 2004) which may disrupt non-covalent bonds, loosening the plant cell wall and making the components more accessible to plant cell wall degrading enzymes (Cosgrove 2000). Apart from plant cell wall related CAZymes, family members of secreted chorismate mutases were identified as PPN-specific genes (Lambert et al. 1999; Huang et al. 2005). These enzymes also most closely resemble bacterial enzymes, again suggesting a critical role for HGT. In spite of the fact that the donors and the exact mechanisms of HGT remain elusive, these events might represent one of the key process that have helped shape the evolution of plant parasitism within RKNs and other plant parasitic nematodes.

6.5 Gene Family and Pathway Conservation and Diversification Among Plant Parasitic and Free-Living Nematodes

The phylum Nematoda is estimated to have originated 650–1,200 million years ago with parasitism having arisen independently at least seven times (Blaxter 1998; Blaxter et al. 1998; Wang et al. 1999). Given this ancient history, it is perhaps not surprising that molecular diversification has been extensive and that the six nematode genomes published to date differ vastly from one another. Total protein-coding genes per genome vary from approximately 11,500 to 23,500, and the genomes share only about 3,000 highly conserved genes that can be identified as 1:1 orthologs in all the genomes. For example, *M. incognita*, *C. elegans*, and *B. malayi* share 3,533 orthologs by orthoMCL (Abad et al. 2008).

One major source of genome variation is the changing composition and size of gene families (Table 6.2). A number of gene families, including those encoding seven transmembrane (7TM) receptors, nuclear hormone receptors, and collagens are observed to be larger in the genomes of free-living nematodes than parasitic nematodes so far sampled. The most striking example of gene family expansion/contraction is in 7TM or G-protein coupled receptors with ~1,200 in *C. elegans*, including many involved in chemoreception (Troemel et al. 1995), versus fewer than 150 in *Meloidogyne*. Additional examples of gene families that are smaller in *Meloidogyne* than *Caenorhabditis* are lysozymes, C-type lectins, chitinases, glutathione S-transferases, and cytochrome P450s possibly indicating that plant parasitic nematodes benefit from spending a portion of their lifecycle within

Table 6.2 Comparisons of protein-coding genes among nematode genomes[a]

Species Estimated Number of	*C. elegans*	*C. briggsae*	*P. pacificus*	*M. incognita*	*M. hapla*	*B. malayi*
Protein Coding Genes	21,193	19,500	23,500	19,212	14,420	11,515
C. elegans Orthologs	–	12,200	13,404	4,309	4,943	5,780
7TM Receptors	1,011–1,280	386–429	NA	108	147	NA
Nuclear Hormone Receptors	284	268	NA	62	76	39
Collagens	154–165	NA	NA	100	81	83
Glutathione-S-transfers	44–51	NA	54	5	NA	NA
Proteases	439	359	NA	339	NA	97
Kinases	411	NA	NA	499	NA	215
Proteins with a Signal Peptide	7,921	5,869	NA	4,438	1,534	2,002

[a] Tabulations from the published genomes of the free-living soil nematodes *C. elegans* (*C. elegans* Sequencing Consortium 1998) and *C. briggsae* (Stein et al. 2003), the human parasite *B. malayi* (Ghedin et al. 2007), the root knot nematodes *M. incognita* (Abad et al. 2008) and *M. hapla* (Opperman et al. 2008) and a necromenic nematode associated with beetles, *P. pacificus* (Dieterich et al. 2008)

root tissue that may provide protection from a diversity of both biotic and abiotic stresses (Abad et al. 2008; Opperman et al. 2008). Besides the CAZymes discussed above, gene families expanded in *M. incognita* relative to *Caenorhabditis* include fucosyltransferases and SUMO cysteine proteases. Changes in gene family size can result from gene loss during adaptation to parasitism as well as gene family expansion or contraction in any individual species lineage. In most cases, there is not yet a rich-enough picture of nematode genome evolution to determine whether the observed gene family size in a species is due to an expansion in one lineage or a contraction in another. For instance, comparing the two characterized genomes in the *Caenorhabditis* genus, 202 gene families differ in composition at least two fold, with 118 enriched in *C. briggsae* and 84 enriched in *C. elegans*, and each of these nematodes is non-parasitic and derived from a soil environment (Stein et al. 2003).

Continued molecular evolution in individual branches of the nematode phylogenetic tree has also created innumerable genes which are not recognized as belonging to any widely conserved gene family. For *M. incognita*, comparison to seven other predicted proteomes (three nematodes, one insect, and three fungi) by OrthoMCL indicated that 52% of root knot genes products had no homolog in the other species (Abad et al. 2008). Likewise, when the *M. hapla* predicted proteome was used to search the Pfam database of protein domains, 66% of gene products lacked any match (Opperman et al. 2008). Characterization of these more rapidly evolving genes has resulted in the finding of tens-of-thousands of nematode-specific protein families, varying from phyla-specific to species-specific (Parkinson et al. 2004; Wasmuth et al. 2008; Yin et al. 2009). In both plant and animal parasitic nematodes, among the most rapidly evolving genes appear to be those that contain a signal peptide (Table 6.1) and are secreted from cells (Harcus et al. 2004). Accelerated evolution could be due to selective pressure from the host or to relaxation of functional constraints. In plant parasitic nematodes, secretory products from the oesophageal glands are known to play roles in migration (e.g. CAZymes) and feeding site formation (as described in other chapters in this book). In *M. hapla*, 832 proteins were identified with a signal sequence but lacking a transmembrane domain. 434 of these are entirely novel and are candidates for playing a role in host-parasite interactions.

While a direct comparison of the *M. incognita* and *M. hapla* genomes has not yet been published, the concurrent analyzes of each genome in 2008 identified a number of conserved pathways which were compared to the previously published nematode genomes (*C. elegans, C. briggsae, B. malayi*) (Abad et al. 2008; Opperman et al. 2008). Comparisons to *C. elegans* are of particular importance because of the species pre-eminence as a system for the study of animal development and the extensive information available about its genetics, genomics, and cellular anatomy. Recognition of pathways or portions of pathways that are conserved may jump-start inquiries into the molecular physiology and development of plant parasites. For example, *M. incognita* encodes orthologs of all the *C. elegans* innate immunity signaling pathways except *trf-1* but lacks almost all of the *C. elegans* antibacterial and antifungal proteins. *B. malayi* shares a similar pattern to *M. incognita*.

Sex determination is specified in *C. elegans* by chromosome (XX female and XO male), whereas in *Meloidogyne* it is environmentally specified with males produced by stresses such as overcrowding. While *M. incognita produces* males, they play no genetic role in reproduction which is by mitotic parthenogenesis. Of 24 genes which play a role in *C. elegans* somatic and germline sex determination and dosage compensation, 11 are identifiable in *M. incognita* (e.g. *sdc-1, tra-1, tra-3,* and *fem-2*) and several were also identified in *M. hapla* (*tra-1* and *tra-2*) suggesting some components of these pathways are ancestral to many nematodes.

Another important development pathway in *C. elegans* is that responsible for dauer formation, an alternative larval stage (L2d) induced by harsh environmental conditions which is non-feeding, long-lived, stress-resistance, and capable of dispersion. Dauer larvae are then able to resume development and reproduction when favorable conditions return. Many dauer formation (*daf*) genes also influence lifespan in *C. elegans*. Parallels have been made between dauer larva and the infective stage of plant and animal parasitic nematodes, though similarities in global gene expression have not been observed (Elling et al. 2007). Of 20 cloned dauer pathway genes in *C. elegans*, 17 putative orthologs are identified in *M. hapla* (14 of which are highly conserved) indicating the likelihood that much of the molecular machinery is intact in root knot nematodes though its function in the lifecycle is unknown.

Many of the genes involved in the RNA interference (RNAi) pathway are also conserved among nematodes. RNAi is the cellular process widely observed in eukaryotes by which double stranded RNAs (dsRNAs) cause gene silencing of matching sequences by post-transcriptional mechanisms including messenger RNA degradation and disruption of translation. The key triggering role played by dsRNA was first described in *C. elegans* (Fire et al. 1998). RNAi occurs by an endogenous biochemical pathway which includes the dicer complex that processes long dsRNAs into 21 nucleotide small interfering RNAs (siRNAs) and the RNA-induced silencing complex (RISC) that guides siRNA-mRNA base pairing and message degradation (Mello and Conte 2004). *C. elegans* also amplifies the RNAi signal via an RNA-dependent RNA Polymerase (RdRP) complex. Orthologs for most genes in the Dicer, RISC, and RdRP complexes are identifiable in *M. incognita* and *M. hapla*. However, consistent with analyzes of *B. malayi* and *Haemonchus contortus*, no *Meloidogyne* homologs were observed for *C. elegans* genes involved in cellular uptake of dsRNAs and systemic RNAi (*sid-1, sid-2, rsd-2, rds-6*) suggesting that this function is either absent or the genes involved are too rapidly evolving to be recognizable.

6.6 Tools for Functional Genomics and Genetics

RNAi is a useful research tool for determining gene function and has potential application in commercial nematode control through transgenic plant-delivered dsRNA. Introduction of dsRNA by microinjection, soaking, and feeding has been

used to knock down expression of all *C. elegans* genes and phenotypic effects have been observed for several thousand (e.g. Fraser et al. 2000). Of the 2,958 *C. elegans* genes having a lethal RNAi phenotype, 1,083 have orthologs in *M. incognita* (Abad et al. 2008). Phenotypic effects in *M. incognita* by soaking pre-parasitic J2s in solutions of dsRNA were first described in 2005 (Bakhetia et al. 2005) and have been followed by numerous publications (reviewed in Rosso et al. 2009) but more recent studies have highlighted the challenges of non-specific phenotypic effects from long dsRNAs that challenge earlier observations and interpretations (Dalzell et al. 2009). Studies have also demonstrated reduction in *Meloidogyne* infection in plants expressing dsRNAs directed against nematode genes. Tobacco expression of dsRNA for a splicing factor protein decreased *M. incognita* gall formation and nematode reproduction (Yadav et al. 2006) and *Arabidopsis* expression of dsRNA for the candidate parasitism gene 16D10 decreased infectivity of multiple *Meloidogyne* species (Huang et al. 2006). Yadav and colleagues selected their RNAi gene candidates based on orthology to essential *C. elegans* genes, whereas Huang and colleagues selected a gland secreted protein that may play a role in feeding-site formation. The *M. incognita* and *M. hapla* genome projects now make available nearly complete catalogs of root knot nematode genes that can be used to identify targets of interest for RNAi studies.

In addition to the variable results observed for phenotypic effects with RNAi directed at root knot and other plant parasitic nematodes, demonstration of mRNA reduction by methods including quantitative rtPCR has also been variable (Dalzell et al. 2009). One possible explanation for phenotypic effects in the absence of measured whole organism mRNA reduction is that RNAi is non-systemic so that not all tissues are silenced. The finding of parasitic nematodes genes that play roles in dsRNA uptake like *sid-1* and other genes in *C. elegans* would aid in interpreting when and where silencing can occur. Perhaps the most intriguing and persuasive demonstration of RNAi in root knot nematodes comes from a positive selection approach to silencing (Gleason et al. 2008). This study characterized a candidate avirulence gene in *M. javanica* (*Cg-1*) thought to be responsible for root knot resistance mediated by the *Mi-1* gene in tomato. Avirulent *M. javanica* J2s were soaked in dsRNA corresponding to the sequence of *Cg-1* and then inoculated onto an *Mi-1* tomato. Unlike controls, J2s exposed to *Cg-1* dsRNA were able to infect the tomato and to retain their virulence over eight generations, presumably by inheritance of the silencing effect in at least a portion of the population. Continued selection on resistant tomato (Motelle, *Mi-1/Mi-1*) was required to maintain strong virulence induced by *Cg-1* silencing. Similar inheritance of silencing has been observed in *C. elegans* (Vastenhouw et al. 2006).

In addition to its applications to RNAi, the availability of genomes from *Meloidogyne*, in particular the *M. hapla* sequence is also enabling much more sophisticated genetic approaches to gene identification. In parallel with the sequencing of *M. hapla*, polymorphisms between the VW8 and VW9 strains were identified by amplified fragment length polymorphism (AFLP) and used to construct a genetic map covering fifteen linkage groups (Opperman et al. 2008).

Subsequent to the generation of the reference genome from VW9, next generation sequencing has been used to generate shotgun coverage from VW8 allowing the rapid identification to a large number of single nucleotide polymorphism (SNP) markers that are being used to enrich the *M. hapla* genetic map. Mapping of genes based on behavioral differences between *M. hapla* strains is in progress (Bird et al. 2009).

6.7 Future Prospects Sequencing of Parasitic Nematode Genomes

Plant nematology is entering an era of comparative and functional genomics that can contribute to new methods for effective and sustainable plant parasite control. In addition to the two root-knot nematode genomes, a draft genome with three-fold coverage is available for the soybean cyst nematode *H. glycines*, the most important pathogen of U.S. soybeans (Genbank accession 170570910). Additional coverage of the *H. glycines* genome has been generated by 454 pyrosequencing from two strains allowing for SNP identification (Bekal et al. 2008). Currently in progress is a genome project for the potato cyst nematode *G. pallida* (www.sanger.ac.uk/sequencing/Globodera/pallida).

Current state of the art sequencing technology, so called "next generation" approaches from Illumina, Roche 454, and Applied Biosystems (SOLiD), allow an increase of several orders of magnitude in sequence that can be generated per unit time or unit cost versus classic Sanger dideoxy chain-termination (Morozova and Marra 2008). Single runs on these massively parallel instruments can generate multi-fold coverage of a 100 million nucleotide genome in one day. The major drawback of these approaches to date has been short read lengths that do not allow for *de novo* assembly of genomes of the size range found in nematodes (Bekal et al. 2008). "Next generation" sequencing may soon be superseded by "third generation" sequencing approaches based upon monitoring of single DNA molecules that combine even greater density of parallel sequencing with longer read-lengths. One method, from Pacific Biosciences, relies upon real-time monitoring of DNA polymerase (Eid et al. 2009) while another method, from Oxford Nanopore, combines an exonuclease activity with transit of released nucleotides through a nanopore (Clarke et al. 2009). Both technologies are aimed at the goal of driving the cost of generating a human genome below $ 1,000. Anticipation of these technologies has resulted in proposals for sequencing projects on a scale unthinkable even five years ago such as generating genomes for 10,000 vertebrate species (Genome 10K Community of Scientists 2009). The rapidly falling cost of sequencing will make acquisition of assembled genomes from any species of interest, including nematodes, common place in coming years. The greater challenge will be the creative use of this information by nematologists and other scientists.

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Chapter 7
Transcriptomes of Plant-Parasitic Nematodes

Joachim Jacob and Makedonka Mitreva

7.1 Introduction

The most rapid and cost-effective approach for gene discovery in eukaryotic genomes, including plant parasitic nematodes (PPN), has been the generation of expressed sequence tags (ESTs). The term EST refers to any sequence derived from an expressed transcript. ESTs are obtained in a high-throughput fashion from mRNA, mostly *via* the construction of complementary DNA (cDNA). While ESTs have been the major resource for genome-driven research on parasitic nematodes to date there are several shortcomings of EST data: (1) they are single-pass unedited sequence data and therefore prone to base-calling errors, although this can be minimized with enough redundancy in the data set; (2) generally ESTs do not completely cover the gene's coding sequence (hence the term 'tag') and therefore represent only gene fragments, (3) construction bias, which is mainly an issue in the conventional clone-based cDNA libraries since short inserts tend to clone more efficiently than do longer ones.

Through careful selection and preparation of the starting material, EST libraries can contain temporal and spatial expression information. For example. EST libraries can be made from different developmental stages, or different tissues (although obtaining the latter from small animals such as nematodes can be cumbersome). In contrast to complete genome sequencing, which gives us insight into what the organism *is capable of*, ESTs give us insight into which parts are *active* during a certain time period, condition or in a certain tissue.

EST analysis has been the foremost strategy used to discover genes in plant parasitic nematodes, mainly because there was a lack of *a priori* molecular knowledge of these nematodes. Furthermore, there are firmly established protocols which guarantee success and there is a relative abundance of tools and software to analyse EST datasets. Finally, analysis of EST data is considered a proven approach in novel gene discovery, a crucial fact for parasitic nematode researchers. Hence, there has

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been a constant increase in the number of ESTs available in public databases, including ESTs originating from PPN.

EST data and genomic data are complementary as demonstrated by the common practice of aligning EST sequences to genomes to facilitate genomic annotation. This mapping can identify splicing events, verify open reading frames, confirm exon/intron boundaries and more. However, complete genome sequences are not currently available for most nematodes, and the ESTs have been used mainly as: (1) probes for expression profiling using cDNA-based microarrays (e.g. Elling et al. 2007b), (2) databases to interpret proteomics derived from polypeptides (e.g. Robinson and Connolly 2005), (3) a basis for phylogenetic analysis, (4) for genome DNA amplification (Jacob et al. 2009), and (5) for endosymbiont discovery (Haegeman et al. 2009). The genomes of two PPN species have been sequenced (Abad et al. 2008; Opperman et al. 2008) and with ever-improving molecular and sequencing technologies (see Sect. 7.6), it is easy to envision many more being sequenced in the near future. For all of these genome projects EST collections have been, or will be, indispensable with regards to gene prediction and annotation.

At the time of writing, about 60 nematode species are represented in the dbEST database, which is a division of GenBank housing ESTs (online at http://www.ncbi.nlm.nih.gov/dbEST/). Approximately 16% of these are plant-parasitic nematode originated ESTs, with an overrepresentation of sedentary endoparasites (Fig. 7.1). The majority of the parasitic nematode expressed sequence tags have been produced by the parasitic nematode group at the Washington University Genome Center (www.nematode.net; Wylie et al. 2004; Martin et al. 2009).

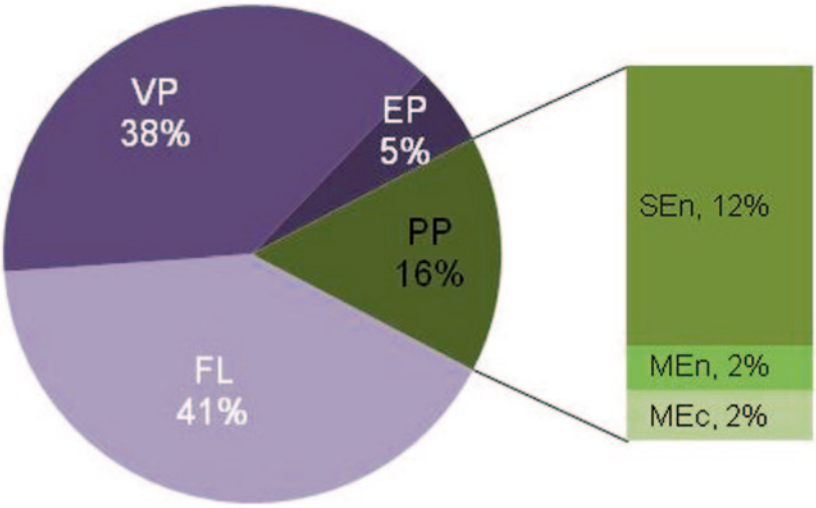

Fig. 7.1 Distribution of ESTs per species of origin (gbEST division of GenBank, February 2010). EP, *entomopathogenic nematodes*, PP, *plant parasitic-*, FL, *free-living-*, VP, *vertebrate parasitic nematodes*, SEn: *Sedentary endoparasites,* MEn: *Migratory endoparasites,* MEc: *Migratory ectoparasites*

7.2 Intra-Specific Transcriptomics Has Proven a Powerful Approach to Identify Parasitism-Related Genes

All PPNs rely on physical as well as biochemical adaptations to successfully parasitize their plant host. The biochemical adaptations involve protein and non-protein (e.g. hormones, Vanholme et al. 2004) factors secreted by the nematode, when the nematode resides outside the plant tissue, inside the plant tissue as well as in direct contact with the specialized feeding cell. Understanding these biochemical factors will definitely improve our comprehension of the parasitism process, but identifying them is by no means a trivial task. Direct comparison of expressed genes encoding secreted proteins in preparasitic and parasitic stages can help identify the protein factors related to parasitism. Besides the widespread application of 'sequence-based' approaches to achieve this (the merits of which we will discuss further below), the 'protein-based' approach has been more successful in identifying truly novel parasitism proteins. The protein-based approach takes secretions themselves as a basis to identify the proteins contained in it and elucidates their corresponding 'parasitism genes'. The protein-based approach was used first in the hunt for parasitism genes, resulting in identification of PPN secretions having activities such as cellulase, pectinase and proteinases (Giebel 1973). Antibodies raised against secretory granule proteins from the pharyngeal glands and screening for immunolocalization to the glands succeeded for the first time in identifying a parasitism gene responsible for the cellulase activity in the secretions (De Boer et al. 1996a, b; Smant et al. 1998). Due to advances in mass-spectroscopy techniques, a recent study succeeded in directly identifying proteins from induced secretions of the root-knot nematode *Meloidogyne incognita*. This study reached an unprecedented depth, identifying dozens of novel putative parasitism genes with expression in the pharyngeal gland cells (Bellafiore et al. 2008). However, the success of such studies relies on the availability of sufficient secretions and of gene sequences that can be used to aid identification of the genes from which the sequenced peptides are derived.

Hence, a valuable alternative and complementary approach to the protein-based approach is the sequence-based approach, which uses EST data to analyse gene expression to discover parasitism genes. The results of EST analyses can be divided into (a) identifying homologues of identified parasitism genes in novel species, (b) analyzing the differences in gene expression or gene content between parasitic versus non-parasitic stages or species and (c) directly analyzing the transcriptome of the tissues producing the secretions.

The first studies of this kind analysed differential gene expression between cDNA pools constructed of parasitic second stage juveniles and other stages (by 'RNA fingerprinting' and 'cDNA-AFLP'). This led to identification of novel parasitism genes (Ding et al. 2000; Qin et al. 2000). In order to identify genes which contributed to the earlier observed enzymatic activities of the secretions, clones of a whole nematode cDNA library were expressed and functionally screened for expected enzymatic activities. A cellulase enzyme was identified using this ap-

proach (Yan and Marr 2005). Furthermore, screening expression clones of cDNA libraries with antibodies raised against PPN secretions has also allowed identification of other parasitism genes (Fioretti et al. 2001; Prior et al. 2001). Following an exceptional approach, one study generated cDNA of total host roots infected with nematodes and subsequently subtracted the pool with cDNA of non-inoculated host root and nematode egg cDNA. This study also yielded a few novel parasitism genes that were subsequently shown to be expressed in the gland cells of *Heterodera glycines* (Tucker et al. 2005).

The availability of *H. glycines* (soybean cyst nematode) ESTs from different developmental stages enabled generation of the first commercially available PPN micro-array which allowed transcript abundance of different life stages to be measured and, combined with an extensive EST analysis, novel putative parasitism genes were identified (De Boer et al. 2002; Elling et al. 2007a, 2009). In virtually all cases, however, combined prediction of a signal peptide for secretion with confirmed expression in the gland cells remains the litmus test for a gene to be assigned as a parasitism gene candidate. Additionally, its presence in (induced) secretions can be confirmed (Vanholme et al. 2004). Soon it became apparent that cataloguing sequences of parasitism genes alone would not improve our understanding of the parasitism process, since many parasitism genes lack homologous sequences (the so called 'pioneers'). However, some parasitism genes of PPNs bear homology to genes previously thought to be restricted to other organisms, such as (plant-pathogenic) bacteria and fungi, and animal parasitic nematodes, and even plants (Olsen and Skriver 2003; Bird and Opperman 2009). This reflects the unique and diverse molecular tools PPN use for successful parasitism. Once a parasitism gene is discovered in one PPN species, homologues can be identified in other PPNs, based on hybridization-based screening of cDNA libraries, PCR amplification with degenerate primers on cDNA pools or searching for homologous genes in EST datasets (Rosso et al. 1999; Goellner et al. 2000; Gao et al. 2001; De Boer et al. 2002; Mitreva et al. 2004a).

It was recognised early that cDNA libraries enriched in genes specifically expressed in the gland cells would be of more value than cDNA libraries made from whole nematodes. In the first attempt to deploy this technically challenging strategy, a cDNA library from the gland cell region was screened with probes originating from the tail region. Clones that did not hybridise to "tail" probes were analysed, resulting in the identification of the nematode parasitism gene chorismate mutase (Lambert et al. 1999). Following advances in technology, specific cDNA libraries were later on constructed from microaspirated gland cell contents. Gao et al. (2001) further developed this technique by constructing a gland-cell specific cDNA library combined with suppression subtractive hybridization using intestinal cDNA. Further optimization of normalisation of gland cell-specific libraries was achieved through the use of secretion signal selection (Wang et al. 2001), differential hybridising the cDNA library on a macro-array with intestinal cDNA (Gao et al. 2003; Huang et al. 2003), and the use of solid-phase subtractive hybridization using intestinal cDNA (Huang et al. 2004). Obtaining ESTs from these and other nematode cDNA libraries contributes to an ever growing pool of transcriptomic data available

in databases. Thorough comparisons of these EST data and focused bioinformatics searches based on expected characteristics of parasitism genes and expected functions achieved further identification of parasitism genes (Popeijus et al. 2000; Dautova et al. 2001; Jones et al. 2003; Jones et al. 2004; Mitreva-Dautova et al. 2006; Vanholme et al. 2006; Roze et al. 2008). Although bioinformatics EST analyses can provide a priority list of candidate parasitism genes, these genes need to be verified. As such, EST analysis is always only a first step in parasitism gene discovery. Expression of a certain candidate needs to be confirmed and localized, for example through *in situ* hybridization e.g. (Mitreva-Dautova et al. 2006) or immunolocalization e.g. (Doyle and Lambert 2002). Its expression needs to be confirmed in tissues related to parasitism, which are the pharyngeal glands, the amphids, the rectal glands, the secretory-excretory system or the epidermis. Subsequent functional characterisation of the candidate genes has proved to be much more challenging than the identification.

It must be noted that all of the above mentioned intra-specific studies (studies restricted to one species) have been performed on sedentary nematodes, as they cause the greatest economical losses and have a scientifically very interesting parasitic behavior. Little parasitism gene research has focused on PPN species with other types of parasitic behaviour, although some progress has been made (Uehara et al. 2001; Mitreva et al. 2004b; Kikuchi et al. 2005; Jacob et al. 2008). Nevertheless, the accumulated knowledge and insights from parasitism genes identified in sedentary PPN may provide a basis for the identification of parasitism genes in migratory PPN (Opperman and Bird 1998).

7.3 Expressed Sequence Tags, the Most Versatile Source of Molecular Data for Plant Parasitic Nematodes

7.3.1 Conventional Cloning-Based EST Libraries

The construction of an EST library can be a very tedious job. It involves the following steps: the RNA fraction is extracted from (a) certain tissue(s) or a time point (life-stage) from a certain species, and is reversed transcribed to cDNA. The cDNA is cloned into bacterial cells, plated and randomly picked clones are selected for growth and plasmid extraction. The sequencing of such clone-based EST libraries is done using Sanger sequencing (for details see Mitreva and Mardis 2009). The characteristics of EST libraries vary considerably, depending on the protocol used and the inclusion of controlled and uncontrolled systemic biases. For example, inconsistencies in the amplification steps, primers used for cDNA synthesis, the system used for cDNA synthesis, cDNA size fractionation, techniques such as subtraction and normalization, and the choice of cloning vectors are all factors that can contribute to systematic biases. In addition to these 'intentionally' introduced variations, further deviations arise from differing quality and quantity of starting material, reverse transcriptase reaction

efficiency, ligation efficiency, difference in cloning 'capacity' of sequences (e.g. toxins) and other unforeseen events during the library construction process. Therefore, direct comparison of different EST libraries, if they are not constructed using identical protocols, should be done with necessary precaution and appropriate controls.

Novel 'next-generation' sequencing techniques allow the cloning step to be bypassed, and to sequence the cDNA pool after adapter ligation and (often) amplification steps. These cDNA construction protocols (e.g. Mitreva and Mardis 2009) deal with some of the biases introduced by conventional EST library construction protocols and lead to more consistent transcriptome data sets (Simon et al. 2009) (see Sect. 7.6).

7.3.2 Analytical Processing of ESTs

Due to the high-throughput nature of EST generation, it is not feasible to check the properties of each sequence manually. This implies that a roadmap of analysis should be set which defines the goals of discovery. Nevertheless, some analyses are common for each EST data set, irrespective of species or goal, and are described here. The first step performed with a raw EST data set is to clean the data of unwanted sequences. Vector and adaptor sequences (depending on the method of generation) need to be removed. Also the removal of short sequences (90 bp is a common cut-off because it codes for 30 amino acids, which is a good length for sequence similarity searches) and low quality/complexity sequences guarantees an increase in the proportion of sequences containing useful information. Depending on the purpose of the ESTs, ribosomal sequences, mitochondrial sequences or prokaryotic sequences (which would be considered a source of contamination amongst eukaryotic ESTs) may be removed. However, by doing so, sometimes valuable information is lost and so these steps are not universally applied. Due to the very nature of the EST generation process, a lot of redundancy exists in an EST data set. Genes with a high expression level will generate more EST sequences than genes with a low expression level. This redundancy is desirable, to a certain extent, to increase base accuracy (countering the high level of sequencing errors) and to increase transcript coverage. Nevertheless, normalization steps applied during library construction can decrease the different representation between genes from many orders of magnitude to just a few orders of magnitude. However, a difference will always exist. In addition, these normalization steps have proven difficult and costly, but increase the portion of lowly expressed genes in the EST data.To deal with the remaining level of redundancy, a computationally demanding step called clustering is typically performed. During computational clustering of an EST data set (e.g. by using the freely available program TGICL, Pertea et al. 2003), each sequence is compared to all other sequences in the dataset and when a certain degree of identity is found between ESTs, these ESTs are considered to be derived from the same gene. A consensus sequence is constructed from this alignment of these ESTs, resulting in a so-called contig sequence. After

the clustering process, the original data set of processed ESTs is transformed into a much reduced data set consisting of contig sequences with higher average sequence lengths and singleton sequences (ESTs that did not cluster with any other EST). Generally, contig sequences are further grouped into clusters, which contain contig sequences derived from the same gene, yet represent different splice isoforms or alleles. The resulting sequences are often referred to as unigenes. However, when calculating the number of discovered genes in an EST dataset, one important bias needs to be corrected for: frequently several ESTs represent non-overlapping parts of the same gene, resulting in the number of unigenes being higher than the real number of genes sampled. When ESTs from the same gene fail to cluster together, this phenomenon is usually described as fragmentation or under-sampling. This can be detected by comparing the sequences to the complete genome sequence of *Caenorhabditis elegans*, or by using statistics through the tool ESTstat (Wang et al. 2005). Of course, when the genome sequence of the species under consideration is available, the fragmentation level can be calculated exactly. On average, a typical degree of fragmentation is between 8 and 18% (Mitreva et al. 2004a, b, 2005; Jacob et al. 2008; Karim et al. 2009). For example, a fragmentation error of 12% means that 12% of the unigenes represent genes which have already been covered by one or several other unigenes in the data set. Hence, a better estimate of the number of genes represented in a certain EST dataset is the total number of unigenes subtracted by the estimated fragmentation level. As a guide, prior experience has shown that on average 10,000 ESTs represent ~4,000 unique genes (Fig. 7.2). The overall result of these first generally applied processing steps is the generation of a data set which is greatly reduced

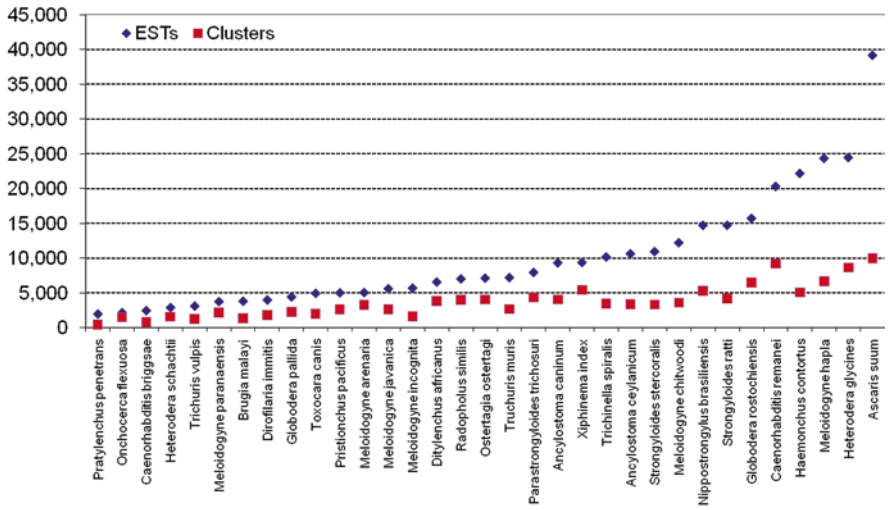

Fig. 7.2 Novel gene discovery in parasitic nematodes. Preprocessing results of several nematode EST libraries

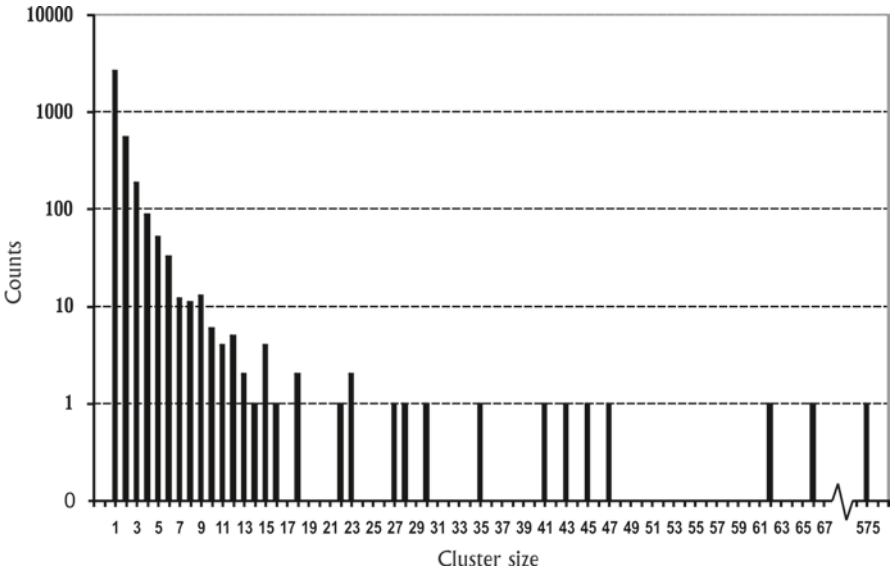

Fig. 7.3 Example of a cluster size distribution graph (7007 ESTs of *Radopholus similis*). The clustering resulted in approximately 3,600 'singletons', while an aberrantly large cluster of 575 ESTs is visible at the other spectrum of the graph. (Taken from Jacob 2009)

in size, but with conservation of information content. The clustering results can be visualized in a graph displaying cluster size (the number of ESTs that were used to construct that cluster sequence) versus cluster numbers (Fig. 7.3). Generally speaking, the larger the cluster size, the higher the expression of the gene from which the ESTs were derived (Audic and Claverie 1997). But a note of caution must be added here. Firstly, a strong length bias confounds gene expression results based on EST counts, when compared to microarray data (Munoz et al. 2004). Secondly, sometimes technical artifacts or impurities can cause an aberrantly large cluster of ESTs. As an example of the latter, in a *Radopholus similis* EST dataset, 8% of all unigenes were derived from mitochondrial rRNA (Jacob et al. 2008). Interestingly, many of these systemic biases in cDNA libraries can be detected through visualization and application of statistics. For example, enrichment of sequences with a certain length (visible in length distribution graphs) can point to the inclusion of normalization steps (Liu and Graber 2006). Plotting histograms of GC content can differentiate between sequences of different sources (Jacob et al. 2008). And the observation of aberrantly large clusters in a cluster size distribution may point to overrepresentation of certain genes, mostly due to artifacts during library construction (Kikuchi et al. 2007) (Fig. 7.4).

The purpose of most EST projects is to discover new genes. To maintain high rates of new gene discovery, the acquisition of stage- and tissue-specific biological samples as starting material is an alternative, valuable approach. In cases where there have been 2 or more stage-specific libraries sampled (e.g. L1, L3, adult) from

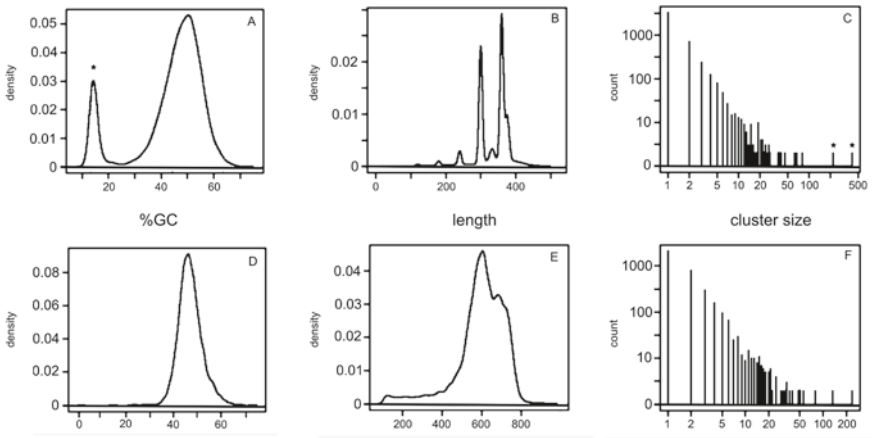

Fig. 7.4 Visualization of some EST library statistics. Each graph of the upper row can be compared to the graph beneath it to demonstrate the versatility in characteristics of EST data. A and D visualize the GC content distribution: a huge enrichment of AT-rich sequences is visible in A (*star*) as compared to D. This enrichment was due to mitochondrial transcripts (Jacob et al. 2009); B and E visualize the length distribution: several discrete enrichments of sequences with certain lengths is visible in B as compared to E. C and F are cluster size distribution graphs: in C, two aberrantly large clusters are visible (*stars*), as compared to the more smoothed graph in F

a nematode species, the majority of clusters are represented in only one library (Mitreva et al. 2004a, 2005).

7.3.3 Web-Based Access to Plant Parasitic Nematode EST Data and Tools to Support Analysis

It is a generally applied policy to deposit generated ESTs into Genbank's dbEST. However, a similar repository does not exist for clustered sequences and their associated analysis. Currently, nematologists have a number of specialised sites available for sequence analysis, some of which also store the results of various analyses. Wormbase (www.wormbase.org; Harris et al. 2010) is an extensive database with curated information about every gene in the model *C. elegans* and other *Caenorhabditis* species; Nembase (www.nematodes.org; Parkinson et al. 2004c) offers access to parasitic nematode sequences and related tools such as visualization of clusters by stage or expression; and Nematode.net (www.nematode.net; Martin et al. 2009) provides rapid access to genome data and tools for parasitic and free-living nematodes. These websites provide important information. For example, Nematode.net provides cluster and contig information for nearly 40 nematode species (the 'Nemagenes') with additional datasets being added as they become available. This site allows browsing of EST clusters, retrieval of the ESTs that comprise a cluster and performance of BLAST searches by clade, species and library. In addition, the EST clusters are associated with Gene Ontology

(GO) categorizations and can be visualized via the Amigo Browser (The Gene Ontology 2008). The GO is a naming convention for classification of function and location of genes (as 'terms') to improve transferability and understanding of gene sets. This allows for a global overview of the most important processes occurring in the species or tissue the EST dataset is derived from. Associations with the KEGG biochemical pathways are graphically displayed using NemaPath (Wylie et al. 2008). The NemaPath viewer provides the ability to display comparative metabolomics, which is extremely useful for detecting taxonomically restricted pathways. The nematology community is fragmented by nature and has generated resources focused on animal-, human-, plant-, entomo- pathogenic and free-living nematodes. However in recent years substantial progress has been made towards the integration of these specialised databases. For example, Nematode.net provide users a link to all related *C. elegans* gene pages from each hosted contig, and Wormbase users are able to jump to the proper contig page within Nematode.net; 27 Nematode.net parasitic nematodes species are represented in Wormbase.

Nembase (http://www.nematodes.org/bioinformatics/index.shtml) also has a number of tools that can be used to analyse an EST dataset (Parkinson and Blaxter 2003; Parkinson et al. 2004a; Wasmuth and Blaxter 2004). These and other publicly available tools are intended to be used by researchers that are not bioinformatics experts. For example, ESTExplorer (Nagaraj et al. 2007) is an online tool that performs all standard analyses on an EST dataset that is uploaded to the site. It will perform preprocessing, clustering, conceptual translation, annotating and mapping to pathways through homology. In addition, EST2Secretome (Nagaraj et al. 2008) includes all the previous analyses, but is extended by a membrane domain prediction and signal peptide for secretion prediction module. There are a number of other tools that are not necessarily developed for analysis of nematode ESTs but are general and applicable to any EST dataset. For example, BLAST-2GO (Conesa et al. 2005; http://www.blast2go.org/), is a web-based tool that can take (preferentially) clustered EST datasets and provide GO associations for each sequence through homology searches, along with a graphical representation of the results. As a derived approach, GO term distributions may be compared between different EST datasets or complete gene sets (for example of *C. elegans vs. parasitic nematodes*) by WEGO (Ye et al. 2006). Development of bioinformatics tools evolves fast, not only in the field of nematology. To comply with a growing need, websites such as Biocatalogue (http://www.biocatalogue.org) or EMBRACE (http://www.embraceregistry.net/) monitor the status of numerous webtools, offering one single searchable access point with extensive documentation. In the following paragraphs, several of the above tools will be mentioned. It is not our purpose to explain the functioning of these tools, but rather to provide important considerations and analysis approaches for nematode EST datasets. One last important remark: performing an EST analysis (as with any scientific research) needs appropriate positive and negative controls, as outputs of programs cannot be blindly trusted.

7.4 Functional and Structural Characterization of ESTs: Understanding the Molecular Basis of Parasitism

The main reasons for analysing transcriptomes of plant-parasitic nematodes are to obtain an insight into the molecular biology of the parasite, to identify genes with roles in the host-parasite interaction, or to find targets for possible pest control. As a widely applied first step, homologues of each unigene are identified in different nucleotide and/or protein databases, through several BLAST searches with appropriate cut-offs. By carefully comparing unigenes to databases of certain selections of sequences (e.g. the non-redundant (nr) database containing all sequences, a *C. elegans* database, and a nematode-specific database), interesting unigenes can be readily identified. The results can be visualised using publicly available tools (e.g. Simitri, Parkinson and Blaxter 2003). A different approach is to BLAST each sequence of the unigene dataset to the nr database, and analyze the species distribution of the resulting hit list (Jacob et al. 2008). The advantage of this approach is the fact that one does not depend on *a priori* arbitrary choices of database construction. Unigenes which have homology only to sequences from prokaryotes as revealed by BLAST, may result from contamination or—as was identified in *R. similis*-may result from an endosymbiotic prokaryotic species (Jacob et al. 2008; Haegeman et al. 2009). A similar approach may also allow identification of nematode-specific, parasitic nematode-specific, and even plant-parasitic nematode-specific unigenes. Such analyses have shown that some parasitism genes may be shared between animal- and plant-parasitic nematode species (see Sect. 7.5). But some unigenes are also found shared by PPNs and plants, and PPNs and pathogenic prokaryotes, which served as a basis for the horizontal gene transfer theory for some of the parasitism genes. To assign functions to these unigenes, the GO framework is widely applied. Determining and comparing GO terms is easily done through BLAST2GO and WEGO (see above).

However, all the analyses described above depend on the availability of known homologous sequences in current databases. Unfortunately, each nematode EST analysis shows that a large proportion of the unigenes lack homology to any known sequence (typically 40–45%, Parkinson et al. 2004b). This can arise for several reasons: the EST may be derived from DNA rather than RNA (contamination), the EST may be derived from the non-coding UTR of a gene, the EST may be derived from a non-coding RNA (such as miRNAs or rRNA) or the EST may in fact represent a genuine novel gene sequence. To resolve this question and identify ESTs derived from genuine protein encoding genes a protein-coding prediction program can be applied to the ESTs. A variety of approaches are used by these programs ranging from simple, such as searching for the longest ORF (eg NCBI's ORFinder), to complicated prediction programs which use a multitier approach and are trained by input of coding sequences, either of related species (e.g. Prot4EST through ESTScan trained by *C. elegans* sequences Wasmuth and Blaxter 2004), or preferably by full length gene sequences manually extracted from the EST dataset (e.g. FrameD; Schiex et al. 2003). It should be bourne in mind that the outputs of these

programmes are predictions: the fidelity of these predictions may be assessed on unigenes known to be derived from the coding part of mRNA assessed by, for example, BLASTx homology to known protein sequences. Obtaining more accurate translations may be worth the effort, as most further analyses are performed on the amino acid (protein) rather than the nucleotide sequences.

Analyses at the protein level include associations with known domains and networks, as well as identification of the parasite's so-called secretome. Special attention has been paid to the secretome (which is the whole of proteins in an organism secreted from any cell's surface), due to the fact that the majority of parasitism genes need to be secreted out of the gland cell into the host plant to exert their effect. The identification of secretome factors is achieved by signal peptide prediction on translated unigenes by SignalP3.0 (Bendtsen et al. 2004), in combination with the lack of predicted transmembrane motifs (which keep the secreted product in the cell membrane) e.g. by TMHMM (Sonnhammer et al. 1998). It is imperative for this step that the translations are of good quality. Secreted proteins can represent up to a quarter of the proteome of an organism. In EST datasets, usually around 3–8% of the unigenes are identified to encode for secreted proteins (Vanholme et al. 2006; Jacob et al. 2008) but this underrepresentation is likely in part due to the fragmented nature of the EST data (in which complete 5' ends typically are underrepresented). Of course, not all of the identified secreted genes contribute to the parasitome. The final step to narrow down the list of parasitome factor candidates, is the combination of several different analyses. We have found it particularly useful to generate a large file or database, in which each row starts with the unigene name and is followed by the results of all different analyses. As such, unigenes can be readily filtered according to different criteria, for example: expressed in parasitic but not free-living stages (BLAST search on stage specific EST databases), having sequence similarity only to parasitic-nematodes, being protein-coding (preferably full-length), being positive for signal peptide prediction, and lacking a transmembrane domain. In this way, a priority list may result from the EST analysis, which may serve as a basis of directed wet-bench research.

7.5 Pan-Phylum Transcriptomics: An Approach that Reveals Broadly Conserved and Taxonomically Restricted Molecular Features in Nematoda

Sequence data from other nematodes, such as human-, animal-parasitic and free-living provide a wealth of information that may help to better understand PPNs at a molecular level. The first global analyses of all nematode sequence data was undertaken using 265,494 ESTs originating from 30 species (including 28 parasites), clustered into 93,645 genes and grouped into ~60,000 gene families (Parkinson et al. 2004b). This data collection suggested that many biochemical pathways are conserved among nematodes despite their diversity. However, taxonomically restricted

pathways were also identified. For example, Spiruria (animal parasitic nematodes) and Dorylaimia (plant migratory ectoparasitic nematodes) had fatty acid biosynthesis pathway-1 but lacked pathway-2 enzymes completely, whereas Tylenchina (mainly sedentary endoparasites) had only pathway-2 enzymes, and Rhabditina (free-living nematodes) had both. Enzymes involved in inositol metabolism and N-glycan degradation enzymes were notably abundant in Tylenchina, but less evident elsewhere. As the complete genome of *C. elegans* encodes many N-glycan degradation enzymes, this suggests that this pathway is particularly highly expressed in Tylenchina plant parasites.

A second systematic approach detected pan-phylum highly conserved proteins and regions. Nematodes likely diverged from other animals over 600 million years ago, during which time they adapted a number of phyla-specific features. Nematode proteins have experienced drastic changes (Stein et al. 2003; Parkinson et al. 2004b) related to functional diversification, speciation, and species adaptation (Givnish et al. 2000; Kocher 2004; Panhuis et al. 2006; Peng and Huang 2006; Jang et al. 2007). Among these are the nematode-specific proteins, which are of crucial importance for understanding nematode evolution and parasitism (Lilley et al. 1999; Davis et al. 2004; Curtis 2007), and proteins that are shared among the pathogen and the host but have become sufficiently diverged in the host as to be functionally absent or altered. Recent pan-phylum studies have focused on identification and characterization of these 2 groups of proteins using over 214,000 predicted polypeptides from 32 nematode species. Yin et al. (2009) identified 758 protein families conserved across the phylum Nematoda or nematode subgroups. These 758 nematode-specific families (5,199 proteins) do not share homology to known Pfam protein domains but a majority of them (462 protein families) are essential for nematodes as they are conserved among free-living and parasitic species that span the phylum Nematoda, and since the *C. elegans* member is most likely to have a severe RNAi phenotype. However, of the 296 parasite-specific families, 90% originated from species with the same trophic ecology, indicating that the number of conserved parasitism-related proteins (e.g. secreted proteins) broadly involved in nematode interactions with hosts may be relatively few (Wang et al. 2010).

Furthermore, a systematic analysis of homologous families having both parasite and host protein members, identified molecular features such as insertions and deletions specific to the parasites (Wang et al. 2009). Wang et al. (2009) identified indels restricted to PPN, of which a subset is conserved across all studied PPN species. These indels are in proteins involved in electron transport and lipid/fatty acid/steroid metabolism, likely related to the adaptation of PPN to their specific lifestyle (different stages capable of surviving aerobic and anaerobic environments). These detected common indels can be one of the reasons for those observed biochemical changes and suggest that PPN are likely to use indels as a strategy for their adaptation in addition to horizontal gene transfer (Wang et al. 2009).

Similarly, smaller scale research lead to identification of the occurrence of plant-specific domains in some parasitism genes (Bellafiore et al. 2008), possibly due to the intimate contact between the PPN and their plant hosts.

In summary, pan-phylum analysis is an useful extension of inter-specific analysis. The described analysis along with other published reports conserved characteristics of gene expression (e.g. pan-intestinal expression, Yin et al. 2009) and more general studies (Mitreva et al. 2006; Wasmuth et al. 2008), will eventually underline the molecular aspects of the parasitic interaction between PPN and their hosts. Today, the general processes by which PPNs parasitize their hosts are much clearer than a decade ago, but numerous issues still remain unresolved and comparative genomic approaches will play a crucial role in this.

7.6 The Future of Plant Parasitic Nematode Transcriptomics

To date, genomic and molecular studies of PPNs have mainly involved conventional cloning-based cDNA libraries sequenced using Sanger sequencing technology on capillary electrophoresis instruments. Molecular techniques for obtaining biological material, performing RNA extraction and construction of cDNA libraries with small amounts of starting material are continuously evolving and improving. This, coupled with the extraordinary progress in sequencing chemistry and instrumentation, decreased sequence cost along with improved bioinformatics tools is expected to greatly accelerate PPN research, resulting in comparative transcriptomics and genomics being the dominant approach to understanding these parasites at a molecular level. Several next generation sequencing technologies provide unique opportunities for high-throughput transcriptomic and genomic explorations of PPNs in far more detail than previously possible (Droege and Hill 2008; Mardis 2008) and at a substantially lower cost per sequenced base than using conventional capillary (Sanger) sequencing. For example, the 454/Roche (Margulies et al. 2005) is an optimal platform for in-depth analysis of de-novo sequenced transcriptomes, and the Illumina and SOLiD systems are highly attractive for resequencing projects aimed at transcriptome sequencing at unprecedented depth, resulting in an increased ability to evaluate the coverage of annotated features of the genome and of candidate processed transcripts, confirm existing or identify new splice junctions, trans-spliced leader sequences, and polyadenylation tracts just to name a few.

New sequencing methodologies that are appropriate for de-novo transcriptome sequencing (454/Roche) further increase the importance of EST clustering to reduce redundancy and increase sequence quality and length. Large sequence datasets produced on different sequencing platforms including traditional Sanger/ABI and 454/Roche pyrosequences use distinct tools for the analysis of their respective sequences including different methods for base calling and detection of high quality regions, trimming of linkers, screening for low complexity regions and contaminants, and returning high-quality sequences (Mitreva and Mardis 2009). The generated sequences are often of varying length and quality and their integration requires algorithms that can handle the differences. For example, for the animal parasitic nematode *A. caninum*, a hybrid assembly of >88,000 ABI ESTs (average read length of 560 bp, total of 53 Mb) and over 1.5 million FLX 454/Roche sequences (average read length of 246 bp, total of 348 Mb), was generated by 454 Life Sciences'

Newbler assembler followed by the addition of ABI reads (unpublished data). This dataset represented four life stages, and the complete clustered dataset grouped into ~47,000 contigs of which 66% were comprised only of FLX platform sequences and lacked representation in the 88,000 ABI reads, indicating the advantage of a method without cloning bias. However, consistent with published results (Mitreva et al. 2005) and despite the high depth of coverage, most contigs were stage-biased with only 26% having representation from more than two stages (Wang et al. 2010). Furthermore, the shorter average read length also altered analysis from traditional ESTs so that only 22% of contigs had BLAST matches in *C. elegans* as opposed to 53% of clusters from 9,331 ABI ESTs (Mitreva et al. 2005). While small contig length also resulted in a very high level of estimated fragmentation (30%) using *C. elegans* as a reference genome, using the 458 core eukaryotic genes (Parra et al. 2007) 94% coverage of the *A. caninum* transcriptome was obtained, a level that is impossible using conventional cloning-based cDNA libraries.

There are numerous similar examples that provide evidence for the big impact new technologies have in different research areas. This is inevitable in plant parasitic nematology as well. There are already two plant parasitic genomes available (Abad et al. 2008; Opperman et al. 2008), and sequencing field populations is expected to accelerate, as one would be interested in comparisons to investigate genome-wide polymorphisms. However, such genomic variations are related to specific gene expression changes, therefore an RNA-seq approach (sequencing of a cDNA pool using a high-throughput sequencing method) to profile and compare expression patterns is an important part of the equation. Finally, as the amount and type of information hosted grows, so does the importance of the different interfaces serving this information. Specialized web databases containing transcriptomics data and the results of their analysis will be increasingly important to effectively disseminate results. Some of the requirements to achieve this are (1) build the infrastructure to sustainably accommodate the upcoming exponential increase in sequence and expression data from plant parasitic nematodes, (2) improve accessibility and integration allowing the user to make meaningful connections between the genetic, genomic and expression information, (3) upgrade the interface to provide easy and effective navigation, and (4) ensure that new datasets are available to the community on a timely basis. In this way, bioinformatics analyses can continue to give rise to new insights and feed the wet-lab research to improve crop resistance to plant-parasitic nematodes.

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Chapter 8
Arabidopsis as a Tool for the Study of Plant-Nematode Interactions

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8.1 Why *Arabidopsis* Was the Best Choice for Molecular Approaches to Plant-Nematode Interactions: A Historical Perspective

In the 1980s, the cruciferous weed *A. thaliana* was chosen as a model for molecular genetics because of its rapid life cycle and well-advanced genetics combined with a small genome size, leading to the first complete plant genome sequence in 2000 (Meyerowitz 2001). Before 1990, several groups became interested in the possibilities of using *Arabidopsis* as a host for phytopathogens. *Arabidopsis* was shown to be susceptible to infection with the bacterial pathogens *Pseudomonas* and *Xanthomonas*, and the downy mildew fungus *Peronospora* (now *Hyaloperonospora*) *parasitica* (reviewed by Dangl 1993). The first report of complete nematode life cycles in *Arabidopsis* was in 1991 (Sijmons et al. 1991) and included several root-knot and cyst nematodes as well as a migratory nematode species. A large European Concerted Action network on plant-nematode interactions was put in place in 1991. Strongly focused on *Arabidopsis* and molecular genetics, this network intensified collaborations between many groups working on this model system across Europe (and also increased contacts with non-European groups), allowing exchange of protocols and knowledge and thus boosting research on plant-nematode interactions. Several joint publications were produced and the project ended with a book that summarized the progress made in the field (Fenoll et al. 1997). In parallel, in 1992 an international *Arabidopsis* Pathogenesis Network (ARAPANET) was created to stimulate communication among phytopathologists using *Arabidopsis* as a plant host. The inspiring ARAPANET and European Concerted Action meetings which included sweeping discussions provoked an increased interest in the use of *Arabidopsis* as a model host to study plant-nematode interactions in the 1990s.

At that time *Arabidopsis* was the only plant species with a well characterised genome (reviewed by Meyerowitz 1987; Meyerowitz and Somerville 1994). Available

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resources included genomic sequences and many molecular markers in integrated maps, a large EST collection obtained from various organs and physiological and environmental conditions and emerging platforms for transcriptomic analyses. *Arabidopsis* also had the largest mutant collection in the plant world, including rapidly increasing collections of molecularly tagged mutants, thanks to efficient protocols to produce and analyse large numbers of transgenic lines. Thus, *Arabidopsis* provided the best resources to tackle the molecular genetic basis for almost any plant process. In spite of its short growing cycle, *Arabidopsis* sustained successful reproduction of several nematode species (see below) and therefore their common or differential effects could be compared on a single model plant, whose thin, translucent roots allowed the details of the first stages of the interaction between plant and nematode to be followed *in vivo* (Wyss and Grundler 1992; Wyss et al. 1992). Last, but not least, researchers anticipated that accumulated knowledge on various plant processes, as well as broad know-how on managing and manipulating *Arabidopsis* in many labs working on other subjects, would enhance progress in research on plant-nematode interactions. These expectations predominantly became reality.

8.2 Findings that Were Possible Because of *Arabidopsis*

In contrast with research on other pathogens, the focus in molecular genetics of *Arabidopis*-nematode interactions was first concentrated not on the interaction itself or on host defence responses but on the molecular description of feeding sites induced by sedentary endoparasitic nematodes. This approach aligned plant nematode research with the areas of cell differentiation and development. There were obvious questions amenable to molecular genetic approaches stemming from the previous cytological studies as it was clear that nematode-feeding cells must undergo massive changes in gene expression during their development.

Gene expression in feeding cells is dramatically different compared to normal root cells but these changes are very local and therefore difficult to analyze. To monitor changes in gene expression *in situ* at the infection site, there is no easier way than to use *GUS*- (β-glucuronidase) or *GFP* (green fluorescent protein)-reporter fusions. A huge collection of transgenic lines containing these reporters is available in *A. thaliana* and scientists have enthusiastically exploited this resource. Goddijn et al. (1993) were the first to use this technique for the analysis of candidate gene expression in giant cells and syncytia. In addition to identifying genes upregulated in nematode feeding sites, these studies showed for the first time that some genes are downregulated in feeding structures. More detailed studies have confirmed that this phenomenon is widespread. It was also recognised that the use of GUS reporters offered the potential for the discovery of ‘novel’ genes. Therefore, a large collection of transgenic *Arabidopsis* plants was generated, all containing a *GUS* gene functioning as a promoter tag. The promoterless *GUS* gene is only activated when it is inserted in the genome in the neighborhood of a gene that is active in the feeding cells. The big advantage of this system is that expression in the whole plant can be

assayed and different nematodes and timepoints can easily and rapidly be compared (Barthels et al. 1997). The initial disadvantage that the tagged genes were difficult to identify ceased to be a major issue with the publication of the full sequence of the *Arabidopsis* genome in 2000. Several interesting findings arose from particular tagged lines including the roles of *MAP65* (see below) and *RPE* (Favery et al. 1998) in feeding site biology. This technique was particularly useful for identifying weakly expressed genes (which may have vital roles in feeding site biology) such as those encoding the transcription factors JAZ1/TIFY10a (Grunewald et al. 2009a) and WRKY23 (Grunewald et al. 2008). These genes were unlikely to have been detected using other methods of expression analysis. There remain many promoter-reporter gene fusions, constructed to investigate other processes, which can be readily used to gain insight into detailed expression patterns of particular genes and gene networks in nematode feeding sites.

The study of the molecular basis underlying the unique morphological features of nematode feeding structures has benefited enormously from *Arabidopsis*, and there are many examples that highlight progress made in this area. Four examples of the utility of *Arabidopsis* as a model system are provided here: the cell cycle modifications linked to the differentiation of syncytia and giant cells, interactions between nematode effectors and plant targets, the role of auxin on feeding site differentiation and transcriptomics.

8.2.1 Cell Cycle Changes in Feeding Cells

Genes involved in control of the cell cycle had been identified from several plant species in the early 1990's. However, it was the availability of transgenic lines expressing *GUS* or *GFP* from the promoters of these genes in *A. thaliana* that allowed unique insights into the importance of the cell cycle and cytoskeleton changes during feeding cell formation to be obtained (see also Chaps. 17 and 18 for more details). Sedentary endoparasitic nematodes form different types of feeding cells: root knot nematodes induce repeated mitosis without cytokinesis giving rise to giant cells while cyst nematodes stimulate cell wall degradation and protoplast fusion of neighbouring cells to produce a syncytium (see Chaps. 5 and 4, respectively).

Cytological analyses demonstrated the presence of multiple large nuclei in feeding cells, suggesting the activation of the cell cycle (Huang and Maggenti 1969; Endo 1971). Using reporter-*GUS* fusions with a cell cycle marker for division competence (*CDC2a*, now *CDKA;1*) and for the G2-phase (*CYC1At,* now *CYCB1;1*) Niebel et al. (1996) showed that both markers are transcriptionally activated in the very early stages of giant cell and syncytium development. This triggered a more comprehensive analysis of the full spectrum of cell cycle related genes and of the importance of DNA synthesis and mitosis for feeding cell development (de Almeida et al. 1999 & Chap. 18).

In situ analysis and protein subcellular localization can be performed on any plant using *in situ* immunolocalization as long as a suitable antibody is available. In

A. thaliana however, this technique can easily be combined with existing transgenics containing a *GFP*-protein fusion (or alternatives such as YFP, yellow fluorescent protein) for in vivo observation of cytoskeleton changes in developing giant cells and syncytia (de Almeida Engler et al. 2004 and Chap. 19).

The simplicity of combining different genotypes by crossing *Arabidopsis* transgenics and mutants brought deeper insight into the molecular processes occurring in nematode feeding sites. It was shown that the microtubule-associated protein MAP65-3-YFP colocalises with microtubules (using MAP4 microtubule binding domain-GFP) in normally dividing cells and in giant cells. Joining the *map65*-mutation with the MAP4-GFP reporter illustrated the failure of phragmoplast microtubules to complete cytokinesis in normal root cells while MAP65 was proven to be essential for nematode-induced giant cell ontogenesis in *Arabidopsis* (Caillaud et al. 2008). The actin cytoskeleton (visualized with fimbrin actin binding domain 2-GFP translational fusion) in giant cells normally consists of thick cables throughout the cortex but amorphous actin with much thinner and shorter bundles in the cytoplasm. Using this protein GFP-reporter in RNAi-plants with lower levels of actin-depolymerizing factor2 revealed that this results in F-actin stabilization and giant cells with thicker actin cables in the cytoplasm impeding their development and that of the feeding nematodes.

8.2.2 Nematode Effector Analysis

The study of endoparasitic nematode molecules involved in plant infection has followed several parallel approaches and numerous nematode proteins were identified that may play important roles in various phases of the nematode life cycle (see Chaps. 12, 13 and 14). Ultimate proof for their causal role could be obtained by demonstrating that mutant nematodes, lacking functional copies of these genes, have an impaired lifecycle. However, neither direct genetics—through generation of mutant nematode lines—nor reverse genetics—by production of knock-outs for specific nematode genes- are available as yet for plant parasitic nematodes (although RNAi offers potential for the latter area—Chap. 10). Other approaches have therefore been used to assign roles to putative nematode effectors during infection. These include studying the effect of a nematode-encoded protein in the host plant, which might partially mimic the real function during infection, and delivering anti-nematode molecules to the parasites through their feeding in transgenic plants. Using these approaches, *Arabidopsis* transgenic and mutant lines have been very helpful in determining putative functions for nematode-secreted proteins.

The first nematode secreted proteins for which *Arabidopsis* tools were used to gather functional information were those related to plant cell wall modifications. Numerous secreted cell wall remodelling enzymes (including endo-glucanases, pectinases and expansins) have been identified in cyst and root-knot nematode secretions and their study has provided much insight into various stages of nematode establishment in the root. This subject is reviewed in Chap. 12 and we have selected

one example that illustrates the value of *A. thaliana* for this type of research. Yeast two-hybrid analysis of a *H. schachtii* Cellulose Binding Protein (CBP) with an *Arabidopsis* root library identified a plant pectin methyl esterase (PME3) as a specific interacting plant protein (Hewezi et al. 2008). Overexpression of either the nematode Hs-CBP or the plant PME3 in *Arabidopsis* caused increased root growth and greater levels of infection by *H. schachtii*, whereas the opposite effects were observed in *Arabidopsis pme3* mutants. These results suggested that the secreted Hs-CBP directly interacts with plant PME to facilitate nematode establishment within the root (Hewezi et al. 2008).

There are two other recent examples that illustrate how *Arabidopsis* genetic and molecular resources can help provide insight into the functional roles of nematode-secreted peptides (see also Chap. 14). The *Heterodera glycines HgSYV46* gene encodes a potentially secreted protein containing a CLE domain (Wang et al. 2005). CLE peptides are a family of plant-specific small secreted peptides with crucial roles in cell to cell communication during development (Fiers et al. 2007; Mitchum et al. 2008). One such plant CLE is encoded by *CLAVATA3* and it is involved in maintaining the cell division/cell differentiation balance at the *Arabidopsis* apical meristem. The *H. glycines* peptide, when expressed in *Arabidopsis*, was able to partially complement the loss of function *clv3-1* mutant phenotype, while its overexpression in wild type plants mimicked *AtCVL3* overexpression, arresting meristem development (Wang et al. 2005). This seminal finding indicates that Hg-CLE has functional interactions with the plant membrane receptors of this ligand in the *Arabidopsis* apical meristem; it might therefore also interact with similar receptors from root cells and impact the cell division/differentiation of the syncytium initials, influencing feeding site development.

Another nematode peptide with a domain similar to CLE is encoded by the *Meloidogyne incognita 16D10* gene (Huang et al. 2006a). Overexpression of 16D10 peptide in *Arabidopsis* promotes root proliferation, but it does not complement the *clv3-1* phenotype. The search for the *Arabidopsis* receptors of 16D10 by yeast two-hybrid screens and immunoprecipitation identified SCARECROW-like transcription factors. This finding was surprising, since plant CLE peptides are thought to function through their interactions with leucine-rich repeat receptor-like kinases (LRR-RLKs) at the apoplastic side of the plasma membrane, and not through physical interactions with nuclear proteins, raising the question as to whether 16D10 might reveal novel mechanisms for cell-cell signalling through CLE peptides in plants. In addition, the *Meloidogyne* 16D10 peptide has a close relationship with the *Arabidopsis* CLEs involved in maintaining division competence of procambial cells and restricting their differentiation into xylem elements. The *M. incognita* peptide might inhibit tracheid fate in the initial root procambial cells, thus opening a window for them to become nematode feeding cells. Supporting this hypothesis, it was found that the differential transcriptome of very young *Arabidopsis* giant cells has some similarity with the transcriptome of the very early stages of cell cultures differentiating towards tracheids (Barcala et al. 2010). Another point of interest is that some plant peptides contain post-translationally modified amino acids. For instance, nano-LC-MS/MS analysis of *Arabidopsis* meristems has determined that

the active CLV3 form is a 13-aa arabinosylated glycopeptide (Ohyama et al. 2009), raising the question of whether the nematode-secreted CLE peptides are also modified and, if so, where this modification occurs. Finally, the *Arabidopsis* genome codes for hundreds of cystein-rich small secreted peptides with demonstrated or potential signalling functions (Silverstein et al. 2007), many of which belong to still uncharacterized gene families. If nematodes secrete other plant-mimicking peptides, tools will be available to help in their analysis.

8.2.3 Auxin as a Driving Force in Feeding Site Formation

The auxin-insensitive tomato mutant (*diageotropica*) was shown to be much less susceptible to root-knot and cyst nematode infection (Richardson and Price 1982; Goverse et al. 2000) indicating the importance of auxin for the induction and expansion of the feeding sites. Although this was a hugely important finding, developing research in this area in tomato was not possible due to the lack of resources. By contrast, in *Arabidopsis* there were a range of different mutants available, deficient in auxin biosynthesis, perception and transport, that could be analyzed for their effect on the nematode infection process. The *DR5-GUS* reporter was used to characterize the early, local and transient increase of auxin in giant cells and syncytia (Karczmarek et al. 2004). Finally, the role of proteins involved in polar auxin transport (*PIN*-genes) in initiation and expansion of syncytia was established using *PIN-GUS* and *PIN-GFP* transgenics for resolving the temporal and spatial expression patterns and the protein localization patterns respectively of the different PIN proteins (Grunewald et al. 2009b). Details on the role of auxin and other hormones in feeding cell development are provided in Chap. 16.

8.2.4 Exploring the Response of the Plant Transcriptome to Nematode Infection

The pronounced effects of nematode infection in the thin, translucent roots of *Arabidopsis* allow easy isolation of the infected tissue from the surrounding root cells. In plants infected with *H. schachtii*, hand dissection or microaspiration of syncytia is far more straightforward than in most crop plants. For root-knot nematodes, galls can be dissected at all stages without difficulty, but giant cells are quickly embedded within these galls. Differential display was the first technique used to compare gene expression in dissected feeding sites and control roots of *A. thaliana* (Hermsmeier et al. 2000; Vercauteren et al. 2001). The availability of genome and transcriptome sequence data made it possible to assign the relatively small tags to the corresponding genes, something that was far more difficult in non-model species.

More recently, the implementation of high-throughput expression analysis tools such as microarrays has enabled a much more comprehensive assessment of plant

genes differentially regulated upon nematode infection (see Chap. 9). As techniques have become more sophisticated, the analysed tissue has changed from whole roots over dissected galls to microaspirated cytoplasm and Laser Capture Microdissected syncytia or giant cells. In addition to monitoring the effects of nematode infection on the whole transcriptome, an alternative use for arrays is to address specific hypotheses. Examples include investigations into the role of a variety of transporters for giant cell function (Hammes et al. 2005) or to examine the role of starch accumulation and degradation in syncytium performance (Hofmann et al. 2008).

Of course these types of investigations can also be performed on feeding cells from other plants, as was convincingly shown for syncytia induced in soybean (Ithal et al. 2007). However, in 2007 little functional information was available for the soybean genes present on the array. Only about 27% of the 1765 soybean genes that were differentially expressed in syncytia 2 days after inoculation could be identified through significant homology to genes in public databases (Ithal et al. 2007), though this limitation will gradually become less of a problem as full genomes for crop species including soybean and tomato are made publically available.

By contrast, several DNA microarrays representing part of the *Arabidopsis* gene set were used in 2003 to study cyst-nematode (Puthoff et al. 2003) and, soon after, root-knot nematode infected roots (Hammes et al. 2005; Jammes et al 2005; Bar-Or et al. 2005). More recent studies have used chips that represent the whole set of *Arabidopsis* Open Reading Frames combined with NFS-specific RNA obtained from various infection times of both cyst and root-knot nematodes (Szakasits et al. 2009; Barcala et al 2010). Comparisons between these NSF transcriptomes or with the many other *Arabidopsis* transcriptomes that have been analysed provide insights into particular processes that occur during nematode feeding site development. For example, the syncytium transcriptome appeared similar to seed and pollen transcriptomes (Szakasits et al. 2009), while giant cells showed similarity to crown-galls and to suspension cells differentiating towards vascular elements (Barcala et al. 2010). Another advantage of *A. thaliana* genome-wide gene expression studies compared to most crop species is that for many processes complete gene families have been identified and studied and gene annotation is increasingly dense and accurate. Sequences are accessible allowing (semi)quantitative expression analysis of each individual member of the gene family and in numerous cases transgenic plants with reporter constructs and mutant lines are available to address gene functions.

8.3 High Expectations that Never Quite Made True

In spite of the wealth of information that *Arabidopsis* has allowed us to gather in the last two decades, researchers had expected to exploit this model system in other ways that, however, have not rendered as much insight as was promised. Perhaps the two best examples are the use of mutants and promoter dissection.

8.3.1 Mutant Analysis

In the early nineties, one obvious approach in the new field of molecular plant nematology was to explore mutagenised *Arabidopsis* populations looking for alterations in susceptibility to nematodes, with the aim of finding resistant lines that would allow identification of new genes involved in plant-nematode interactions. A few laboratories reported screening for these traits, with some success (Baum et al. 2000; Vercauteren et al. 2002) but others tried this approach without making significant progress.

Several mutants altered in specific genes which were thought or known to be involved in feeding site formation have also been tested. Although there have been some remarkable findings using *Arabidopsis* mutants, they have mostly failed to define specific signal transduction pathways and gene circuits important in the plant-nematode interaction and in feeding site differentiation in a way comparable to other biological processes, which have been unravelled to a greater extent using this approach. In consequence, plant-nematode interactions have benefited comparatively little from the powerful predictive value of genetic analysis in *Arabidopsis*. Some key exceptions should, however, be noted. For example, as discussed above, analysis of auxin transport mutants has been very informative (Goverse et al. 2000; Grunewald et al. 2009b and Chap. 16). Ethylene overproduction and ethylene insensitive mutants (Wubben et al 2001, 2004 and Chap. 16) have also been useful in establishing the role of these two hormones, particularly during cyst-nematode infections. Mutants in cell wall modifying enzymes, often sustaining poor nematode development or reproduction, particularly in *H. schachtii* but also in *M. incognita* infections (Wieczorek et al 2008 and Chap. 19) have also helped assigning a functional role in syncytia to particular proteins.

There could be several reasons for this limited success. Screening mutagenized populations for decreased nematode susceptibility is technically difficult, as infection rates are low (and often highly variable) in *Arabidopsis* (particularly in some accessions) and large individual plant numbers are therefore necessary to draw conclusions from a resistance test. But it is also possible that there is too much inherent resilience in this system to allow it to be readily amenable to genetic analyses. For example, a high degree of redundancy in the plant targets for the nematode effectors or in other plant proteins needed for feeding site differentiation or maintenance would impose the need for loss-of-function of several genes simultaneously in order to disturb the infection process in a detectable way. This applies to gene family redundancy and also to the possibility that parallel, alternative pathways with distinct gene networks could be involved in feeding site differentiation. Several of the *Arabidopsis* mutants reported to have diminished nematode susceptibility exhibit highly pleiotropic phenotypes and often display poor development and aberrant roots, precluding to a great extent the interpretation of the nematode-related phenotypes. Examples include mutants with severely compromised root development or lacking essential components of basic metabolic pathways needed for plant growth. Conditional mutants produced through inducible gene expression systems where

the time and site of the gene loss-of-function can be controlled might be another way of exploring gene circuits in a more conclusive way.

8.3.2 Promoters with Specific NFS Expression for Crop Engineering

Most known nematode-responsive promoters were identified in *Arabidopsis*, either as conventional promoter-reporter gene fusions, or through blind screens in promoter- and enhancer-tagged collections. These have proved valuable in understanding many aspects of the compatible interaction (see above; there are also many examples in other chapters). One of the main expectations from the very early days of molecular plant-nematode interaction studies in *Arabidopsis* was that it would be possible to use this model for the identification or construction of a "feeding site-specific" promoter, which would be a key instrument for driving, in a highly specific way, the expression of RNAs which would generate nematode resistance in crops. The longer term strategy was to identify or develop these specific nematode responsive promoters in *Arabidopsis* and then transfer them to crop species.

We are still a long way from being able to engineer promoters to make them strictly nematode-responsive. All promoters identified as nematode-responsive to date are also expressed in other plant tissues and/or induced by environmental or physiological conditions. Though a synthetic regulatory sequence which responds only to nematode effectors (or is only expressed in feeding sites) is in theory possible, such a sequence has not been developed to date. There have been a number of attempts to narrow down the expression domains of nematode responsive promoters, and in some instances, regions linked to nematode responsiveness have been identified. This area has been extensively reviewed by Gheysen and Fenoll (2002) and is not covered in great detail here. An extensive study made in tobacco for the CaMV35S promoter indicated that different parts of this promoter have distinct responses and that the responses differ for cyst and root-knot nematodes (Bertioli et al. 1999). Since then, several others have been analysed. These include $Hs1^{pro\text{-}1}$ (Thurau et al. 2003) in sugar beet hairy roots and *Arabidopsis*, *HaHSP17.7* (Escobar et al 2003) in *Arabidopsis* and tobacco, *AtCel1* in *Arabidopsis* (Sukno et al. 2006) and *ABI3* (de Meutter et al. 2005) in *Arabidopsis*. None of these studies have ever rendered a true nematode feeding-site specific promoter but they have identified relatively short regulatory sequences that could either be engineered further to restrict expression outside feeding sites or used as alternatives to the broad and strong expression of the 35S promoter (see below).

Another key question is to what extent *Arabidopsis* promoters are useful for engineering nematode resistance in crops. Few promoters identified and studied in *Arabidopsis* have been checked in other plants, and in most cases these are other models rather than crops. Conversely, a small number of heterologous promoters have been introduced into *Arabidopsis* to test whether they maintain their

nematode-responsive behaviour so that they can be analysed in the easier system. One example is the promoter of the sunflower *Hahsp17.7G4* gene which encodes a small heat-shock protein involved in sunflower late embryogenesis and in some stress responses (Almoguera and Jordano 1992). A minimal 87 bp version of this promoter was sufficient for nematode induction both in tobacco and in *Arabidopis.* Nucleotides essential for nematode-responsiveness were also identified (Barcala et al. 2008). Therefore, in this case, the sequence elements needed for nematode-responsiveness seem to be similarly interpreted in tobacco and *Arabidopsis* and hence the model plant can be used to identify transcription factors involved in its regulation. Another case of promoter functional equivalence in different species was found in a viral promoter that drives virion-sense transcription of late genes in the geminivirus maize streak virus (Escobar et al. 2010). Also in this case, tobacco and *Arabidopsis* seem to respond in a similar way to nematode infection, activating this promoter in giant cells and syncytia and nowhere else in the roots. Again, gene regulatory sequences function in a similar way in nematode feeding sites of the two species.

The most broadly used promoter for driving gene expression in plants is the CaMV 35S promoter. This promoter was reported to be down-regulated in both *Arabidopsis* and tobacco feeding sites (Goddijn et al. 1993), thus limiting its use in anti-feeding site approaches to nematode resistance. However, other studies indicated different expression patterns in *Arabidopsis* (Urwin et al. 1997a) using a different reporter gene. Today, many studies -mostly performed in *Arabidopsis*- show that both anti-nematode and anti-feeding site constructs under the control of the 35S promoter are successful, demonstrating that this promoter is indeed active in feeding sites (*e.g*. Urwin et al 1997b; Huang et al 2006b; see also Chap. 10). However convenient this or other constitutive promoters are, next generation transgenic crops would benefit from more targeted transgene expression, for which more specific promoters would be needed. The tests for such optimized promoters will require an extensive production of transgenic lines which can only be tackled in a plant like *Arabidopsis*. Approaches such as pathway engineering through gene stacking or using high hierarchy regulatory genes like transcription factors can be more easily studied in this model prior to crop transformation.

8.4 When It Would Be Better to Use Other Model Systems

Model organisms are useful for better understanding general processes and *Arabidopsis* is no exception with regard to plant-nematode interactions. The specific details of important crop nematode diseases or interactions of nematodes with specific plant species are beyond the scope of *Arabidopsis* research in this field. One major drawback of research on *Arabidopsis*-nematode interactions is that no resistance genes against plant parasitic nematodes have been identified in *Arabidopsis*. Therefore there is no opportunity to compare compatible and incompatible plant reactions.

Relatively few nematode species have been tested in *Arabidopsis*. Complete life cycles can obtained on *A. thaliana* for several root knot nematode species including *M. incognita* & *M. arenaria* (Sijmons et al. 1991), *M. javanica* (Wu et al. 1998), *M. hapla* (Boiteux et al. 1999), cyst nematode species (*H. schachtii*, *H. trifolii*, *H. cajani*; Sijmons et al. 1991), the reniform nematode *Rotylenchulus reniformis* (Urwin et al. 2000) and the migratory nematodes *Pratylenchus penetrans* (Sijmons et al. 1991), *P. coffeae* and *Radopholus similis* (Elsen et al. 2001). Despite the fact that the migratory nematodes *P. coffeae* and *R. similis* can complete their life cycle in *A. thaliana*, nematode reproduction is low in the thin roots and it is very difficult to obtain reproducible results (Carlens et al. 2005). However, there are other plant species that can be regarded as models for particular approaches on plant-nematode interactions, including tomato, potato, soybean, Lotus, rice, maize, *Brachypodium* and *Selaginella*.

Corn, soybean, potato and tomato are important crops but they either have very complex genetics or they are difficult to transform (or both). Nevertheless, soybean has been instrumental as a host for the study of incompatible plant-nematode interactions. The soybean-soybean cyst nematode (SCN) system is an outstanding model to compare gene expression during compatible and incompatible reactions because well defined SCN populations exist that cause either a resistant or a susceptible response on the same soybean genotype. Laser capture microdissection of syncytia during compatible and incompatible interactions has identified differences in gene expression in a developing versus a degenerating syncytium, even before any sign of collapse was evident in the latter (Klink et al. 2009, 2010, Chap. 9). Furthermore, comparative analysis of gene expression in virulent versus avirulent nematode populations revealed different transcriptional profiles even before the nematodes infected the roots (Klink et al. 2009).

Lotus japonicus has been assessed as a model to investigate parallels and differences between rhizobial symbiosis and nematode parasitism. For example, it was demonstrated that cytokinin plays a positive role in both nodule (by *Rhizobium*) and gall (by *Meloidogyne*) development (Lohar et al. 2004). More recently, significant differences in susceptibility to root knot nematode infection were shown when comparing different *L. japonicus* ecotypes (Poch et al. 2007). This finding might have attractive implications for the genetic dissection of host pathways involved in nematode parasitism and possibly in comparison with microbial symbiosis.

Rice is the only major crop that has a relatively small genome, simple genetics and that can be readily transformed using *Agrobacterium tumefaciens*. It is therefore no surprise that it has emerged as a novel monocotyledon model for plant molecular biology. At the same time, nematodes are being recognized as a growing problem on rice, particularly root knot nematodes in aerobic rice growing conditions (Mew et al. 2004; Chap. 24). New molecular tools are emerging that facilitate research on rice. For example, Caldana et al. (2007) have developed a quantitative RT-PCR platform for high-throughput expression profiling of 2500 rice transcription factors. This platform could be used to acquire a detailed insight into how expression patterns of plant transcription factors change after nematode infection.

8.5 Future Prospects: Will *Arabidopsis* Still Be the Best or Only Choice?

The original reasons for working with *Arabidopsis* (well characterised genome, cheap and complete transcriptomics, simple transformation, accumulated tools and knowledge in all plant biology fields) will still be relevant when considering whether to use *Arabidopsis* in the future. Although it is not a crop, *A. thaliana* can be used as a first rapid test system to evaluate the efficacy of constructs that aim to generate nematode resistance. Examples of this approach include the analysis of several proteinase inhibitors (Urwin et al. 1997b, 1998, 2000) and, given the ease with which it can be transformed compared to the majority of crop species, *Arabidopsis* will certainly continue to be used for this type of research.

Linking *Arabidopsis* genomics with nematode genomics will bring new breakthroughs in identifying candidates for plant targets of nematode effectors, and for proof-of-concept using nematode or plant gene silencing (through RNAi against plant or nematode genes). The ease of transformation and increasingly dense gene function annotations will allow investigation of host and pathogen gene function in the infection process.

Genome-wide analyses of protein-protein interactions (interactomics) in *Arabidopsis* is being tackled using collections of yeast two-hybrid tests and protein arrays (http://signal.salk.edu/interactome.html). For particular protein pairs, *in planta* bi-molecular fluorescence complementation (Hu et al. 2002) methods are known to work well in *Arabidopsis* (reviewed by Ohad et al. 2007) and these can be applied to proteins of plant or nematode origin, The first successful application of this last format showed an interaction between a plant nuclear protein and a nematode secreted peptide (Huang et al. 2006b). Another bimolecular fluorescence complementation assay was performed by Hewezi et al (2008) to demonstrate a physical interaction inside plant cells between the nematode CBP secreted protein and its putative plant target, a pectin methylesterase. The same laboratory has recently shown that *Arabidopsis* spermidin synthase 2 interacts with the soybean cyst nematode effector 10A06 (Hewezi et al. 2010) in onion epidermal cells.

Another promising tool is the development of conditional mutants for plant genes whose molecular effects are restricted and highly specific, for instance, to feeding cells or to the areas of the plant that the nematode invades. Silenced or overexpression lines are common tools that have been used extensively to investigate gene function. However, over expression of the gene of interest or of the silencing factor is often driven by strong, constitutive promoters. This can produce aberrant and pleiotropic phenotypes as a result of a profound mis-regulation that commonly disturbs the un-infected plant and not just the plant-nematode interaction. These drawbacks can be partly overcome by the use of chemically inducible, cell-specific expression dual systems (Brand et al 2006). The dual systems allow the use of an *Arabidopsis* promoter to, upon induction (and thus at the desired time and place before or after nematode infection), alter gene expression specifically in the native expression domain of this promoter. Combining this approach with the appropri-

ate mutant backgrounds will render detailed, sound information with much more physiological and developmental value than before. Systems for promoter-specific, inducible gene expression are starting to be used in *Arabidopsis* but are currently less available in other plant species. Similarly, artificial microRNAs (amiRNAs) that can target several sequence-related genes at a time, reducing or abolishing their expression (Schwab et al. 2006), have been designed for *Arabidopsis*. Numerous collections of suppressed lines are being developed and will sooner or later be publically available. They may prove useful to overcome problems caused by gene redundancy in dissecting the molecular mechanisms underlying nematode feeding site formation.

An additional benefit of using this model system would perhaps be mining for useful genes by looking at natural variation. Though technically difficult, the availability of many collections of recombinant inbred lines from crosses of various *Arabidopsis* ecotypes should help unveiling quantitative trait loci (QTLs) for reduced susceptibility to nematodes. It is well known that some *Arabidopsis* accessions are better hosts than others, but a systematic study of these observations has not been published as yet. This ecotype variability, together with the fact that mutant screens have not rendered clear-cut tolerance/resistance genes, makes it probable that these traits are quantitative in nature, and therefore could be disclosed by conventional QTL analyses in available recombinant inbred lines (which often show transgressive values for various traits as compared to their parental genotypes). Even new alleles and allele combinations of already known genes can provide novel phenotypes in terms of nematode resistance or tolerance. The increasing number of wild *Arabidopsis* genotypes whose genomes are being sequenced allows directed searches for novel alleles in virtually any gene. The potential of systematic searches of natural alleles has been demonstrated for various biological processes (reviewed by Alonso-Blanco et al. 2009).

Powerful tools are available in *Arabidopsis* for characterisation of allele series for selected genes of interest in plant-nematode interactions. These include mutant collections which can be molecularly screened with great ease, such as various versions of tagged insertion mutants or TILLING (Targeting Induced Local Lesions in Genomes; Colbert et al. 2001) collections. Commercial microarray-based technologies are also boosting and becoming cheaper for *Arabidopsis*, thus allowing systematic and more and more detailed transcriptomic analyses of interactions of nematodes with this model plant. Most *Arabidopsis* resources can be obtained at the *Arabidopsis* Information Resource hosted in the USA (http://www.arabidopsis.org/index.jsp) and at the Nottingham Arabidopsis Stock Centre in Europe (http://arabidopsis.info/).

If there is a single plant species where biological processes can be modelled based on large quantities of experimental data from a range of disciplines, approaches and complexity levels, it is *Arabidopsis*. It is hard to predict how much more we will learn from *Arabidopsis* in the coming years. It seemed unlikely that cell biology would be widely used to explore in *Arabidopsis* in the early 1990s, due to the very small size of both the individual and its cells, making it difficult to use immuno-techniques and *in situ* hybridization compared to other species with

larger organs and cells. However, the development of fluorescent fusion proteins changed this picture allowing precise, functional studies on protein subcellular localization and dynamics. Only in *Arabidopsis* were these fusion proteins developed at a genomic scale. Thus, looking at the past, it seems probable that if any new approach or technique is developed in the fields of molecular genetics, genomics, or systems biology, *Arabidopsis* will be the first plant species to benefit from it.

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Chapter 9
Transcriptomic and Proteomic Analysis of the Plant Response to Nematode Infection

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9.1 Parasitic Nematode Interaction with Plants

The rhizosphere is a niche environment exploited by a wide variety of microorganisms and plant roots are continuously subjected to a plethora of biotic stresses including being fed upon by plant-parasitic nematodes (Grunewald et al. 2009). Plant-parasitic nematodes can be ecto- or endoparasites and either sedentary or migratory. In the latter case, the nematodes feed upon and quickly destroy plant cells. Sedentary endoparasitism has evolved in the order *Tylenchida*. Nematodes that employ this feeding strategy become immobile after initiating a permanent feeding site. The most studied plant-parasitic nematodes are the sedentary endoparasitic root-knot (*Meloidogyne* spp.) and cyst (*Globodera* and *Heterodera* spp.) nematodes. These nematodes have evolved complex interactive relationships with host cells to form highly specialized nematode feeding sites (NFSs) called giant-cells (GCs) and syncytia, respectively, in infected plant roots from which they withdraw nutrients to sustain a sedentary parasitic lifestyle (Curtis 2007). Understanding the complex signal exchange that occurs during infection of plants is an important parameter for defining those processes that govern parasitic nematode interactions with plant hosts. In order to establish feeding sites, nematodes use secreted effector proteins to manipulate the endogenous molecular and physiological pathways of their hosts. To date, significant progress has been made to identify stylet-secreted effector proteins originating from the pharyngeal glands which assist nematode invasion, migration, and feeding site formation in root tissues (Curtis 2007; Abad and Williamson 2010). On the plant side, molecular studies have shown that physiological changes are accompanied by extensive alterations in plant gene expression (reviewed by Gheysen and Mitchum 2009; Caillaud et al. 2008; Li et al. 2009). However, the functional role in feeding site formation of many of the genes with altered expression remains a mystery. An approach that combines the use of proteomic technology with genet-

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ics is expected to further increase our knowledge of protein function in the plant response to nematode infection. The integration of existing functional genomic data with proteomic analyses will help elucidate the complete picture of the parasitic interaction.

9.2 A Historical View of Methods Used to Study Transcriptional Changes During Plant-Nematode Interactions

The first attempts to identify and clone differentially expressed genes during plant-nematode interactions identified the first GC-induced gene, *TobRB7,* from a tobacco root cDNA library (Opperman et al., 1994). The methodologies available at the time were restricted to differential screening of libraries which required large amounts of RNA, presenting a challenge to obtain enough infected biological material for analysis. Despite this challenge, 11 differentially expressed genes were successfully identified in a study using 1 mg of RNA isolated from tomato roots infected with *Meloidogyne incognita* (Van der Eycken et al., 1996). In a separate report, differentially expressed genes encoding catalases were identified in potato infected with *Globodera pallida* (Niebel et al., 1995). In all studies, timepoints represented late infection stages when the nematode was nearing completion of its life cycle.

The limitation of obtaining enough material enriched in NFSs and the ability to obtain enough RNA for gene expression analyses was soon overcome by the development of PCR-based techniques for transcript amplification. This step forward demanded less starting biological material and allowed for the dissection of tissues limited to the infection area at earlier infection stages than were analyzed in previous studies. From approximately 50 mg of hand-dissected mature tomato GCs, 11 µg of RNA was isolated and 297 differentially expressed transcripts were identified (Wilson et al. 1994). Other studies isolated tissues enriched for NFSs from *Medicago* and tomato roots during the early stages of infection (12–72 h post-inoculation; hpi) with *Meloidogyne* spp. or soybean infected with *Heterodera glycines* (Potenza et al. 2001; Lambert and Williamson 1993; Khan et al. 2004).

PCR-based techniques for transcript amplification, such as differential display and cDNA-AFLP, were also employed for studying plant gene expression changes in response to nematode infection. One of the first studies based on differential display with syncytia enriched material identified 15 differentially expressed transcripts from only 400 ng of RNA using 10 primer combinations in soybean infected with *Heterodera glycines* at 24 hpi (Hermsmeier et al. 1998). Several studies rendered similar numbers of identified clones when meticulous selection and enrichment of the infection structures were used to isolate starting material (Hermsmeier et al. 2000; Vercauteren et al. 2001). One of the most significant differential display analyses, yielding 27 unique sequences, was conducted on microaspirated *Meloidogyne javanica* GCs at 25 days post infection (dpi) (Wang et al. 2003). The

only technical limitation of this analysis was that high turgor pressure precluded the collection of cytoplasm from GCs younger than 15 dpi. In contrast to differential display, only one study based on cDNA-AFLP has been reported. In this study, 15 differentially expressed transcripts were identified during the incompatible interaction of sugar beet (carrying the resistance gene *HS1*$^{pro\text{-}1}$) with *Heterodera schachtii* (Samuelian et al. 2004).

While global analyses of gene expression were ongoing, other analyses focused on characterizing the activation of specific plant gene promoters or enhancers in NFS, based on promoter:gene reporter fusions or enhancer traps employing β-glucuronidase (GUS) or green fluorescent protein (GFP) (Gheysen and Fenoll 2002; Li et al. 2009). The use of luciferase (LUC) as a reporter for studying gene expression changes in NFSs has been limited to genes with transient expression patterns, such as cell cycle genes (Goverse et al. 2000). Although the identification of minimal regulatory sequences from these nematode-activated promoters is scarce, a few promoters such as *TobRB7, AtPYK10, AtPYK20*, and *CaMV35S* have been studied by deletion analysis (Bertioli et al. 1999; Nitz et al. 2001; Opperman et al. 1994; Puzio et al. 1999). Furthermore, putative *cis* elements associated with NFS expression have been proposed based only on *in vitro* analyses (Escobar et al. 1999). Attempts to identify GC activated *cis*-elements determined that *HSEs* are indispensable for the activation of *HSPs* in GCs (Escobar et al. 2003; Barcala et al. 2008).

More recently, technological advancements for single cell isolation such as laser capture microdissection (LCM) and microaspiration, coupled with DNA microarray technology and linear amplification of RNA, have constituted a substantial step forward in the understanding of global transcriptional changes occurring in developing NFSs during plant-nematode interactions.

9.3 Microarray Analysis of Nematode-Infected Root Tissues

DNA microarrays allow the simultaneous analysis of expression changes from a large number of genes, and in some cases, such as in *Arabidopsis*, they are representative of the whole genome. Although this holistic approach constituted considerable progress in the understanding of the transcriptional changes occurring during plant-nematode interactions, microarrays of other plant species such as tomato and soybean have provided a partial picture of transcriptional regulation after nematode infection. This poses a limitation for cross-species comparison of gene expression changes within galls, GCs and/or syncytia. In the coming years, this is expected to change as the genome sequences of several plant species, including tomato and soybean (O'Rourke et al. 2009), have been completed and full genome microarrays become available. Meta-analyses of the microarray data will likely identify commonalities of transcriptional regulation among genes and pathways in galls and syncytia formed in different plant species.

9.3.1 Cyst-Nematodes

The first microarray study to identify plant gene expression changes during cyst nematode parasitism utilized an *Arabidopsis* Affymetrix GeneChip containing probesets for 8,200 genes, representative of approximately one-third of the total genome (Table 9.1; Puthoff et al. 2003). In this study, a comparative analysis between *Heterodera glycines*-infected *Arabidopsis* roots (incompatible interaction) and *H. schachtii*-infected *Arabidopsis* roots (compatible interaction) at 3 dpi was conducted. Infected whole roots were used as the starting material for RNA isolation and 116 genes were identified to be differentially regulated in response to *H. schachtii*. Only 12 genes were found to be differentially regulated by *H. glycines*, but these were also differentially regulated in response to *H. schachtii*. Interestingly, genes uniquely regulated by *H. glycines* were not identified. In fact, the lack of an active defense response at the transcriptional level suggested that the inability of *H. glycines* to parasitize the non-host plant *Arabidopsis* was more likely due to an inability of the two species to communicate (Puthoff et al. 2003). The microarray analysis confirmed previous studies demonstrating that during the compatible interaction the nematode alters the regulation of genes involved in hormone responses, cell wall modification, and cell cycle for syncytium development. Moreover, the downregulation of genes by the nematode is likely to be just as important as upregulaton of specific plant responses for successful infection.

The next phase of microarray studies were conducted on the *H. glycines*-soybean interaction using infected whole root pieces to first probe partial cDNA microarrays (Khan et al. 2004; Alkharouf et al. 2006) and later the Soybean Affymetrix GeneChip during both compatible (Ithal et al. 2007a; Klink et al. 2007a) and incompatible (Klink et al. 2007a; Klink et al. 2010) interactions. These studies (Table 9.1) identified a number of genes involved in primary metabolism, biosynthesis of phenolics, cell wall modification, cell signaling, and transcriptional regulation. A clear trend was the general activation of plant defense genes in response to *H. glycines* during a compatible interaction. In addition, it was found that soybean responds differently, both qualitatively and quantitatively, to avirulent and virulent populations of the *H. glycines* prior to feeding site establishment (Klink et al. 2007a).

9.3.2 Root-Knot Nematodes

The number of microarray based studies for root-knot nematode interactions are limited compared to that of cyst nematode-plant interactions (Table 9.1; reviewed in Li et al. 2009). The first studies were performed on either infected whole roots or hand-dissected galls at early-middle and late infection stages using different oligonucleotide array platforms including Affymetrix, CATMA, and 50-mer MWG Biotech (Hammes et al. 2005; Jammes et al. 2005; Fuller et al. 2007) or cDNA arrays (Bar-Or et al. 2005). Only two plant species, *Arabidopsis* and tomato, have been analyzed in response to root-knot nematodes during a compatible interaction.

Table 9.1 Summary of plant gene expression analyses in response to cyst and root-knot nematodes based on microarrays

Nematode	Population	Plant species	Infection timepoints and isolation methods			Analysis	Reference	Database
			GCs	Syncytia	Biological material			
Heterodera schachtii, H. glycines	OP50, HG-type 1.2.3.5.6.7; *H. schachtii* on cabbage	*Arabidopsis thaliana* (Col-0)			Excised whole root pieces 3 dpi	Affymetrix Arabidopsis GeneChip ~8,200 genes	Puthoff et al. (2003)	Data presented in paper
Heterodera glycines	NL1-RHg, HG-type 7	*Glycine max* Kent			Excised whole root pieces 2 dpi	Soybean cDNA microarray ~1,300 genes	Khan et al. (2004)	http://bioinformatics.towson.edu/SGMD/MicroarrayExps/2D_KentPaper.htm
Heterodera glycines	NL1-RHg, HG-type 7	*Glycine max* Kent			Excised infected whole root pieces 6, 12, 24 hpi; 2, 4, 6, 8 dpi	Soybean cDNA microarrays ~6,000 genes	Alkharouf et al. (2006)	http://bioinformatics.towson.edu/SGMD/Publications/KentTmeline/index.htm
Heterodera glycines	PA3, HG-type 0	*Glycine max* Williams 82			Excised infected whole root pieces 2, 5, 10 dpi	Affymetrix Soybean GeneChip	Ithal et al. (2007a)	ArrayExpress Accession #E-MEXP-808
Heterodera glycines	NL1-RHg, HG-type 7; TN8, HG-type 1.3.6.7	*Glycine max* Peking			Excised whole root pieces 12 hpi, 3 and 8 dpi	Affymetrix Soybean GeneChip	Klink et al. (2007a)	Soybean Genomics and Microarray Database; http://www.towson.edu/nalkharo/SGMD/SupplementalSites/GmWholeRootIC/
Heterodera glycines	PA3, HG-type 0	*Glycine max* Williams 82		LCM 2, 5, 10 dpi		Affymetrix Soybean GeneChip	Ithal et al. (2007b)	ArrayExpress Accession #E-MEXP-876
Heterodera glycines	NL1-RHg, HG-type 7; TN8, HG-type 1.3.6.7	*Glycine max* Peking		LCM 3, 8 dpi		Affymetrix Soybean GeneChip	Klink et al. (2007b)	Soybean Genomics and Microarray Database; http://bioinformatics.towson.edu/SGMD3/
Heterodera schachtii	*H. schachtii* on mustard	*Arabidopsis thaliana* (Col-0)		Micro-aspiration, 5, 15 dpi		Affymetrix Arabidopsis (ATH1) GeneChip	Szakasits et al. (2009)	http://bioinf.boku.ac.at/pub/Szakasits2008/

Table 9.1 (continued)

Nematode	Population	Plant species	Infection timepoints and isolation methods			Analysis	Reference	Database
			GCs	Syncytia	Biological material			
Heterodera glycines	NL1-RHg, HG-type 7	*Glycine max* PI88788		LCM 3, 6, 9 dpi		Affymetrix Soybean GeneChip	Klink et al. (2010)	Soybean Genomics and Microarray Database; http://bioinformatics.towson.edu/SGMD3/
Meloidogyne incognita		*Arabidopsis thaliana* (Wassilewskija)			Hand-dissected galls 7, 14, 21 dpi	CATMA array	Jammes et al. (2005)	Array Express (accession number E-MEXP-233; http://www.ebi.ac.uk/arrayexpress)
Meloidogyne incognita		*Arabidopsis thaliana* (Col-0)			Hand-dissected galls 21 dpi	Arrays printed with the MWG 25,000 Arabidopsis 50mer oligonucleotide set.	Fuller et al. (2007)	TAIR (http://www.arabidopsis.org/tools/bulk/go/index.jsp)
Meloidogyne incognita		*Arabidopsis thaliana* (Col-0)			Whole infested roots 7, 15, 30 dpi	Affymetrix Arabidopsis ATH1	Hammes et al. (2005)	MIAME: www.mged.org/Workgroups/MIAME/miame.html
Meloidogyne javanica		*Arabidopsis thaliana* (Col-0)	LCM, 3 dpi		Hand-dissected galls 3 dpi	*Arabidopsis* synthetic 70-mer oligonucleotides set, version 3 from Qiagen-Operon obtained from Dr. David Galbraith (University of Arizona).	Barcala et al. (2010)	Array express http://www.ebi.ac.uk/microarray-as/aer/entry
Meloidogyne javanica		*Solanum lycopersicum* Mill cv. Moneymaker	LCM, 3, 7 dpi		Hand-dissected galls 3, 7, 14 dpi	TOM1 microarray slides from the Center for Gene Expression Profiling (CGEP) at the Boyce Thompson Institute	Portillo et al. (2009; unpublished)	Data presented in paper

These analyses (Table 9.1) have revealed interesting information regarding different aspects of transcript regulation across the infection stages. For example, only 11.4% of the genes differentially expressed at 5 dpi in tomato galls were common to 10 dpi galls, suggesting qualitative differences in gene expression throughout infection. In addition, the amplitude of variation among the common genes was higher at 10 dpi than at 5 dpi, which indicates a more vigorous response at the later stage (Bar-Or et al. 2005). Certain groups of genes, such as those with functions in cell wall and cytoskeleton remodeling and hormone–associated genes are similarly regulated in galls in different plant species and experiments. In contrast, the group of genes related to transcriptional regulation and defense, show more heterogeneity in their responses. For example, in tomato galls most pathogenesis-related (PR) genes were induced, including a defensin and a harpin-induced gene (*hin-1*) (Bar-Or et al. 2005). Similarly, *Arabidopsis* microarray studies of *M. javanica* galls at 3 dpi and *M. incognita* galls at 21 dpi found that most of the biotic stress genes were up-regulated in hand-dissected galls (Fuller et al. 2007; Barcala et al. 2010). In contrast, genes encoding PR proteins and several WRKY coding transcription factors, mainly involved in plant defense against pathogens, were found to be repressed in an *Arabidopsis* microarray study of *M. incognita* galls at either 7, 14, or 21 days post-infection (Jammes et al. 2005). The discrepancies among studies may be attributed to differences in the infection stages and the reference tissues used in each study, or perhaps subtle differences in the plant responses to the nematode populations used.

To date, there have been few attempts to compare gene expression of hand-dissected galls or whole root pieces containing syncytia with that of isolated GCs or syncytia at the same early infection stage in the same plant system; however, these studies have revealed important differences (Ithal et al. 2007b; Barcala et al. 2010). Normalization of expression data obtained from different microarray platforms and further modifications in the interpretation of data that could account for the lack of experimental uniformity will be necessary in order to exploit the available microarray datasets for a better understanding of plant-nematode interactions. This is an endeavor that will no doubt require cross-disciplinary expertise in bioinformatics.

9.3.3 Analysis of Isolated Feeding Cells

Giant-cells are embedded in a voluminous root structure called a gall that forms as a result of hyperplasia of surrounding cells (Gheysen and Fenoll 2002). The volume contribution of five to eight GCs to the total volume of a gall is quite small at least at early developmental stages. Similarly, a syncytium, which can form a complex of up to 200 cells, is still only a small fraction of the total root cell population. Therefore, for detailed information of the molecular changes occurring within these specialized cells (GCs and syncytia), their specific isolation is crucial. The first attempts to isolate GCs was by hand-dissection from tomato galls from late stage infection (1–2 months) (Wilson et al. 1994). Since then, different methodologies

have been employed or developed for the isolation of individual plant cells or cell types for microanalysis of plant-microbe interactions (He et al. 2005; Ramsay et al. 2006). Some methods, such as microfabricated nanocutting devices of high precision (Chang et al. 2006) have only been used in animal systems. However, for GC and syncytium isolation, laser-capture microdissection (LCM) and microaspiration with a modified pressure probe attached to an oil-filled microcapillary (Fig. 9.1a, b, respectively; Ramsay et al. 2006; Portillo et al. 2009) have rendered interesting results (Table 9.1).

One of the first demonstrations of the strong dilution effect of GC-specific transcripts in whole galls by qPCR was performed from microcapillary-aspirated tomato GCs at 25 dpi (Wang et al. 2003). Again, cytosolic material was extracted only from late infection stages, possibly because a high turgor pressure precluded the aspiration of younger cells. LCM established a clear improvement, as GCs were isolated as early as 48 to 92 hpi, when their first morphological features are clearly distinguishable in sections, either in paraffin-embedded or in optimal cutting tem-

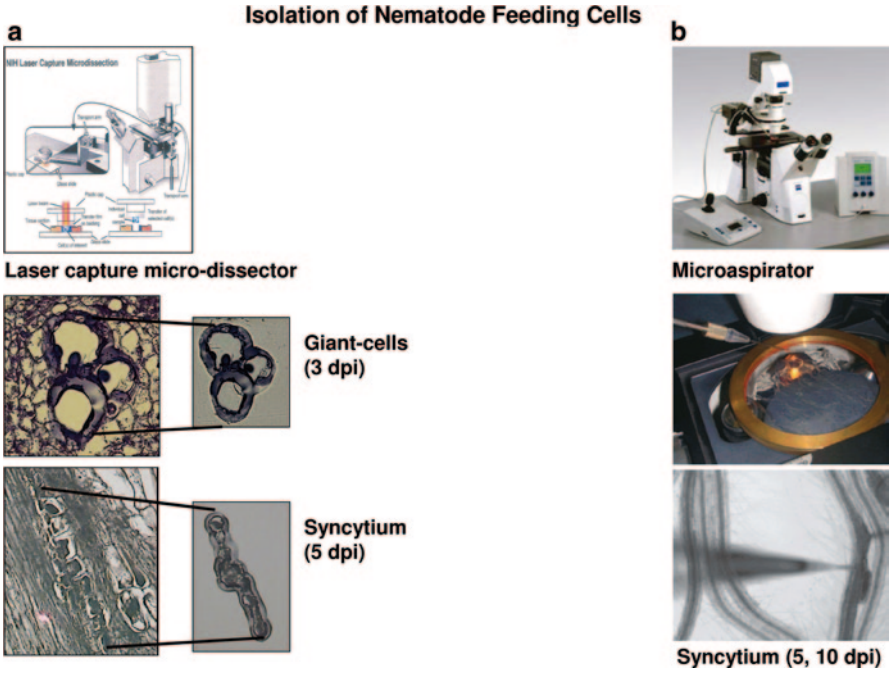

Fig. 9.1 Schematic representation of two methods used for isolation of nematode-feeding cells. **a** upper panel, laser capture microdissector device; second and third panels represent the capture of giant cells (GCs) and syncytia respectively. **b** upper panel, microaspirator device; second panel, metal ring fixed under an inverse microscope (Zeiss, http://www.zeiss.com) holds a thin glass plate covered with medium enclosing the roots; third panel, a microcapillary is guided towards the roots by a micromanipulator (Eppendorf, http://www.eppendorf.com) for piercing a single syncytium. (Szakasits et al. 2009)

perature media (OCT) from cryosections (Ramsay et al. 2004; Fosu-Nyarko et al. 2009; Portillo et al. 2009). As little as two collection caps containing 100 LCM GCs, was sufficient for successful PCR amplification of 4 out of 7 genes tested, including a loading control. Transcripts from a *MAPK* gene and the *LeCycD3;3* and *LeCycD3;2* genes were clearly detected (Ramsay et al. 2004). However, it is important to note that the mRNA recovery and its amplification, as well as an adequate RT-PCR product of a particular gene does not in itself guarantee the structural integrity of RNA after LCM (Fig. 9.2; Portillo et al. 2009). Thus, sensitive techniques, such as electropherograms, are recommended to test the quality and integrity of the amplified RNA (aRNA) before using it for transcriptomic analysis. One parameter crucial for achieving good integrity RNA from cryosections is the quantity of starting material. For *Arabidopsis* and tomato GCs, 200–300 GCs isolated at 3 and/or 7 dpi have been shown to yield high quality RNA for subsequent microarray analysis. Whether RNA integrity also depends on the fixation and embedding protocols used for galls is something that remains to be determined as there are currently only two

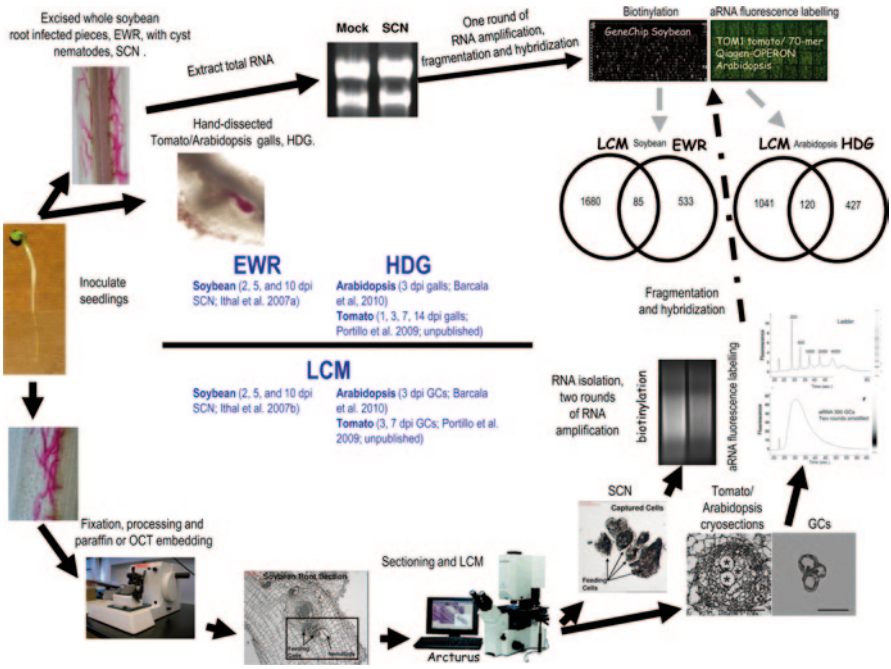

Fig. 9.2 Diagram representing the steps required for the RNA extraction of excised whole soybean root pieces (EWR), infected with cyst nematodes (SCN) and hand dissected galls (HDG) formed by root-knot nematodes for subsequent microarray hybridization, upper part. Steps required for laser micro-dissection of giant cells (GCs), and soybean syncytia (SCN), RNA amplification and quality checking for microarrays hybridisation, lower part. A Venn diagram representing the common genes in the intersection of EWR, *versus* LCM-SCN, as well as HDG *versus* LCM-GCs. OCT, optimal cutting temperature media; LCM, laser capture micro-dissection

LCM GC studies published, both from cryosectioned galls (Portillo et al. 2009; Barcala et al. 2010).

The use of giant-cell cytoplasm as starting biological material for further transcriptomic analysis provided more accurate information on the relative levels of gene expression in GCs, either by classical PCR, qPCR or microarray analysis (Ramsay et al. 2004; Barcala et al. 2010), and has also allowed the identification and isolation of particular genes by differential display and library construction (Table 9.1; Wang et al. 2003; Fosu-Nyarko et al. 2009). Additional confirmation of a strong dilution of the GC transcripts in galls was provided from microarray data of LCM GCs isolated at 3 dpi compared to their corresponding hand-dissected galls (Barcala et al. 2010) (Fig. 9.2). This effect was clearly enhanced for genes with lower expression changes (fold change values of −1 to −3 and 1 to 3) in GCs as compared to uninfected cells from vascular tissue. In these fold change ranges, most of the differentially expressed GC transcripts were not detected in the whole gall transcriptome (Barcala et al. 2010). Similarly, this trend was confirmed in tomato at the same and even later infection stages (3, 7 dpi; Portillo et al. (2009); unpublished). Only 120 genes out of 1,161 differentially expressed in GCs were shared with those of the gall transcriptome in *Arabidopsis* (Fig. 9.2) and the tendency was similar in tomato. Reliability of the comparison was high as both analyses were performed using the same microarray platform with exactly the same experimental design, hybridization steps and data processing. One of the most striking differences between galls and GCs was the identification of a high number of down-regulated genes in GCs that were not detected as being differentially expressed in galls. Furthermore, the categories of secondary metabolism and biotic stress included a high proportion of 'gall and GC distinctive genes', but with opposite expression patterns (repressed in GCs, but up-regulated in galls). In addition, only eight out of the more than 100 genes encoding transcription factors differentially expressed in GCs were co-regulated between GCs and galls. In contrast, genes related to cell wall modification, such as expansins (EXPA6, EXPA1 and EXPA2), were mostly up-regulated co-regulated genes in GCs and galls. These data suggest that genes typically involved in defense mechanisms against pathogen attack (Dixon et al. 2002), such as those involved in the phenylpropanoid pathway were probably shut-off by the nematode exclusively in GCs, but not in the rest of the gall tissues (Barcala et al. 2010). This interpretation is supported by the identification of nematode pathogenicity factors such as secreted chorismate mutase and calreticulin which may be directly involved in plant defense suppression (Doyle and Lambert 2003; Jaubert et al. 2005).

Sensitivity in detecting gene expression changes specific to feeding cells induced by cyst nematodes was also increased when LCM and microaspiration approaches were employed to isolate the contents of syncytia (Fig. 9.2; Klink et al. 2005; Ithal et al. 2007b; Klink et al. 2007b; Szakasits et al. 2009). Microarray analyses using RNA isolated from laser-microdissected syncytia resulted in a substantial increase in the number of differentially expressed genes that were identified compared to microarray analyses using total RNA isolated from nematode-infected whole root pieces (Table 9.1; Ithal et al. 2007b; Klink et al. 2007b). Although both approaches

identified genes in common (Ithal et al. 2007b), the fold-change in expression of these genes in the LCM study was on average 26-fold higher and an additional 1,680 genes were identified. A similar increase in sensitivity was demonstrated when microaspirated syncytium cytoplasm was used for microarray analyses (Szakasits et al. 2009). In contrast to microaspiration studies at 5 dpi, which identified 18.4% of the total number of genes represented on the GeneChip as upregulated and 15.8% as downregulated (Szakasits et al. 2009), an analysis of infected whole roots at 3 dpi only identified 1% (upregulated) and 0.6% (downregulated) of the total genes represented as being differentially regulated (Puthoff et al. 2003).

A direct comparison between soybean gene expression changes identified using infected whole root pieces with those from syncytia after LCM, revealed only a small percentage of genes in common (Fig. 9.2; Ithal et al. 2007a, b; Klink et al. 2007b). Many of the co-upregulated genes included those belonging to the multibranched phenylpropanoid pathway which leads to the production of a diverse number of secondary metabolites in plants, including flavonoids, anthocyanins, and secondary cell wall components, genes involved in cell-wall related processes such as those coding for expansins and extensins, and genes that code for proteins involved in general stress responses including peroxidases, glutathione S-transferases, harpin-induced gene family members, and disease resistance-responsive family proteins (Ithal et al. 2007a, b; Klink et al. 2007b). In the LCM study reported by Ithal et al. (2007b), JA biosynthesis genes and other genes associated with abiotic and biotic stress responses including senescence-associated proteins, wound and osmotic stress responsive genes, and pathogen responsive receptor-like kinases were downregulated in syncytia. Consequently, the upregulation of genes involved in general plant defense identified from studies of infected whole root pieces may include a response of the plant to the intracellular migration and early establishment of feeding sites by the nematodes; components of which may be later suppressed by the pathogen as syncytia develop, similarly to GCs.

Although LCM has proven to be an effective tool to study gene expression in nematode feeding cells, GCs and syncytia are not clearly distinguished in sections at very early differentiation stages (12–48 hpi). This is partly due to the intrinsic characteristics of the developing feeding cells, and partly due to the tissue processing steps prior to LCM. In general, before microdissection, GCs and syncytia can be recognized in sections by their dense cytoplasm, sometimes slightly plasmolysed due to the fixation and dehydration treatments (Barcala et al. 2010). In addition, LCM requires mild fixation treatments to preserve macromolecules, but it produces a poorer preservation of the histological structures (Ramsay et al. 2004, 2006; Portillo et al. 2009). It is recommended to omit histochemical staining during sample processing to minimize RNA degradation (Ramsay et al. 2004). In addition, GCs and syncytia at very early developmental stages do not show unambiguous morphological features. Thus, the isolation of GCs and syncytia during the early stages of differentiation will require the development of new strategies, such as the combination of reporter lines activated at early infection stages during GC and syncytia differentiation to aid in the identification of the developing cells before LCM is

applied. The combination of LCM coupled to epifluorescence microscopes could allow for the isolation of emergent GCs and syncytia from their precursor cells that still do not show unequivocal morphological characters.

9.4 Next Generation Sequencing Technology to Study Plant Responses to Nematode Infection

Next generation rapid sequencing technology has been used only once for the study of the plant response to nematode infection (Hewezi et al. 2008). In this study, small RNA molecules were purified from total RNA isolated from cyst nematode-infected *Arabidopsis* roots by size fractionation and 100,000 sequence reads were obtained using 454 sequencing technology. Of 16 miRNAs checked after *H. schachtii* infection, 14 were altered at 4 dpi and 7 were altered at 7 dpi (Hewezi et al. 2008). Such approaches open the possibility of using different plant species from which scarce sequence data are available. Cross-species comparisons of data could perhaps also reveal physiological responses in galls and syncytia conserved among species or identify responses unique to each plant species-nematode interaction.

9.5 Proteomic Analysis of the Plant Response to Nematode Infection

9.5.1 Application of Proteomics to Investigate Plant-Microbe Interactions

The term proteome refers to the complete set of proteins present in a cell, organ or organism at a given time (Wilkins et al. 1995). Advances in proteomics have been made possible due to improvements in protein separation by two dimensional-gel electrophoresis (2-DE) (Görg et al. 2000), multidimensional liquid chromatography (MudPIT) (Washburn et al. 2001), peptide sequencing by mass spectrometry (MS) (Steen and Mann 2004; Venable et al. 2004), and bioinformatics (Apweiler et al. 2004). However, progress in defining proteomes is expected to proceed at a slower pace than genome sequencing (Jorrin et al. 2006). The application of proteomics in plant pathology is being used to characterize pathogen virulence factors, as well as to identify changes in protein levels in plant hosts upon infection (Kav et al. 2007). It is well known that nematode feeding site ontogeny is a reflection of extensive gene expression modification in infected root cells (Caillaud et al. 2008; Li et al. 2009). In contrast, strategies aimed at studying the proteomic plant response to nematodes are still in their infancy. Technical advances in the quality and reproducibility of 2-DE gels, software packages to process digitized images of gels, the development of non-gel based high-throughput protein separation techniques, and

analytical mass spectrometry should facilitate an increase in proteomic approaches to study plant-nematode interactions (Chen and Harmon 2006; Domon and Aebersold 2006). In order to establish a reliable, specific proteomic study, cell-specific analysis is a prerequisite as tissues are usually composed of heterogeneous cell populations and molecular analysis of biological samples as a whole may be of limited value. LCM and microaspiration provide powerful new tools to extract proteins from feeding sites for molecular analysis of the plant-nematode interactions. Although transcriptomic analyses have already been performed (Sect. 9.3.3), proteomics studies utilizing LCM to analyze the plant responses underlying GC and syncytium formation are still at a very early stage.

9.5.2 *Understanding Plant-Nematode Interactions in Light of Proteomic Studies*

The current knowledge on plant-nematode interactions is primarily based on genome and transcriptome analysis with few studies focused on the proteomic profiles of plants infected by nematodes. One of the first studies to examine changes in protein expression in response to nematode parasitism compared *Globodera rostochiensis* infected and uninfected roots of potato carrying the *H1* resistance gene. The presence of the nematodes in the root system did not cause any changes at 6 and 21 hpi, or at 3 and 6 dpi. Surprisingly, variations were observed in young leaves of infected plants (Hammond-Kosack et al. 1990). In contrast, Callahan et al. (1997) reported different results on one- and 2-DE analysis of resistant and susceptible cotton (*Gossypium hirsutum* L.) root protein extracts. Several polypeptides were differentially expressed in response to root-knot nematode infection. A novel 14 k Da polypeptide was more abundantly expressed in young galls of the resistant isoline at 8 dpi (Callahan et al. 1997). The profile of genes or proteins induced by the nematodes during feeding site formation can generate potential targets for reverse genetics. For example, the tomato expansin gene *LeEXPA5* found to be expressed during *Meloidogyne javanica* parasitism, facilitates cell expansion *in vivo* and was presumed to be important for the expansion of GCs. Consistently, the ability of nematodes to complete their life cycle on *LeEXPA5*-antisense transgenic roots was reduced (Gal et al. 2006). Thus, proteomic approaches coupled with functional genomics tools presents a powerful approach for the identification of targets for the development of transgenic crops resistant to nematodes.

Root proteomics aimed at discovering plant defense-related proteins in roots have been studied in nematode-resistant cotton and coffee cultivars infected with *Meloidogyne paranaensis* and *M. incognita*, respectively. A 2-DE analysis comparing infected versus non-infected roots identified a class III chitinase of *C. arabica*, known to be involved in defence responses to pathogens (Jaubert et al. 2002). Another differentially expressed protein in cotton was a quinone reductase 2 (QR2) that catalyzes the divalent reduction of quinones to hydroquinones to protect plant cells from oxidative damage (Sparla et al. 1999). These findings emphasize the importance of root

proteomics in the isolation of resistance and defense-related proteins against nematodes. Ultimately, transcriptomic, proteomic and metabolomic analyses will need to be integrated to elucidate the complex nature of the plant response to nematodes.

9.6 Conclusions

A vast list of differentially expressed genes identified from comparisons of either infected roots *versus* uninfected roots or from hand-dissected root nematode induced structures, is available in different databases and publications. Furthermore, the combination of precise cell-specific isolation techniques, such as LCM and microaspiration, together with holistic approaches for gene expression analysis based on microarrays, differential display, and EST sequencing have identified a diverse catalogue of genes differentially expressed in GCs and syncytia. Nevertheless, information pertaining to which genes have restricted expression in NFS is limited. Similarly, there is still fragmented information on comparative analyses among different experiments and plant-nematode interactions. Although in their infancy, the application of next generation sequencing technologies and proteomics analysis promises to provide more functional information on the sophisticated interactions between nematodes and their host plants.

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Chapter 10
C. elegans as a Resource for Studies on Plant Parasitic Nematodes

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Abbreviation

RNAi RNA interference

10.1 Introduction

Although significant progress in the molecular characterisation of plant-parasitic nematodes has been made during the last decade, their obligate biotrophic life cycle, enduring for up to eight weeks, and small size still remain a major obstacle. The conditions required for cultivation often make it difficult to obtain large numbers of nematodes for experimentation, with collection of the early parasitic stages of sedentary endoparasites being particularly demanding. Despite their disparate modes of existence plant parasitic nematodes share many common and highly conserved biological processes with the free-living soil Rhabditid nematode *Caenorhabditis elegans*. The rapid three-day life cycle, 2–3 week life-span, ideal size (1–1.5 mm) and high number of progeny (up to 350) per hermaphrodite allows laboratory maintenance of high numbers of these nematodes on agar plates seeded with a lawn of *Escherichia coli*. Furthermore the anatomical and genomic simplicity readily lends itself to technical laboratory manipulation, including a variety of genetic and molecular studies. *C. elegans* therefore provides a powerful laboratory model in which to investigate highly conserved biological processes of interest in target plant-parasitic nematodes including neurobiology, metabolism, development, moulting and reproduction.

C. elegans has been widely exploited as a laboratory model organism for over forty years (Brenner 1974) and was the first multicellular eukaryote to have its 97 Mb genome completely sequenced (C. elegans Consortium 1998). The *C. elegans*

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genome project has had many practical and technological applications to the mapping, sequencing and annotation of root knot nematode genomes. Complete sequences are now available for the 86 Mbp genome of *Meloidogyne incognita* (Abad et al. 2008) and 54 Mbp genome of *Meloidogyne hapla* (Opperman et al. 2008). Genome sequencing is now currently underway for the potato cyst nematode *Globodera pallida* and soy bean cyst nematode *Heterodera glycines* (publically available at http://www.sanger.ac.uk/sequencing/Globodera/pallida/ and http://www.ncbi.nlm.nih.gov/). Annotation and analyses of the 19,099 predicted protein encoding *C. elegans* genes are still in progress. Over 60% of these genes have a homologue in other species, facilitating their study in the target species (Kamath et al. 2003). Furthermore, the completion of genome sequencing for *Caenorhabditis briggsae* (Stein et al. 2003) and *Pristionchus pacificus* (Dieterich et al. 2008), along with sequencing projects currently underway for *Caenorhabditis remanei* and *Caenorhabditis japonica* promise to greatly assist the annotation of the *C. elegans* genome, enhance the understanding of the evolutionarily conserved features of the Nematoda Phylum and provide reference genomes for plant-parasitic species (Coghlan et al. 2006).

10.1.1 Resources Available for C. elegans Researchers

A number of physical and database resources have been developed by the *C. elegans* community to allow large-scale studies of gene expression and function. These tools are also proving invaluable for gaining insight into the function of gene orthologues from plant parasitic nematodes. Many *C. elegans* mutants targeting a wide range of alleles are now available for biological research, with some genetically determined phenotypes easy to observe. These are publicly available through the *C. elegans* Genetics Centre, USA, supported by the National Institutes of Health—National Center for Research Resources (http://www.cbs.umn.edu/CGC/). The ability to freeze and store *C. elegans* also increases the availability and ease of use of both wild type and mutant worms (Wood 1998). The development and application of high throughput RNA interference (RNAi) screens (Fire et al. 1998; Timmons and Fire 1998) has also significantly expanded the data available on *C. elegans* gene function in a number of biological processes, including fat regulation, ageing, RNAi, neuromuscular junction and nervous system development and detoxification (Ashrafi et al. 2003; Fraser et al. 2000; Hamilton et al. 2005; Hansen et al. 2005; Kamath et al. 2001, 2003; Kim et al. 2005; Kim and Sun 2007; Lee et al. 2003; Schmitz et al. 2007; Sieburth et al. 2005; Timmons et al. 2001). Libraries containing RNAi clones targeting almost 90% of *C. elegans* genes are commercially available at Geneservice Ltd., UK (http://www.geneservice.co.uk/products/rnai/Celegans.jsp). Large-scale RNAi screens in *C. elegans* have shown that phenotypes are more likely to be observed for genes that have an orthologue in another eukaryote (Britton and Murray 2006). Until reliable methods are available to examine gene function directly in target parasitic nematodes, *C. elegans*

remains a suitable alternative, particularly where putative orthologues exist in the free-living nematode (Blaxter et al. 2000).

Aided by a complete genome sequence, several transcriptomic and proteomic studies have yielded gene and protein expression profiles in *C. elegans* under given genetic, environmental and temporal conditions. Microarrays have been successfully used by *C. elegans* researchers to probe gene expression in specific tissues including the germline (Reinke et al. 2000), pharynx (Gaudet and Mango 2002), muscle (Roy et al. 2002) and neurons (Cinar et al. 2005; Colosimo et al. 2004; Fox et al. 2005; Kunitomo et al. 2005; Portman and Emmons 2004), and to investigate changes in gene expression associated with development (Hill et al. 2000; Jiang et al. 2001), ageing (Golden and Melov 2004; Lund et al. 2002; McCarroll et al. 2004; McElwee et al. 2004; Murphy et al. 2003) and other processes. A variety of proteomics studies have also been carried out as recently reviewed (Shim and Paik 2010). Although protein analysis of individual tissues has not yet been achievable in *C. elegans,* global and sub-proteomic expression changes have been investigated during development (Krijgsveld et al. 2003; Madi et al. 2003a; Mawuenyega et al. 2003), reproduction (Bantscheff et al. 2004; Chu et al. 2006; Lin and Reinke 2008) and ageing (Dong et al. 2007; Jones et al. 2010; Madi et al. 2008) as well as under a variety of conditions including temperature (Madi et al. 2003b) and RNAi exposure (Baek et al. 2008; Duchaine et al. 2006; Lacourse et al. 2008; Vinther et al. 2006). Furthermore, a shot-gun proteomics approach has been useful for the discovery of previously unpredicted coding sequences (Merrihew et al. 2008).

The development of an efficient transformation system for *C. elegans* (Mello et al. 1991) has allowed promoter-reporter constructs to be used to study gene expression in transgenic worms (Chalfie et al. 1994; Hope 1991). Green fluorescent protein (GFP) remains the reporter of choice, allowing observation of expression patterns in living worms. The more recent success of ballistic transformation methods for *C. elegans* has allowed higher throughput analysis of expression patterns (Praitis et al. 2001). A first version of the *C. elegans* promoterome has been generated, with approximately 6,000 cloned promoter regions readily transferable to Gateway destination vectors to drive expression of GFP in fusion with protein-encoding open reading frames (ORFs) available in the ORFeome resource (Dupuy et al. 2004; Hope et al. 2004). Individual promoterome clones or the complete library set are commercially available from Geneservice Ltd, Cambridge, UK (http://www.geneservice.co.uk). The advance of a Gateway-compatible yeast one-hybrid system (Y1H) has enabled studies of differential gene expression controlled by specific *C. elegans* transcription factors to identify protein-DNA interactions (Deplancke et al. 2004). This system allows high throughput functional genomic analysis and can be used in conjunction with the *C. elegans* promoterome resource (Dupuy et al. 2004; Reece-Hoyes et al. 2007). A two-hybrid interactome map of the *C. elegans* germline has been generated (Walhout et al. 2002) and compared to both a phenome map of the germline obtained by RNAi (Kim et al. 2005) and a transcriptome map obtained by clustering worm genes across 553 expression profiling experiments (Piano et al. 2002). Investigation of the *C. elegans* interactome network using high-throughput yeast two-hybrid screening (HT-Y2H, (Li et al. 2004)) shows high potential for the

identification of protein-protein interactions (Boulton et al. 2002; Wawersik and Maas 2000). Continuing use of *C. elegans* as a heterologous system for studying gene function and regulation will be important to fully exploit the parasite sequence data and further our understanding of parasite biology (Britton et al. 1999; Britton and Murray 2002; Couthier et al. 2004; Kampkotter et al. 2003; Kwa et al. 1995).

There are a large number of internet-based resources which act as a public repository for electronic storage of information regarding *C. elegans* as well as a means to acquire physical research tools readily available to the community. The ever-expanding and evolving Wormbase (http://www.wormbase.org Release WS207, (Rogers et al. 2008) remains the most efficient means to readily access functional genomic data. Wormbase contains sequence data from the public databases EMB and Genbank as well as from the Welcome Trust Sanger Institute (SANGER) and Washington University Genome Sequence Centre (WASHU, (Harris et al. 2003)). Sequence data are annotated with genetic maps, literature details and functional data deposited by SANGER, Cold Spring Harbour Laboratory (CSHL) and California Institute of Technology (CALTEC). The most recent version contains fully integrated *C. briggsae* genomic sequence as well as genome wide microarray data, RNA interference (RNAi) data, expression data, proteome-wide two-hybrid interactions, antibody reagents, three-dimensional structures, eukaryotic orthologous gene groups from NCBI, EST and protein alignments, 5′ and 3′ untranslated regions (UTRs) for all genes with mRNA or EST data and a browsable ontology for worm anatomy (Harris et al. 2003). Furthermore, the site directory enables searches to be performed within a specific type of information such as Sequences; Cells and Gene Expressions; Genetics; Strains and Phenotypes or by customising complex sequence-related queries (WormMart). To allow routine large-scale data analyses, both the data and software of Wormbase can be downloaded for local use. A new release of the database, containing additional data as well as update and refinement of existing data, is assembled every fortnight by the Sanger worm informatics group (Rogers et al. 2008).

Web resources now exist to catalogue and integrate new sequence data from parasitic nematodes and make them accessible to the user community. In 2000, the Genome Center at Washington University (GC) joined a consortium including the Nematode Genomics group in Edinburgh, and the Pathogen Sequencing Unit of the Sanger Institute to obtain new sequence data via the rapid and cost-effective generation of Expressed Sequence Tags (ESTs) from a number of human, animal and plant-parasitic nematodes. Over 530,000 ESTs and 1.2 million genome survey sequences have now been generated from over 44 nematode species, including 14 plant-parasitic, 23 animal-parasitic as well as seven other free-living nematodes (http://www.nematode.net/Species.Summaries/index.php). A total of 79,978 ESTs have been generated from various developmental stages of *Meloidogyne* spp., 16,229 ESTs from *Globodera* spp. and 27,259 ESTs from *Heterodera* spp. (Martin et al. 2009). With a large amount of EST data also available from numerous animal-parasitic nematodes, comparisons with sequence data from *C. elegans* are allowing the identification of nematode-specific genes as well as genes specifically associated with a parasitic lifestyle. Nematode.net was implemented to offer user-

friendly public access to data produced by this project (http://www.nematode.net). Two complementary databases (NEMBASE—developed by the Nematode Genomics group in Edinburgh and NemaGene—developed at the GSC) aim to integrate the sequence data in a biological context. NEMBASE (http://www.nematodes.org) can be searched via sequence annotation, sequence similarity (via a web-based WU-BLAST search tool that allows basic and complex querying) and stage-specificity (searching for explicit expression patterns).

NemaGene EST clusters have now been generated for 15 species, including cyst and root knot nematodes. Putative functions have been assigned based on extrapolation from homology. Conserved domains were identified using the Interpro protein domain database and clusters were then mapped onto the Gene Ontology (GO) classification scheme (Eilbeck et al. 2005) and to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database of biochemical pathways (http://www.genome.ad.jp/kegg) via NemaPath (Wylie et al. 2004). Currently 33 species (30 parasitic and three free-living) of nematodes have been mapped. NemaGene EST cluster entries are now integrated with *C. elegans* gene pages from WormBase in cases where a relationship is believed to exist, allowing easy navigation between Nematode.net and WormBase. Another addition to Nematode.net is the inclusion of the NemFam collection of nematode-related, conserved regions of proteins that are not found in public protein databases (Finn et al. 2008). NemFam was constructed using over 214,000 polypeptides from 32 nematode species (27 of which are parasites of vertebrates or plants). EST data mining has started to reveal the close relationship between *C. elegans* and parasitic nematodes. Phylogenetic analysis with small subunit ribosomal RNA places *C. elegans* in Clade V, along with the parasitic strongylid nematodes such as *Haemonchus contortus*, *Nippostrongylus brasiliensis*, *Necator* spp. and *Ancylostoma* spp. (Blaxter et al. 1998).

Analysis of the available EST data has shown that 50–70% of sequences from Clade V species have significant similarity to *C. elegans* predicted proteins (raw BLAST score $\geq$ 50) (Parkinson et al. 2004). Even nematodes from the more distantly related Clade I, such as *Trichinella* spp. and *Trichuris* spp., show homology to *C. elegans* in around 45% of their genes (Parkinson et al. 2004). More recent comparisons of the genome sequence from the Clade III nematode, *Brugia malayi*, with that of *C. elegans* and *C. briggsae* have found that ~48% of predicted proteins from *B. malayi* have homologues in either *Caenorhabditis* species (Ghedin et al. 2007). OrthoMCL clustering of the predicted protein sequences from the *M. incognita* genome sequence also found that homologues were present in other species for 48% of these sequences (Abad et al. 2008). Of these homologue groups 71% showed significant homology to *C. elegans* with 14% being nematode specific and only 2% being species-specific. Many of these genes have RNAi lethal phenotypes, supporting the correlation between sequence conservation and severe RNAi phenotype. Similarly, almost half of the genes to which a function has been assigned in *M. hapla* (based on BLAST searching) have the highest sequence identity to *C. elegans* genes. Where no homology has been found between predicted protein sequences from *Meloidogyne* genomes and those in *C. elegans* some homology has then been found with ESTs from other plant- and animal-parasitic nematodes.

Whilst *C. elegans* does not provide a useful model for predicting the function of these particular genes, their absence in its genome helps identify them as potentially having roles in parasitism.

10.2 *C. elegans* As a Model Nematode

10.2.1 Neuronal Biology and Chemoreception

Root localisation by newly hatched pre-parasitic root knot and cyst nematode J2 larvae is dependent on a gradient of diffusing stimuli from the plant root. The chemotactic response of the nematode towards the penetration site is coordinated by integration of input from chemoreceptive and mechanoreceptive neurons. These sensory sensilla are located at the anterior end of the nematode and their structure is conserved in a wide-range of plant-parasitic nematodes including *M. incognita*, *H. glycines* and *G. rostochiensis* (Perry 2005). Despite differences in lifestyle between plant-parasitic nematodes and *C. elegans*, it is likely that both share a common chemoreceptive circuitry and suite of neurochemicals, with species-specific differences in the sensitivity of the sensory organs to the appropriate mechanical and chemical cues. The neuronal network in *C. elegans* has been extensively studied and characterised at the cellular level. An adult *C. elegans* hermaphrodite contains 302 neurons, comprising approximately one third of the total somatic cells. Precise information about the identity, gross morphology and synaptic connectivity of every neuron in the adult hermaphrodite has been discovered (Schafer 2005; White et al. 1986). Six primary sensory neurons have been identified at the amphids in *C. elegans* and their roles in sensory perception determined, including perception of pheromones, water-soluble chemicals and temperature as well as odour attraction and repulsion (Tsalik and Hobert 2003).

Gene knockout experiments and the creation of precise cell lesions have allowed the expression patterns of a number of gene families with functions in neuroreception to be analysed. These gene families have included glutamate receptors (Brockie et al. 2001), G protein subunits (Jansen et al. 1999) and serotonin receptors (Tsalik and Hobert 2003). A genome-wide touch-neuron-specific screen coupled with GFP reporter constructs has identified and verified the expression of a number of genes (Zhang et al. 2002). Several other studies have identified important gene sets expressed in different components of the chemosensory system of *C. elegans*, including the thermosensory neurons, inhibitory motor neurons and cholinergic motorneurons involved in backward locomotion (Cinar et al. 2005; Colosimo et al. 2004; Fox et al. 2005). Genes expressed in ciliated sensory neurons have also been analysed using the targeted expression of an epitope-tag poly-A binding protein (Kunitomo et al. 2005). Ablation of individual neurons with the use of a laser microbeam has allowed the identification of several phenotypes associated with specific neurons. These abnormalities have included defects in chemotaxis, egg laying, movement

and locomotion (Schafer 2005). At the cellular level many aspects of neuronal function, such as sensory transduction, synaptic plasticity and G-protein-mediated neuromodulation are broadly conserved between nematodes (Schafer 2005). A number of genes with chemosensory functions have been identified from the altered response of odorant (*odr*) mutants of *C. elegans* to specific chemical cues (Bargmann and Mori 1997). Analyses of (*odr*) mutants in *C. elegan*s have identified a family of guanylyl cyclases, which function in olfactory discrimination and signalling downstream of odorant receptors (L'Etoile and Bargmann 2000). Gene members of the guanylyl cyclase family appear to be highly conserved between *C. elegans* and *H. glycines*, in both sequence and expression patterns (Yan and Davis 2002; Yu et al. 1997).

In addition to the identification and characterisation of neuronal cells and their synaptic connectivity, the study of neurotransmitters and their specific expression also bears direct relevance on nematode control. Despite the simplicity of the *C. elegans* nervous system, the diversity of neurotransmitters and their specific expression may be compared to that of more complex organisms such as vertebrates. Neuropeptides function as ubiquitous signalling molecules in the nervous system and 250 potential neuropeptides have currently been identified in *C. elegans* (Li 2005; McVeigh et al. 2006). At least 68 of these are FMRFamide-related peptides (FaRPs) encoded by 29 known *flp* genes (Li 2005; McVeigh et al. 2005). Expression patterns of *flp* genes have been analysed using GFP reporter constructs (Kim and Li 2004; Li et al. 1999), loss of function and over-expression experiments (Rogers et al. 2003). These *flp* genes are expressed in at least 50% of neurons (including motor, sensory and interneurons) and are involved in multiple behaviours such as feeding, defecation and reproduction (Kim and Li 2004; Li 2005; McVeigh et al. 2005). BLAST searching within EST databases has revealed the high conservation of individual FLPs and FLP motifs across the Nematoda Phyla, suggesting a fundamental role in nematodes of all lifestyles (McVeigh et al. 2005). Transcript to five *flp* genes with high homology to *C. elegans* have been identified in *G. pallida* (Kimber et al. 2001). Analyses of the recently completed genome sequence of *M. incognita*, has also identified 19 *flp* genes, 17 of which have predicted orthologues in *C. elegans* (Abad et al. 2008). Where current tools for expression analyses in the J2 larvae of plant-parasitic nematodes is limited by their size and tractability, *C. elegans* provides a suitable heterologous system for the expression of target genes as well as a system in which to study target orthologues.

Both serotonin and dopamine act as transmitters in the *C. elegans* nervous system by binding to specific biogenic amine receptors. Dopaminergic signalling has been shown to mediate various behaviours including locomotory response to food (Sawin et al. 2000), feeding and egg laying (Schafer and Kenyon 1995; Weinshenker et al. 1995). The *C. elegans* genome encodes two types of dopamine receptors, DOP-1 and DOP-2 (Komuniecki et al. 2004) and their expression has been assessed by GFP reporter constructs (Tsalik and Hobert 2003). The expression of DOP-1 appears to be restricted to the tail neuron axons, while that of DOP-2 is more specific to interneurons. The presence of dopaminergic neurons has also been demonstrated in cyst nematodes (Sharpe and Atkinson 1980). The neurotransmitter serotonin also

affects locomotion, egg-laying, and pharyngeal pumping (Avery and Horvitz 1990; Horvitz et al. 1982; Schafer and Kenyon 1995; Trent et al. 1983). Three serotonin receptors have been identified in *C. elegans* (SER-1, -4, -7), with GFP expression localised to pharyngeal muscle, head interneurons and pharyngeal motorneurons respectively (Hobson et al. 2003). The expression of SER-7 during pharyngeal pumping, suggests that SER-7 agonists may be useful for an anthelmintic design. BLAST searches with translated nucleotide sequences (tBLASTx) at http://www.sanger.ac.uk and http://www.ncbi.nhl.nih.gov have found possible orthologues for *dop-1*, *dop-2*, *ser-1*, *ser-4* and *ser-7* in the genome sequence for both *G. pallida* and *M. incognita*.

Approximately 115 neurons in *C. elegans* appear to be cholinergic (Rand et al. 2000). Acetylcholine controls motor function by binding to excitatory/inhibitory transmembrane receptors that are classified as ionotropic (nicotinic) or metabotropic (muscarinic) (Martin et al. 2005). *C. elegans* is paralysed by cholinesterase inhibitors such as aldicarb and by nicotinic acetylcholine agonists such as levamisole (Brenner 1974). Acetylcholine is synthesised by choline acetyltransferase, encoded by the *cha-1* gene, and transported into synaptic vesicles by the vesicular acetylcholine transporter, encoded by the *unc-17* gene. Null mutations for both of these genes are lethal in *C. elegans* (Rand and Russell 1984) and tBLASTx searching at http://www.ncbi.nhl.nih.gov and http://www.sanger.ac.uk has strongly suggested orthologues to both genes in the genome sequences for *M. incognita* and *G. pallida*. Partial mutations in *unc-17* result in uncoordinated *C. elegans* worms containing excess acetylcholine (Brenner 1974; Hosono et al. 1987; Nguyen et al. 1995). Partial mutations in *cha-1* show impairment in a variety of behaviours including coordination, feeding and defecation (Rand and Russell 1984; Rand 1989). Both partial mutants are also defective in egg-laying (Bany et al. 2003).

Four distinct genes (*ace-1-4*) encode acetyl-cholinesterase in *C. elegans* (Combes et al. 2000) and possible orthologues to all four of these genes have also been identified in the genome sequences of *M. incognita*, *G. pallida* and *H. glycines*. Although single mutations in these genes do not affect the phenotype in *C. elegans*, nematodes lacking both *ace-1* and *ace-2* are uncoordinated and mutations in *ace-1*, *ace-2* and *ace-3* are lethal (Culotti et al. 1981; Johnson et al. 1981; Johnson et al. 1988). As a target for some of the most commonly used nematicides (organophosphate and carbamate), acetylcholinesterases of plant-parasitic nematodes are of particular interest. Two acetyl cholinesterase genes have been isolated from the root-knot nematode *M. incognita* (Laffaire et al. 2003; Piotte et al. 1999) and an *ace-2* homologue has been isolated from the cyst nematodes *G. pallida* and *H. glycines*. The *Meloidogyne ace-1* gene is expressed only in eggs, preparasitic juveniles and males, whereas the *ace-2* gene from the same species is expressed in all stages including the sedentary female. The *ace-2* homologue in *G. pallida* and *H. glycines* has been found most highly expressed in the pre-parasitic infective J2 stage, with lowest expression in the early parasitic stages (Costa et al. 2009). Functional equivalence of these orthologues has been demonstrated by the ability of the *G. pallida ace-2*, heterologously expressed in *C. elegans*, to restore a normal phenotype to the uncoordinated *ace-1; ace-2* double mutant (Costa et al. 2009). These studies are providing

an important insight into development of effective controls against plant-parasitic nematodes. Furthermore, the potential for using transgenic methods to deliver aldicarb and levamisole mimetic peptides *in planta* to disrupt chemoreception has been demonstrated (Liu et al. 2005).

10.2.2 Intermediary Metabolism and Cellular Detoxification

As a result of the tough cuticles and egg-shells, along with the inability to collect tissue in appropriate quantities, a relatively small number of biochemical studies have been carried out on plant-parasitic nematodes (as reviewed by Barrett and Wright 1998). Most biochemical studies on nematodes have been on animal-parasitic species and *C. elegans* (Barrett 1997). The intermediary metabolic network is well conserved among nematodes and other eukaryotes, and all appear to catabolise energy reserves by glycolysis/β-oxidation, the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. Orthologues for most of the enzymes involved in the main pathways of intermediary metabolism have been found in the genomes of *C. elegans* and *M. incognita* (Abad et al. 2008; Braeckman et al. 2005). The main carbohydrate reserves are glycogen, trehalose and glucose, which may be utilised under aerobic and anaerobic conditions in all nematode species (Barrett and Wright 1998). Plant-parasitic nematodes also contain energy reserves in the form of neutral lipids, in particular unsaturated fatty acids such as triacylglycerol.

When periodically exposed to anaerobic conditions plant-parasitic nematodes produce ethanol, acetate, lactate, succinate and glycerol as an end product. The presence of an active glyoxylate cycle enables the conversion of both anaerobic end products and lipids to carbohydrates. This pathway is also found in bacteria, algae, fungi and plants and enables these organisms to utilise acetyl-CoA from the β-oxidation of fatty acids for gluconeogenesis. In the glyoxylate cycle two molecules of acetyl-CoA are used for each glyoxylate cycle, with the net production of succinate and malate that can be used for the synthesis of glucose. Expression of both glyoxylate cycle enzymes has been demonstrated in a range of plant-parasitic nematodes, including J2 larvae of *M. incognita* (McCarter et al. 2003) and the egg, L1 and dauer stages of *C. elegans* (Liu et al. 1995, 1997; O'Riordon and Burnell 1990). Dauer larvae have altered energy metabolism, with a higher utilisation of lipid reserves and suppression of aerobic respiration in favour of glycolysis and fermentative metabolism (Vanfleteren and De Vreese 1996). Transcriptomic and proteomic studies have also supported enhanced resistance to oxidative stress and a shift toward microaerobic and anaplerotic metabolic pathways and hypometabolism in dauer larvae (Burnell et al. 2005; Holt and Riddle 2003; Houthoofd et al. 2002; Jones et al. 2001; Madi et al. 2008; McElwee et al. 2004; Wang and Kim 2003).

In order to detoxify lipid peroxidation products generated as a result of reactive oxygen species (ROS) released during the electron transfer chain, nematodes have evolved a number of detoxification pathways, common to other eukaryotes. Free-living nematodes are also under constant assault from a wide range of xenobiotics to

which they are exposed in their heterogenous soil environment. *C. elegans* contains a wide array of detoxification enzymes encoded by 86 cytochrome P450 (CYP) genes, 68 short-chain dehydrogenases (SDR), 72 UDP-glucuronosyl or glycosyl transferases (UGT) genes, 48 glutathione transferase (GST) genes (representatives from Omega, Sigma and Zeta classes) as well as a number of genes encoding superoxide dismutases and glutathione peroxidases (Lindblom and Dodd 2006). The genome sequence of *M. incognita* contains a vast reduction in genes encoding these enzymes with only 27 CYP genes and five GST genes, all from the Sigma class (Abad et al. 2008). These GST sequences from *M. incognita* display high homology with nematode specific (Nu) members of this class identified in *C. elegans* (Campbell et al. 2001). A similar reduction in genes encoding proteins which function in detoxification has also been reported in the genome of *B. malayi* (Ghedin et al. 2007). This reduction is likely to be a consequence of natural selection during evolution of a parasitic lifestyle with exposure to fewer xenobiotics (Abad et al. 2008). Furthermore, there is evidence that these sigma GSTs may have acquired a new role in the protection of plant-parasitic J2 larvae against the plant oxidative burst defence response (Dubreuil et al. 2007). GST expression has been reported in oesophageal glands and the enzyme is present in stylet secretions of *M. incognita* (Dubreuil et al. 2007).

10.2.3 Nematode Cuticle and Moulting

During post-embryonic development, most nematodes are enclosed within an exoskeleton called the cuticle (Cox et al. 1981a, b; Johnstone 1994; Kramer 1994a, 1997). The nematode cuticle not only provides a structure against which longitudinal muscles can contract during locomotion but also a dynamic interface with the external environment. The cuticle performs complex fundamental roles in protection from the environment, physiology, nutrition and excretion (Chen and Howells 1981; Geary et al. 1995; Ho et al. 1990, 1992; Howells and Chen 1981). The cuticle is a tough elastic dynamic and extracellular structure with a complex composition that is synthesised by an underlying ectodermal cell layer termed the hypodermis. The nematode cuticle is composed predominantly of cross-linked collagens, as well as insoluble proteins termed cuticulins and associated glycoproteins and lipids (Blaxter and Robertson 1998; Johnstone 2000). Variations in cuticle structure have been found between different species of nematodes and have encouraged its analysis for phylogenetic purposes (Decraemer et al. 2003). Furthermore, intraspecific differences occurring spatially, temporally and between sexes have been reported (Wang et al. 1998). Differences in cuticular structure may therefore reflect specialist environmental interactions. For example, the recovery of *C. elegans* dauer larvae and activation of plant-parasitic J2 larvae are both accompanied by changes in lipophilicity of the surface (Proudfoot et al. 1991).

The cuticle of *C. elegans* has been the most extensively studied at the morphological, genetic, molecular and biochemical level (as reviewed in Johnstone 1994;

Kramer 1994a, b, 1997; Politz and Philipp 1992). Cuticle synthesis occurs five times during development, once in the embryo and then at the end of each larval stage prior to moulting. Synthesis occurs underneath an existing cuticle, which is displaced during secretion and polymerization of the new cuticle (Kramer 1997). Moulting can be subdivided into three stages including lethargus, apolysis and ecdysis. The moulting process is initiated by a gradual decrease in general activity and feeding, followed by separation of the old cuticle from the newly formed cuticle and subsequent emergence from the old cuticle. The factors regulating nematode moulting and cuticle synthesis remain to be resolved, however evidence suggests the involvement of orphan nuclear hormone receptors, including NHR-23 and NHR-25 (Gissendanner and Sluder 2000 Kostrouchova et al. 2001) liganded by cholesterol and steroid derived hormones (Kuervers et al. 2003; Yochem et al. 1999). More than 150 genes have been demonstrated by RNA interference to be required for the moulting process, including transcription factors, secreted peptides, transmembrane and extracellular matrix proteins as well as proteases and peroxidases (Frand et al. 2005).

The cuticle surrounding the main body of the animal can be sub-divided into the broad dorsal and ventral regions and the narrow lateral regions overlying the seam cells (Blaxter and Robertson 1998). Longitudinal structurally distinct ridges termed alae are positioned on the cuticle of the L1 and dauer larvae and of the adult. Another clearly visible surface specialisation is the annuli, which are present throughout the life-cycle and formed during cuticle secretion (McMahon et al. 2003). The cuticles of many species, including *C. elegans* and *G. rostochiensis*, conform to a four zone format including an epicuticle, cortical, median and basal zone. The lipid-rich epicuticle is covered by a glycoprotein-rich coat. This surface coat is synthesised from the excretory system and gland cells and has been associated with immune evasion in several parasitic nematode species (Blaxter et al. 1992; Page et al. 1992). In reflection of a disparate life-style the epicuticle in *C. elegans* is more likely to function in locomotion and prevention of predatory microbe adhesion. Although *C. elegans* is likely to encounter a very wide variety of bacteria in its daily life few of these seem to have a deleterious effect, thus implying the existence of effective defence mechanisms. For example the coryneform bacterium *Microbacterium nematophilium* often contaminates cultures of *C. elegans*, causing a distinctive swollen tail (known as the deformed anal region or dar phenotype) due to the substantial local swelling of the hypodermal tissue underlying the cuticle. Detailed analyses have demonstrated that a local activation of the MAP kinase cascade is associated with subsequent protection against the pathogen, supporting the existence of an innate immune mechanism (Nicholas and Hodgkin 2002). Furthermore, it has been reported that *srf-3* encodes a multi-transmembrane hydrophobic protein exclusively expressed in secretory cells (Hoflich et al. 2004). This protein is capable of translocating UDP-galactose and UDP-acetyl glucosamine, providing the donor substrate for glycosyl transferase, necessary for the glycosylation of proteins. Indeed, *M. nematophilium* fail to adhere to the cuticle of *srf-3* mutants, suggesting that an alteration of the glycosylation pathway may alter the *C. elegans* cuticle component involved in bacterial adhesion. The structural components of the

cuticle provide rigidity and elasticity and are closely involved in determining body shape and locomotion.

Cuticular collagen is the most abundant component, whilst other non-collagenous components have been termed ‘cuticulin’ (Fujimoto and Kanaya 1973; Lewis et al. 1994). Cuticle collagens are found in all major layers, except for the epicuticle and surface coat, whereas cuticulins are restricted to the cortical layer. Cuticle collagens of *C. elegans* have been studied in great detail and have also been analysed in *Meloidogyne* (Ray and Hussey 1995; Van der Eycken et al. 1994). Collagen proteins are characterised by a repeat of glycine-X-Y where X and Y are more frequently proline and hydroxyproline, respectively. Collagen biosynthesis occurs at the endoplasmic reticulum and involves numerous post-translational modifications and processing steps that in turn are catalysed by specific enzymes. The cuticle collagens in *C. elegans* are encoded by a large gene family of over 180 members grouped into six sub-families (Page and Johnstone 2007) and are subject to a strict pattern of temporal regulation. Interestingly, the collagen gene family seems to be reduced in parasitic nematodes, with only 81 members found in the *M. hapla* genome and 122 members in the *M. incognita* genome (Abad et al. 2008; Opperman et al. 2008). Currently mutants of 21 of the cuticle collagen genes have been identified in *C. elegans* resulting in a variety of body morphology defects including phenotypes described as DumPY short fat worm (Dpy), ROLler, right-hand roller (Rol), BLIster, fluid filled separation of the cuticle layers (Bli), SQuaT (Sqt), Ray AbnorMal (Ram) and LONg (Lon).

The cuticulin proteins have cysteine-rich zona pellucida-like domains and play stage-specific roles in formation of the seam cell derived cuticular alae (Sapio et al. 2005). Many different CUT proteins have been recently defined in *C. elegans*, and their fine spatial and temporal regulation of expression characterised (Sapio et al. 2005). *Cut-1* was the first CUTiculin gene to be identified (Sebastiano et al. 1991) and, along with the product of *Cut-5* is involved in alae formation in dauer larvae. CUT-3 and CUT-5 are required for alae formation in dauer larvae and, along with CUT-6, involved in the maintenance of the correct radial body morphology in this long-lived stage (Muriel et al. 2003; Sapio et al. 2005). CUT-3 is similarly associated with alae formation and body morphology in the L1 larvae, whereas CUT-4 is involved in the adult cuticle formation (Sapio et al. 2005). BLAST searching with translated nucleotide sequences (tBLASTx) at http://www.sanger.ac.uk and http://www.ncbi.nhl.nih.gov has found strong orthologues for *cut-1*, *cut-3*, *cut-4*, *cut-5* and *cut-6* in the genome sequences of *G. pallida* and *M. incognita*. Furthermore, the fine temporal regulation of expression has been revealed in *M. artiellia*, in which *Cut-1* transcript is highly expressed at the first moult but expression reduced in the infective J2 stage (De Giorgi et al. 1997).

10.2.4 Development and Reproduction

Another important feature attributing to the success of nematode free-living and parasitic life-styles is the plasticity of the nematode life-cycle. The ability to form a dor-

mant stage capable of enduring harsh environmental extremes is highly conserved across the Nematoda phylum. *C. elegans* and many other free-living nematodes have evolved a dispersal strategy which uses a fast generation time, large brood size and rapid habitat depletion (Riddle and Albert 1997). Adult hermaphrodites may rapidly colonise a small habitat of rich microbial food resources, eventually exhausting the habitat of its nutrients before re-colonising a new habitat (Barriere and Felix 2007). Such a lifestyle presents the challenge of starvation for the progeny of the founder adults as the habitat becomes depleted and crowded. In response to this *C. elegans*, like many soil nematodes, can undergo one of two larval developmental pathways.

C. elegans has two natural sexes, hermaphrodites with 959 somatic cells and males with 1,031 somatic cells. Males are smaller and thinner than hermaphrodites, and contain a fan-shaped tail composed of structures required for mating. Hermaphrodites produce both sperm and eggs and are therefore capable of reproduction by either self-fertilisation or cross-fertilisation with males (reviewed in Corsi 2006). A hermaphrodite lays about 300 eggs during the entire life. Oocytes are synthesised in a limited region of the ovary called the loop (White 1988). Oocytes enlarge and mature as they pass down the oviduct, before being fertilised in the spermatheca. The embryo begins development inside the uterus of the hermaphrodite, regardless of whether the sperm that fertilised the oocyte originates from the spermatheca of the hermaphrodite or from mating with a male. From the early stage of development the zygote has a chitinous layer, making the egg the most resistant stage of the life cycle and consequently the most difficult stage to embed and section before observation in the transmission electron microscope (Bird and Bird 1991). One of the two chitin synthetase genes of *C. elegans* is abundantly expressed in the adult hermaphrodite, during which life stage eggshells are being synthesised (Veronico et al. 2001). Approximately three hours following fertilisation and coinciding with gastrulation, the egg is deposited through the vulva.

Embryogenesis is characterised by cell proliferation followed by morphogenesis (Wood 1988). Cell division, cell movement and specific cell death proceed in a precise temporal and spatial pattern, giving rise to a permanently fixed number of cells. The first markedly unequal division gives rise to the six founder cells, from which the different cell lineages develop. Morphogenesis is characterised by the cessation of cell division. The embryo begins to elongate and move, and as the different tissues become more or less differentiated the cuticle is formed. The first-stage larva (L1) hatches from the egg at about 14 h following fertilisation. Hatching is an important regulatory event since many embryonic genes function as a consequence of the dilution of the maternal gene product and the requirement for new ones. Larval development proceeds through three additional stages of L2, L3, L4, punctuated by moults, in approximately three days, before reaching reproductive maturity as an adult (Byerly et al. 1976). In response to appropriate environmental cues, indicating that conditions are inadequate for successful reproduction it is able to enter into, and recover from an environmentally-resistant dauer larva at the second moult (Cassada and Russell 1975).

This non-feeding dauer stage, specialised for dispersal and survival of inhospitable conditions, shares some morphological and behavioural similarities with the

infective stage of the parasitic life-cycle (Bird et al. 1999; Blaxter and Bird 1997; Burglin et al. 1998; Jones et al. 1993; Proudfoot et al. 1993). Dauer formation and recovery in *C. elegans* is controlled by specific environmental cues, including temperature, food signal and population density as measured by a secreted pheromone (Golden and Riddle 1984a). Despite their apparent quiescence, several thousand genes continue to be expressed (Wang and Kim 2003). A range of techniques including laser ablation (Bargmann and Horvitz 1991) and mutant analysis (Golden and Riddle 1984b) have identified 32 dauer-formation (*daf*) genes in *C. elegans*. Multiple parallel genetic pathways have been uncovered, with different sets of amphidial neurons monitoring conditions independently and each capable of giving a signal to form dauer larvae. The precise nature of these cues is unknown in plant-parasitic nematodes but are likely to differ from those in *C. elegans*. Possible orthologues for 17 *daf* genes acting downstream in the dauer pathway have been identified in the genome of *M. hapla*, thus indicating that although the response to the environment in parasitic versus free-living nematode is substantially diverged the basic aspect of development is conserved in Nematoda (Opperman et al. 2008).

Despite altered life-styles and specific environmental responses many other important developmental pathways in *C. elegans* are partially conserved in the *Meloidogyne* genome (Abad et al. 2008). Sex determination is another key developmental event which occurs in all nematodes, often in response to environmental conditions, whereby males are favoured under sub-optimal conditions. The most frequent mode of reproduction among nematodes is sexual reproduction (amphimictic), employing males and females (Poinar and Hansen 1983). However, the strategy of asexual reproduction is also well represented among free-living groups from which many parasitic forms appear to have evolved, including hermaphrodism (automixis) and parthenogenesis (amixis). *C. elegans* is capable of both sexual reproduction (amphimixis) and asexual hermaphroditic reproduction, depending on food availability. Sex determination in *C. elegans* is genotypic and occurs early during embryogenesis, whereby true XO males arise spontaneously as rare products of non-disjunction of the X chromosomes in the XX hermaphrodite. Conversely, mating of a XX hermaphrodite with a XO male results in broods containing equal numbers of XX and XO (Zarkower 2006).

The mode of reproduction in *Meloidogyne*, *Heterodera*, *Pratylenchus* and *Helicotylenchus* is amphimictic and parthenogenic, depending on the species (Triantaphyllou 1971, 1983). *M. incognita* reproduces exclusively by mitotic parthenogenesis, with no genetic contribution from males to the production of offspring, whereas *M. hapla* is capable of reproduction by both amphimixis and meiotic parthenogenesis. However, a hermaphroditic *M. hapla* isolate has also been described (Triantaphyllou 1993). The cue for sex determination in *Globodera* and *Meloidogyne* species occurs by the J2 stage but the molecular mechanism remains unknown (Grundler et al. 1991). However, the sex determination pathway in *C. elegans* has been extensively studied (Kuwabara and Kimble 1992; Parkhurst and Meneely 1994). In *C. elegans* the XX-XO sex determination pathway and dosage compensation mechanisms are intimately interlinked, with both pathways being commenced by the same genes (Zarkower 2006). In spite of their different modes of reproduc-

tion, *M. incognita* and *M. hapla* possess possible orthologues of at least one member of each stage of the *C. elegans* sex determination cascade, as well as those of numerous downstream genes repressing and promoting male differentiation and behaviour (Abad et al. 2008; Opperman et al. 2008). The lack of orthologues upstream in the pathway suggests a divergence between the root knot species and *C. elegans* in signals that trigger these sex determination pathways. Indeed, in contrast to sex determination in *C. elegans*, sexual fate in root knot nematodes is environmentally specified with males being produced only under stressful conditions, such as limited nutrition or crowding. It is therefore possible that *M. incognita* uses a similar genetic system for sex determination, but with the male pathway also modulated in response to environmental cues. However, cyst nematodes such as *G. pallida* do not contain sex chromosomes and therefore may be able to delay expression of sex determining genes until an environmental cue is received.

10.3 Application of RNAi in *C. elegans* and Parasitic Nematodes

10.3.1 RNAi Mechanism in C. elegans

Gene silencing using RNAi has proved to be an invaluable tool for the analysis of gene function in *C. elegans* and other organisms. This reverse-genetics approach enables systematic knockdown of gene expression in order to study loss-of-function phenotypes *in vivo*. It has led to the identification of a number of genes involved in essential processes in *C. elegans*. RNAi is a natural cellular phenomenon in which double stranded RNA (dsRNA) is recognised as being foreign, initiating a chain of processes in which both the dsRNA and homologous mRNA is degraded, leading to sequence specific homology-dependent gene silencing. Following its characterisation in *C. elegans* (Fire et al. 1998) the RNAi pathway has been recognised in numerous organisms throughout the Animal, Plant and Fungi Kingdoms (Baulcombe 2004; Cogoni and Macino 1997). It is thought to be a highly conserved mechanism, which initially evolved to combat genomic 'parasites' including viruses and transposable elements that use double stranded RNA in their life-cycles (Bakhetia et al. 2005b).

Early pioneering experiments which discovered that RNAi is induced by dsRNA, its effect is systemic and also heritable have led to the exploitation and development of this natural mechanism as a high-throughput reverse-genetics approach for gene function analysis in *C. elegans* (Tabara et al. 1998, 1999; Kamath et al. 2003; Fraser et al. 2000). It has also been applied in a wide variety of organisms, including protozoa (Ullu et al. 2004), amphibians (Li and Rohrer 2006; Nakano et al. 2000; Oelgeschlager et al. 2000), insects (Brown et al. 1999; Hughes and Kaufman 2000; Kennerdell and Carthew 1998; Zamore et al. 2000) and mammals (Berns et al. 2004; Brummelkamp et al. 2002; Elbashir et al. 2001; Paddison et al. 2004; Paul et al. 2002; Peng et al. 2006; Sorensen et al. 2003; Tiscornia et al. 2004; Ventura

et al. 2004). The basic mechanism of RNAi seems to be conserved in all eukaryotic organisms in which the phenomenon has been investigated, although differences have been found in the systemic nature and heritability of the effect. The approach has also been attempted in parasitic nematodes but with variable levels of success. Inconsistencies in successful knock-down of gene products may be due to a number of reasons, including the mode of delivery of double-stranded RNA to the parasite, the particular stage targeted and possibly more significantly whether the RNAi pathway is fully functional in some parasitic nematodes.

The RNAi pathway, including the necessary molecular components has been extensively studied in *C. elegans* (for a more extensive review see Grishok 2005). The molecular mechanism of RNAi by which dsRNA leads to gene-silencing is believed to be very complex. The original studies in *C. elegans* attempted to characterise the RNAi pathway by identification of RNAi deficient (*rde*) mutants, which were refractory to RNAi (Tabara et al. 1999). Since then genome-wide RNAi assays have been undertaken and integrated with Worm Interactome map (W15) in *C. elegans* to gain a further insight into the mode of action of RNAi and the components involved (Kim et al. 2005; Li et al. 2004). As well as the classical pathway there are thought to be a number of related and overlapping pathways, which are responsible for the silencing of transposable elements and tandem arrays in the germline (Dernburg et al. 1998; Kelly et al. 1997; Ketting et al. 1999; Ketting and Plasterk 2000).

In *C. elegans* the initial import of dsRNA from the gut lumen is facilitated by SID-2, a transmembrane protein expressed at the apical membrane of the intestinal cells. SID-2 subsequently activates another transmembrane protein SID-1, which contains a large amino-terminal extracellular domain and 11 membrane-spanning domains (Feinberg and Hunter 2003; Hunter et al. 2006; Winston et al. 2002). SID-1 promotes passive uptake of dsRNA with longer molecules of 500 bp transported more efficiently than siRNAs (Feinberg and Hunter 2003). Several components of the intracellular vesicular transport are required for systemic RNAi (Saleh et al. 2006). Upon entry into the cell, dsRNA is recognised by a protein complex containing a dsRNA binding protein (RDE-4) and is then cleaved by the multidomain ribonuclease Dicer into short interfering double stranded RNAs (siRNAs), approximately 21–23 nucleotides in length. These, siRNAs, containing 5′ monophosphate groups and two nucleotide overhangs at the 3′ end, are then transported to and incorporated in the RNA-induced silencing complex (RISC).

The assembly of RISC complex is ATP-dependent and its components include the dsRNA binding protein RDE-4, the Argonaute protein RDE-1 and the dicer-related helicase DRH-1 (Tabara et al. 2002). Another member of the Argonaute family of proteins, related to RDE-1 is involved in endonucleolytic cleavage of the mRNA. This key RISC component remains to be identified in *C. elegans*, but 27 candidate Argonaute (AGO) orthologues have been found (Yigit et al. 2006) and may act redundantly in the RISC complex (Grishok 2005). Argonaute proteins share two main structural domains. The PAZ domain, which is also present in the Dicer protein, is a binding pocket that anchors the characteristic two nucleotide 3′ overhang resulting from the digestion of dsRNAs, and a PIWI domain which shows extensive homology to the RNAse III (Grishok 2005; Hock and Meister 2008).

Other identified components of the *C. elegans* RISC include an RNA binding protein VIG-1 and TSN-1, a protein possessing a Tudor domain and five staphylococcal nuclease domains that may be responsible for the exonuclease activity of RISC (Caudy et al. 2003). Once incorporated into the RISC complex the double stranded siRNA are unwound, culminating in the stable association of the anti-sense strands, with the Argonaute effector protein RDE-1 (Yigit et al. 2006). The siRNA single strand directs the RISC complex to the complementary RNA targets for degradation by the PIWI domain of the Argonaute protein RDE-1. Cleavage of the phosphodiester linkage between the target nucleotide sequences that are base paired to siRNA residues 10 and 11 (from the 5′ end) generates products with 5, monophosphate and 3′ hydroxyl termini. Cellular exonucleases complete the degradative process, resulting in knockdown of the encoded protein.

The most remarkable feature of the RNA interference is its potency and persistence. The entry of only a few dsRNA molecules into one cell can lead to systemic silencing in different tissues and transmission to the next generation. This mechanism of amplification is achieved by the synthesis of secondary unprimed (di- or triphosphate terminated) antisense siRNA. In *C. elegans* the primary siRNAs guide ATP-dependent RNA polymerase (RdRP) to homologous mRNAs and synthesise secondary siRNAs in a dicer-dependent manner (Pak and Fire 2007; Sijen et al. 2007). The two RdRPs, EGO- and RRF-1, active in the germline and somatic cells respectively, are thought to be involved in this process and may function in a complex with the RDE-3 polymerase (Chen et al. 2005a). Secondary siRNAs are more abundant than primary siRNAs and induce post-transcriptional silencing more efficiently. Furthermore, whereas primary siRNAs are bound by RDE-1, the secondary siRNAs are bound by a different set of Argonaute proteins, in particular CSR-1. This protein is responsible for the prominent slicer activity induced by secondary siRNAs on the target mRNA, causing post-transcriptional silencing (Claycomb et al. 2009).

Secondary siRNAs bound to the argonaute protein CSR-1 are also thought to enter into the cellular nucleus causing epigenetic effects at the DNA or chromatin level and transcriptional silencing. Inheritance of strong RNAi-induced effects in first generation progeny (F1) and beyond has been reported for a number of genes, with transmission reported to exceed over 80 generations (Vastenhouw et al. 2006). Genes identified as being involved in inheritance of silencing include some which function in chromatin remodelling, suggesting that the mechanism underlying inherited RNAi may act at the transcriptional level (Vastenhouw et al. 2006). Three *rsd* (RNAi spreading defective) mutants are all deficient in transmission of the RNAi effect to the germline. A role in vesicle trafficking is predicted for RSD-3, suggesting that dsRNA may be transported within endocytotic vesicles (Tijsterman et al. 2004). Recent work confirms the role of the endocytic pathway in uptake and translocation of dsRNA (Saleh et al. 2006). Several *C. elegans* gene products with roles in intracellular vesicular transport and lipid modification were found to be essential for systemic RNAi.

It is well known that RNA interference in *C. elegans* can also be generated by genome coded small RNA (miRNA) (Ruvkun 2008). The miRNA are transcribed

by RNA polymerase II to relatively long primary RNA prior to being processed in the nucleus to 70–80 nucleotide long dsRNA by the RNase III enzyme Drosha. It is subsequently exported in the cytoplasm by Exportin and finally processed by the Dicer enzyme into 22 nucleotide duplex miRNA. The pathway of the diced miRNA is similar and partially overlapping with that of diced siRNA. These miRNA mediate translational repression but may also guide mRNA degradation (Wienholds and Plasterk 2005).

There are currently four methods by which dsRNA can be delivered in *C. elegans* to achieve effective RNAi. The original RNAi studies were performed by injection of dsRNA into the gonad or body cavity of worms and examination of phenotypes in the next generation (Fire et al. 1998). Subsequently a technique was developed for feeding worms with bacteria expressing dsRNA (Timmons and Fire 1998) and soaking of worms in dsRNA was also shown to be capable of inducing an RNAi phenotype (Tabara et al. 1998). Since then RNAi has also been achieved by *in vivo* production of dsRNA from transgenic promoters (Tavernarakis et al. 2000). The method of feeding bacterial clones expressing dsRNA still remains the most efficient RNAi (and indeed reverse-genetics) approach for high throughput analysis.

The application of RNAi has greatly assisted functional genomics in *C. elegans* and large-scale screens have now been performed on the vast majority (86%) of the predicted genes in *C. elegans* (Gunsalus and Piano 2005). Large-scale RNAi screens to target nearly 90% of genes on chromosome I have increased the number of genes with known phenotypes from 70 to 378 (Fraser et al. 2000). The same approach has also identified most of the genes on chromosome III as being required for normal cell division in *C. elegans* embryos (Gonczy et al. 2000). Furthermore, 47% of the genes associated with a differential interference induced phenotype have clear orthologues in other eukaryotes, indicating putative gene functions for other species. Numerous other large-scale RNAi screens have since been performed (see Sect. 10.1.2) and as well as discerning gene function in a number of biological processes they are also helping to evaluate off-target effects of RNAi. A genome-wide phenotype screen on *C. elegans* used 200 bp long dsRNA with 80% identity as a threshold for target RNAi specificity (Kamath and Ahringer 2003). Off-target effects have been predicted to occur in *C. elegans* when an mRNA sequence and dsRNA share more than 95% identity over 40 nucleotides (Rual et al. 2007). RNAi feeding libraries are commercially available to the scientific community as individual clones or chromosome sets for *C. elegans* chromosomes I, II, III, IV, V and X (http://www.geneservice.co.uk/products/rnai).

10.3.2 RNAi Mechanism in Animal Parasitic Nematodes

Following the successful development of RNAi as a functional genomics approach in *C. elegans* numerous attempts have been made to develop RNAi in parasitic nematodes. However, applying RNAi directly to parasitic nematodes has proved challenging, largely due to their dependence on a host organism and the technical

inviability of establishing in-vitro cultures. Furthermore, as a result of their small size, microinjection of the infective stages of plant-parasitic nematodes also presents a major technical challenge. In addition, they do not usually consume fluid until they have infected a host. However, RNAi has been achievable for several parasitic nematodes under certain conditions and has displayed potential use as a tool for direct functional genomics in target parasitic species. RNAi has now been tested extensively in a variety of different parasitic nematodes from across three phylogenetic clades. Variable levels of success have been achieved; with a larger number of effective RNAi studies reported in plant-parasitic nematodes. More recent studies have also demonstrated effective delivery of dsRNA to cyst and root knot nematodes *in planta* and the potential for developing this technology in transgenic crops as an application for plant-parasitic nematode control.

The first parasitic nematode in which RNAi was successfully performed was the Clade V rat hookworm *Nippostrongylus brasiliensis* (Hussein et al. 2002). Soaking adult worms in dsRNA targeted against acetylcholinesterase (AchE) A isoform resulted in a 80–90% reduction in enzyme activity of 3 AChE isoforms (A, B and C) over a 6-day period. Despite this significant expression, no phenotypic effects were observed. Shortly after, a microvolume culture system was optimised for the soaking of the Clade III human parasite *Brugia malayi* in dsRNA (Aboobaker and Blaxter 2003). This system was used successfully to target beta- tubulin, RNA polymerase II large sub-unit and a microfilarial sheath protein in adult worms. All transcript levels started to decline between 14 and 17 h after soaking and worms soaked in dsRNA for beta-tubulin and RNA polymerase II large subunit were not viable after 24 h. Targeting the microfilarial sheath protein did not result in a lethal phenotype but a marked reduction in released microfilariae was observed, 50% of which did not have fully elongated sheaths. Transcripts encoding proteins with various expression patterns have now been targeted in a number of animal-parasitic worms from both Clade III and V (as summarised in Table 10.1). RNAi-targeted proteins include those expressed in the body wall, head and pharyngeal musculature, those expressed in the myoepithelial sheath as well as those with more specialised parasitic functions expressed in the pharyngeal glands and intestine. Highly abundant non-parasite specific proteins were selected based on their putative crucial roles in metabolism, cuticle moulting and motility inferred from their annotation in *C. elegans*. RNAi has now been effective in L3 infective stages of the human parasites *Onchocerca volvulus* and *Ascaris suum*, and adult stages of the mouse filarial worm *Litosomoides sigmodontis* belonging to Clade III (Islam et al. 2005; Lustigman et al. 2004; Pfarr et al. 2006). RNAi has also been successful in a number of veterinary important Clade V nematodes including the L1 stage of *Trichostrongylus colubriformis* and L3 stages of both *Haemonchus contortus* and *Ostertagia ostertagi* (Geldhof et al. 2006; Issa et al. 2005; Kotze and Bagnall 2006; Visser et al. 2006).

Electroporation was able to effectively deliver both dsRNA and siRNA targeted against ubiquitin in the L1 stage of the sheep nematode *T. colubriformis* (Issa et al. 2005). Delivery by electroporation resulted in an inhibition of development of the L1 larvae compared to larvae in buffer only, although transcript knockdown was not assessed. Significant reductions in transcript levels were achieved for beta-tubulin

Table 10.1 Summary of genes successfully targeted by RNAi studies performed in animal-parasitic nematodes. Experiments performed on clade III nematodes are shaded and those performed clade V nematodes are unshaded

Target stage	Target tissue	Target gene	Sp.	Method	dsRNA (mg ml^{-1})	Soaking period	Phenotype obs. time-point	Phenotype	Reference
L1	Muscle	Ubiquitin	*T.c* (V)	Soaking Electroporation	2 (2 µM siRNA)	6 hr	6 days	Inhibited development	Issa *et al.* (2005)
L1	Muscle	Tropomyosin	*T.c* (V)	Feeding Soaking Electroporation	2 (2 µM siRNA)	6 hr	6 days	Inhibited development	Issa *et al.* (2005)
L1-L3	Pharynx Intestine Hypodermis	Cathepsin L	*H.c* (V)	Electroporation Soaking	1	24 hr	(6 days)	n.d	Geldhof *et al.* (2006)
L1-L3	Intestine	Ca^{2+} binding protein	*H.c* (V)	Soaking	1	24 hr	(6 days)	n.d	Geldhof *et al.* (2006)
L1-L3	Muscle Intestine	Heat Shock Protein HSP70	*H.c* (V)	Soaking	1	24 hr	(6 days)	n.d	Geldhof *et al.* (2006)
L1-L3	Intestine Muscle	Vacuolar ATPase	*H.c* (V)	Electroporation Soaking	1	24 hr	(6 days)	n.d	Geldhof *et al.* (2006)
L1-L3	Muscle	Paramyosin	*H.c* (V)	Soaking	1	24 hr	(6 days)	n.d	Geldhof *et al.* (2006)
L1-L3	Intestine Muscle	Cu-Zn superoxide dismutase	*H.c* (V)	Electroporation Soaking	1	24 hr	(6 days)	n.d	Geldhof *et al.* (2006)
L1-L3	Muscle	Intermediate filament	*H.c* (V)	Soaking	1	24 hr	(6 days)	n.d	Geldhof *et al.* (2006)
L1-L3	Muscle	Type IV collagen	*H.c* (V)	Soaking	1	24 hr	(6 days)	n.d	Geldhof *et al.* (2006))
L1-L3	Intestine	GATA	*H.c*	Soaking	1	24 hr	(6 days)	n.d	Geldhof *et*
		transcription factor	(V)						*al.* (2006)
L1-L3	Unknown	β-tubulin	*H.c* (V)	Electroporation Soaking	1	24 hr	(6 days)	n.d	Geldhof *et al.* (2006)
L1-L3	Unknown	COPII component	*H.c* (V)	Soaking	1	24 hr	(6 days)	n.d	Geldhof *et al.* (2006)
L3	Muscle	Tropomyosin	*O.o* (V)	Electroporation Soaking	1	24, 48, 72 hr	(24 hr)	n.d	Visser *et al.* (2006)
L3	Muscle	ATP-synthetase	*O.o* (V)	Electroporation Soaking	1	24, 48, 72 hr	(24 hr)	n.d	Visser *et al.* (2006)
L3	Intestine Muscle	Superoxide dismutase	*O.o* (V)	Electroporation Soaking	1	24, 48, 72 hr	(24 hr)	n.d	Visser *et al.* (2006)
L3	Muscle	Ubiquitin	*O.o* (V)	Electroporation Soaking	1	24, 48, 72 hr	(24 hr)	n.d	Visser *et al.* (2006)
L3	Muscle	Transthyretine-like protein	*O.o* (V)	Electroporation Soaking	1	24, 48, 72 hr	(24 hr)	n.d	Visser *et al.* (2006)
L3	Hypodermis	Cathepsin L-like Cathepsin Z-like	*O.v* (III)	Soaking	0.5	18-20 hr	7 days	Viability & moulting	Lustigman *et al.* (2004)
L3	Unknown	Serine protease inhibitor	*O.v* (III)	Soaking	0.5	18-20 hr	7 days	Viability & moulting	Lustigman *et al.* (2004)
L3	Hypodermis	Inorganic pyrophosphatase	*A.s* (III)	Soaking	2.0	24 hr	10 days	Moulting	Islam *et al.* (2005)
L3	Oesophageal glands	Polyprotein allergen	*O.o* (V)	Electroporation Soaking	1	24, 48, 72 hr	(24 hr)	n.d	Visser *et al.* (2006)
L3	Oesophageal glands	17kDa ES protein	*O.o* (V)	Electroporation Soaking	1	24, 48, 72 hr	(24 hr)	n.d	Visser *et al.* (2006)
L3	Unknown	β-tubulin	*O.o*	Electroporation	1	24, 48,	(24 hr)	n.d	Visser *et al.*
			(V)	Soaking		72 hr			(2006)
L3-L4-adult	Unknown	β -tubulin	*H.c* (V)	Soaking	1	24 hr & 6 days	6 days	Motility	Kotze *et al.* (2006)
L3-L4-adult	Unknown	β -tubulin	*H.c* (V)	Soaking	1	24 hr & 6 days	6 days	Motility	Kotze *et al.* (2006)
Adult	Neuronal tissue	Acetylcholin-esterase	*N.b* (V)	Soaking	1	16 hr	(6 days)	n.d	Hussein *et al.* (2002)

Table 10.1 (continued)

Adult	Myo-epithelial sheath	Sheath protein	*B.m* (III)	Soaking	0.08	14-17 hr	24 hr	Microfilarial release Microfilarial sheath	Alloobaker & Blaxter (2003)
Adult	Unknown	RNA polymerase II	*B.m* (III)	Soaking	0.08	14-17 hr	24 hr	Lethal	Alloobaker & Blaxter (2003)
Adult	Unknown	β -tubulin	*B.m* (III)	Soaking	0.08	14-17 hr	24 hr	Lethal	Alloobaker & Blaxter (2003)
Adult	Muscle Female reproductive tract	Actin	*L.s* (III)	Soaking	0.035 to 35 uM	24 hr	48-72 hr	Paralysis	Pfarr *et al.* (2006)

T.c Teladorsagia circumcincta, *H.c* Haemonchus contortus, *O.o* Ostertagia ostertagia, *O.v* Onchocerca volvulus, *A.s* Ascaris suum, *N.b* Nippostrongylus brasiliensis, *B.m* Brugia malayi, *L.s* Litosomoides sigmodontis, *n.d* not determined, *obs.* observation, *sp.* species

and superoxide dismutase 24 h following electroporation of *Ostertagia ostertagi* (Visser et al. 2006). However, the high level of larval death observed, even in the presence of control dsRNA, makes this method currently inappropriate for functional analysis. The majority of successful RNAi studies in *C. elegans* have used soaking to deliver dsRNA but the route of uptake remains unclear. It seems likely that dsRNA molecules are taken up through the oral route rather than the cuticle. Fluorescently labelled dsRNA has been detected in the pharynx and gut of L2 and L4 but not L3 stages of *H. contortus* (Geldhof et al. 2006). Since L3 larvae do not possess a functional oral orifice but remain susceptible to dsRNA this suggests an alternative route of uptake other than ingestion. This suggestion is further supported by the finding that worms treated with ivermectin to paralyse their pharynx are also susceptible to dsRNA effects (Geldhof et al. 2006).

Although most studies have used full-length dsRNA to mediate RNAi, two studies have investigated the effectiveness of using varying lengths of dsRNA. The first successful RNAi study found smaller dsRNA (240 bp) fragments to be more effective at targeting acetylcholinesterase (AchE) A isoform in adult *Nippostrongylus brasiliensis* than targeting the full 1.8 kb length (Hussein et al. 2002). This finding was also supported by another study, which investigated the effectiveness of using 22 bp synthetic dsRNA (siRNA) to target a ubiquitin gene in L1 *T. colubriformis* (Issa et al. 2005). A siRNA derived from the coding sequence resulted in more effective delivery by soaking than did the longer dsRNA molecule designed to target the entire transcript. Interestingly, electroporation was effective at delivering both siRNA and dsRNA to the worms but soaking was only capable of inducing an effect with siRNA. Generally, incubation periods of at least 18 h and typically 24 h have been used for targeted RNAi by soaking life-cycle stages in dsRNA. When adult worms of the human parasite *Brugia malayi* were soaked in dsRNA targeting beta- tubulin, RNA polymerase II large sub-unit and a microfilarial sheath protein the transcript levels started to decline between 14 and 17 h of soaking (Aboobaker and Blaxter 2003).

A variety of methods for the evaluation of RNAi effects in animal-parasitic nematodes have been used, with no standard approach currently in place. The majority of studies have primarily carried out measurement of the target transcript,

by quantitative real-time PCR (RT-PCR). The effect on the corresponding protein has also sometimes been investigated. For example, when an inorganic pyrophosphatase in the L3 stage larvae of *Ascaris suum* was targeted by dsRNA both target mRNA and protein levels were measured by RT-PCR, enzyme assay, immunofluorescence and immunoblotting (Islam et al. 2005), although the number of worms included in this analysis was unclear. Depending on the putative function of the protein targeted particular phenotypic assessments may be made, based on viability, development, morphology or locomotion. For example, although a lethal phenotype did not result when the microfilarial sheath protein was targetted in adults of *B. malayi,* a marked reduction in microfilariae was released, 50 % of which did not have elongated sheaths (Aboobaker and Blaxter 2003).

Off-target effects, as a result of sequence identity between dsRNA and non-target transcripts, have been observed in some RNAi experiments. For example, treatment of L3 *O. volvulus* larvae with control dsRNA resulted in a 24.7–49.8% reduction in moulting (Ford et al. 2005; Lustigman et al. 2004) and adult *B. malayi* parasites soaked in control dsRNA showed a reduced motility (Aboobaker and Blaxter 2003). Measurement of an increased expression of a heat shock protein has shown that high concentrations of dsRNA were also stressful to the filarial parasite *L. sigmodontis* (Pfarr et al. 2006). Non-target effects were also reported in *H. contortus* L3 after 72 h incubation in dsRNA targeting a beta-tubulin gene (Kotze and Bagnall 2006). However, only one published study in animal parasitic-worms has monitored the expression of a large set of non-target genes for off-target RNAi effects (Visser et al. 2006). These off-target effects may be misinterpreted as or mask the true RNAi-induced specific phenotype and emphasise the importance of appropriate dsRNA design and use of appropriate controls.

Following dsRNA exposure and transfer of life cycle stages to dsRNA-free culture medium the effects, if not lethal, on the targeted transcript, corresponding protein and phenotype may persist for several days. RNAi effects may be species-specific, as a result of differences in the RNAi pathway or may also depend on the abundance, turnover and location of the specific transcript. Furthermore, there is no accepted standard concentration for dsRNA exposure at present. One study investigated the effect of soaking adult worms of the rodent parasite *L. sigmodontis* in varying concentrations of 0.035–35 uM dsRNA (Pfarr et al. 2006). It found that the targeted actin transcript was significantly suppressed by all tested concentrations and persisted for at least 72 h, even in the lowest concentrations of dsRNA. Treated worms displayed paralysis between 48 and 72 h after the start of the experiment and released significantly less microfilariae. In another study, when L3 stage of *A. suum* were incubated in dsRNA (2 mg ml^{-1}) for 24 h, target inorganic pyrophophatase mRNA and protein levels remained suppressed for five days after treatment with dsRNA (Islam et al. 2005). After 10 days, a 31% reduction in moulting from L3 to L4 was observed in treated larvae compared to control larvae. However, control larvae were not incubated in the presence of non-specific dsRNA to eliminate the possibility of non-specific treatment effects. Several other studies also report the persistence of RNAi effects on the phenotype for up to six or seven days (Ford et al. 2005; Kotze and Bagnall 2006).

These successful RNAi studies in animal-parasitic nematodes have demonstrated the potential for using RNAi to assign gene-function directly in target species. Attempts have now been made to optimise RNAi systems in a number of parasitic species of medical and veterinary importance. The growing need for standardisation of accepted methods for RNAi studies in these target species is starting to become recognised. However, current approaches for RNAi studies in these target species remain time consuming, expensive, inefficient and limited. Therefore, development of high throughput RNAi screens, as have been established for *C. elegans*, in these target species is unachievable at present. In the absence of large-scale functional genomic screens for these parasitic nematodes, indirect analysis of gene function in *C. elegans* remains the most suitable alternative.

10.3.3 RNAi Mechanism in Plant-Parasitic Nematodes

Applying RNAi directly to plant-parasitic nematodes has also proved challenging, although generally with more success than in animal-parasitic species. Since the first demonstrations (Rosso et al. 2005; Urwin et al. 2002) of successful RNAi in the Clade IV cyst and root knot nematodes more than twenty successful applications have been published (as summarised in Table 10.2). More recently, effective RNAi has also been demonstrated in migratory nematodes (Haegeman et al. 2009; Park et al. 2008). Despite a few studies using electroporation, delivery of dsRNA has generally been performed and achieved by soaking. A major breakthrough has been the development of successful techniques to enhance oral uptake of dsRNA from the soaking solution. The first successful RNAi study in plant-parasitic nematodes was achieved using the neuroactive compound octopamine to stimulate oral ingestion of dsRNA by pre-parasitic J2 of the cyst nematodes *G. pallida* and *H. glycines* (Urwin et al. 2002). Target transcripts included those encoding cysteine proteinases and a C-type lectin homologue. Treated nematodes were subsequently used to infect plants and the resulting phenotypes determined. Soaking of *H. glycines* in dsRNA targeting the C-type lectin homologue resulted in 41% fewer nematodes being collected from the plant 14 days post-infection. Although RNAi targeting of cysteine proteinase transcripts did not reduce the number of parasites that established on plants 14 days post-infection it did alter the sexual fate towards males.

The same method has also been used successfully to induce uptake of dsRNA by J2 of the root-knot nematode *M. incognita* (Bakhetia et al. 2005a; Shingles et al. 2007). Successful dsRNA uptake in this nematode has been induced using resorcinol and serotonin (Dubreuil et al. 2007; Huang et al. 2006; Rosso et al. 2005) and lipofectin in *Bursaphelenchus xylophilus* (Park et al. 2008). Adaptations of the original method, including the addition of spermidine to the soaking buffer and an extended incubation time, have been reported to increase the efficiency of RNAi for the cyst nematode *G. rostochiensis* (Chen et al. 2005b). Uptake of dsRNA from the gut is a proven route to systemic RNAi in *C. elegans*. The systemic nature of RNAi in plant parasitic nematodes following ingestion of dsRNA suggests they

Table 10.2 Summary of genes successfully targeted by RNAi studies performed in plant-parasitic nematodes. Experiments performed on cyst nematodes are shaded and those performed on root-knot nematodes and the migratory nematodes *Bursaphelenchus xylophilus* and *Radopholus similis* are unshaded

Target stage	Target tissue	Target gene	Sp.	Uptake induction	dsRNA (mg ml^{-1})	Soaking period	Phenotype observation time-point	Phenotype	Reference
Egg	Eggs	Chitin synthetase	*M.t*	None	1	1-3 days	3,7 days	Hatch defect	Fanelli *et al.* (2005)
J2	Amphids	ams-1	*G.r*	Octopamine	2-5	24 hr	2 weeks p.i	Host location & invasion	Chen *et al.* (2005b)
J2	Neuronal	Neuropeptides	*G.p.*	None	0.1	18-24 hr	1,2,7 days	J2 motility	Kimber *et al.* (2007)
J2	Neuronal	Neuropeptides	*G.p* *M.i*	None	0.1 (siRNA)	24 hr	25, 26, 27, 28 hr	J2 motility	Dalzell *et al.* (2009)
J2	Intestine	Cysteine protease	*G.p*	Octopamine	2-5	4 hr	2, 4 weeks p.i	No. est. nematodes male/ female ratio	Urwin *et al.* (2002)
J2	Intestine	Cysteine protease	*H.g*	Octopamine	2-5	4 hr	2, 4 weeks p.i	No. est. nematodes Male/ female ratio	Urwin *et al.* (2002)
J2	Intestine	Cysteine protease	*M.i*	Octopamine	2-5	4 hr	3 weeks p.i	No. est. nematodes	Shingles *et al.* (2007)
J2	Muscle	Heat shock protein 70 Cytochrome C Ribosomal protein-23	*H.g*	Octopamine	10	4 hr	4 days	J2 viability	Alkharouf *et al.* (2007)
J2 J3	Muscle	Heat shock protein 70	*B.x*	Lipofectin	1	24 hr	5 days	J2-J3 viability at high temp Motility	Park *et al.* (2008)
J2 J3	Muscle	Myosin heavy chain Tropomyosin	*B.x*	Lipofectin	1	24 hr	5 days	J2-J3 viability at high temp	Park *et al.* (2008)
J2	Hypodermis	C-type lectin	*H.g*	Octopamine	2-5	4 hr	2 weeks p.i	No. est. nematodes Female development	Urwin *et al.* (2002)
J2	Hypodermis	Dual oxidase	*M.i*	Octopamine	2	4 hr	2, 5 weeks p.i	No. est. nematodes	Bakhetia *et al.* (2005)
J2	Oesophageal glands	Cellulase	*G.r*	Octopamine	2-5	24 hr	2 weeks p.i	No. est. nematodes	Chen *et al.* (2005)
J2	Oesophageal glands	Cellulase	*H.g*	Octopamine	2	16 hr	10 days p.i	No. est. nematodes	Bakhetia *et al.* (2007)
J2	Oesophageal glands	Pectate lyase	*H.g*	Octopamine	2	16 hr	10 days p.i	No. est. nematodes	Bakhetia *et al.* (2007)
J2	Oesophageal glands	Chorismate mutase	*H.g*	Octopamine	2	16 hr	10 days p.i	Male/ female ratio	Bakhetia *et al.* (2007)
J2	Oesophageal glands	Unknown, *syv-46*	*H.g*	Octopamine	2	16 hr	10 days p.i	Male/ female ratio	Bakhetia *et al.* (2007)
J2	Oesophageal glands	Unknown, *hg-gp*	*H.g*	Octopamine	2	16 hr	10 days p.i	Male/ female ratio	Bakhetia *et al.* (2007)
J2	Oesophageal glands	Unknown, *dg-13*	*H.g*	Octopamine	2-5	16 hr	10 days p.i	Male/ female ratio	Bakhetia *et al.* (2007)
J2	Oesophageal glands	Unknown, *dg-21*	*H.g*	Octopamine	2-5	16 hr	10 days p.i	No. est. nematodes Male/ female ratio	Bakhetia *et al.* (2007)
J2	Oesophageal glands	Pectate lyase	*H. s*	Octopamine	1	24 hr	6 days p.i	No. est. nematodes	Vanholme *et al.* (2007)
J2	Oesophageal glands	Calreticulin	*M.i*	Resorcinol	4	4 hr		n.d	Rosso *et al.* (2005)
J2	Oesophageal glands	Polygalacturonase	*M.i*	Resorcinol	4	4 hr		n.d	Rosso *et al.* (2005)

Table 10.2 (continued)

J2	Oesophageal glands	16 D10	*M.i*	Resorcinol	1	4 hr	3,7 weeks p.i	No. est. nematodes	Huang et al. (2006)
J2	Oesophageal glands	Glutathione transferase	*M.i*	Resorcinol Serotonin	4	4 hr	6 weeks p.i	Fecundity	Dubreuil *et al.* (2007)
J2	Oesophageal glands	Cellulose-binding protein	*M.j*	Resorcinol	2	16 hr	6, 35 days p.i	No. est. nematodes	Adam *et al.* (2008)
J2	Oesophageal glands	Xylanase	*R. s*	Octopamine	0.5	24 hr	10 days p.i	No. est. nematodes	Haegeman *et al.* (2009)
J2	Spermatocyte	Major sperm protein	*H.g*	Octopamine	2-5	4 hr	2, 3 weeks p.i	n.d	Urwin *et al.* (2002)
J2	Female reproductive tract	Aminopeptidase	*H.g*	Octopamine	2-5	4 hr	3 weeks p.i	No. est. nematodes male/ female ratio	Lilley *et al.* (2005)
J2	Unknown	Avirulence gene, *cg-1*	*M.j*	Octopamine	0.5	48 hr		Virulent on *Mi-1* plant	Gleason *et al.* (2008)

M.i Meloidogyne incognita, *M.j* Meloidogyne javanica, *M.t* Meloidogyne artiellia, *G.p* Globodera pallida, *G.r* Globodera rostochiensis, *H.g* Heterodera glycines, *H.s* Heterodera schachtii, *B.x* Bursaphelenchus xylophilus, *R.s* Radopholus similis, est. established, *n.d* not determined, *sp* species

share similar uptake and dispersal pathways. Alternative routes for the uptake of dsRNA have been explored in plant-parasitic nematodes and have been achieved by soaking intact eggs of *M. artiellia* within their gelatinous matrix in a solution containing dsRNA (Fanelli et al. 2005).

Gene products expressed in a range of different tissues and cell types have now been successfully targeted. The ingested dsRNA can silence genes in the intestine (Urwin et al. 2002; Shingles et al. 2007), muscle (Alkharouf et al. 2007; Park et al. 2008), hypodermis (Bakhetia et al. 2005a; Urwin et al. 2002), female and male reproductive systems (Urwin et al. 2002; Steeves et al. 2006; Lilley et al. 2005), and both subventral and dorsal oesophageal glands (Adam et al. 2008; Bakhetia et al. 2007; Chen et al. 2005b; Dubreuil et al. 2007; Gleason et al. 2008; Haegeman et al. 2009; Huang et al. 2006; Rosso et al. 2005; Vanholme et al. 2007). In *C. elegans* neuronally expressed genes can be refractory to RNAi (Kamath et al. 2003; Timmons et al. 2001), although RNAi effects can be enhanced using a mutant strain defective in the RdRP *rrf-3* (Simmer et al. 2002, 2003). Interestingly, FMR Famide-like (*flp*) neuropeptide genes of *G. pallida* have also been successfully targeted by RNAi, even in the absence of neurostimulants (Dalzell et al. 2010; Kimber et al. 2007). This implies that RNAi-targeted knockdown of the neuronal *flp* genes utilises an alternative dsRNA uptake route. It is possible that the dsRNA may be entering via the secretory/excretory pore, the cuticle or more likely the amphids, which have also been successfully targeted by RNAi in *B. xylophilus* (Park et al. 2008).

These paired anterior sense organs are capable of taking up fluorescein isothiocyanate in (FITC) in both *C. elegans* (Hedgecock et al. 1985) and cyst nematodes (Winter et al. 2002). The sensory neurons of *C. elegans* are capable of taking up 12 k Da but not 19.5 k Da fluorescent dextran conjugates (Hedgecock et al. 1985). Cyst nematode chemosensory dendrites can provide a route for uptake of soluble compounds including peptides from the external environment (Winter et al. 2002). It is therefore a rational assumption that exposed nerve processes could also take up molecules of dsRNA. A gene product encoding a secreted amphid protein of unknown function (*gr-ams-1*) has been targeted by RNAi in *G. rostochiensis* (Chen

et al. 2005b). Although octopamine was included in the soaking medium, *gr-ams-1* was more susceptible to an RNAi effect than a gland-cell-expressed endoglucanase in the same study. Therefore, it is possible that the two genes were being targeted by different routes, with more efficient uptake of dsRNA occurring at the amphids. These differences in efficiency may also be due to a number of other factors including differing spatial expression patterns, level and turnover rate of the endogenous transcripts and length of the dsRNA.

A range in size of dsRNA molecules have proved effective in inducing RNAi in both cyst and root knot nematodes but the optimum size remains unknown and may depend on the targeted gene. Different lengths of dsRNA have been used to target the same gene Gp-flp-6 in *G. pallida* (Kimber et al. 2007). An 88-bp length was unable to induce any silencing. Both 227- and 316-bp dsRNAs silenced the target transcript and resulted in reduced motility, but the shorter molecule consistently induced stronger effects. A more recent study reported the specific knockdown of several *flp* genes in J2 *G. pallida* and *M. incognita* using discrete 21 bp siRNA (Dalzell et al. 2010) as measured by RT-PCR and motility assays. However, another RNAi study targeting both the 42 bp coding region and the full 271 bp transcript of a oesophageal gland peptide in *M. incognita* J2 resulted in a 93–97% reduction for both (Huang et al. 2006). Further studies are therefore required to determine whether there is an optimum size or sequence position within the targeted gene for design of dsRNA fragments. The use of a smaller length of dsRNA may also minimise the risk of off-target effects, due to sequence similarity. (Dalzell et al. 2010).

The timing effects of RNAi have been observed following exposure of J2 s to dsRNA for time periods ranging from four hours to seven days. Four hour incubation periods of J2 in dsRNA may be sufficient for RNAi induction of some genes in cyst nematodes (Alkharouf et al. 2007; Lilley et al. 2005; Urwin et al. 2002) and root knot nematodes (Bakhetia et al. 2005a; Dubreuil et al. 2007; Huang et al. 2006; Rosso et al. 2005; Shingles et al. 2007). However, in general, increasing the incubation time leads to greater transcript reduction and enhanced phenotypic effects (Chen et al. 2005b; Kimber et al. 2007). This likely also depends on the transcript abundance, turnover and location. For RNAi-targetted knockdown of *flp* transcript of *G. pallida*, a minimal incubation period of 18 h was required for producing an aberrant phenotype, with maximal effect after 7 days (Kimber et al. 2007). Incubation periods were extended for up to 24 h for successful knockdown in J2 of cyst nematodes *G. rostochiensis*, *H. glycines* and *H. schachtii,* as well as the migratory species *B. xylophilus* and *R. similis* (Bakhetia et al. 2007; Chen et al. 2005b; Haegeman et al. 2009; Park et al. 2008; Vanholme et al. 2007). In the root knot nematodes an incubation period of 24 h was required for efficient RNAi effect in *M. artiellia* egg masses (Fanelli et al. 2005), whilst a 48 h period has been necessary during one study for targeted knockdown in J2 of *M. javanica* (Gleason et al. 2008).

Similar to the studies carried out in animal-parasitic nematodes a range of techniques have been used to analyse the RNAi response in plant parasitic nematodes. RT-PCR has generally been used to measure abundance of the target transcript in conjunction with a variety of phenotypic responses. One study also measured a re-

duction of cysteine proteinase activity when this gene was targeted in *M. incognita* (Shingles et al. 2007). A direct phenotypic alteration has been detected in *M. artiellia* chitinous layer of eggs, when chitin synthase expression was RNAi targeted (Fanelli et al. 2005). Due to the obligate parasitic life cycle of these species, many RNAi phenotypes can be revealed only after the treated J2 s have been allowed to invade host roots and develop to adulthood. However, subtle phenotypes may remain undetected. Experiments have commonly analysed the impact on the numbers of nematodes able to establish infections and initiate feeding sites or the proportion of cyst nematodes that develop as either males or females. Following exposure to dsRNA an increase in the male-to-female ratio may arise if nematode digestion and subsequent nutrition is impaired (Urwin et al. 2002). A similar effect has also been reported when a gene product involved in syncytial development is targeted by RNAi, leading to a compromised feeding cell delivering a sub optimal nutrient supply (Bakhetia et al. 2007). Effects on female size and fecundity have also been measured, along with the shape and size of developing nematodes at given time points following infection (Dubreuil et al. 2007). More direct phenotypic analysis of RNAi effects have included migration assays to detect impaired motility when the *flp* genes of *G. pallida* were targeted (Dalzell et al. 2010; Kimber et al. 2007).

Once removed from exposure to dsRNA the RNAi-induced transcript knockdown and subsequent effects in J2 are time limited. Following removal of *M. incognita* from a 4 h exposure of dsRNA targeting calreticulin (*mi-crt*) and polygalacturonase (*mi-pg-1*) maximum transcript repression was observed at 20 and 44 h respectively. The transcript levels of both genes had returned to normal after 68 h (Rosso et al. 2005). Similar results have been reported for cyst nematodes. Transcript repression of a β-1,4-endoglucancase in *H. glycines* J2 was observed immediately following a 16 h treatment of dsRNA and returned to normal by 15 days after removal from dsRNA exposure (Bakhetia et al. 2007). When reduced motility was used to monitor persistence of *Gp-flp-12* gene silencing in exposed J2 s there was a small but insignificant recovery after 24 h and significant but not complete recovery after 6 days (Kimber et al. 2007). If the treated juveniles are allowed to infect plants and continue development phenotypic consequences of RNAi may persist for a number of weeks (Bakhetia et al. 2005a, 2007; Chen et al. 2005b; Huang et al. 2006; Lilley et al. 2005; Urwin et al. 2002). RNAi is effective in *C. elegans* by continual exposure to dsRNA. Delivery of dsRNA to plant parasitic worms in the feeding cell would similarly prolong the effective exposure and maximise the RNAi effect.

Since the first demonstration of RNAi in plant parasitic nematodes the optimum goal has been to bioengineer delivery of dsRNA from the feeding cell to target specific essential nematode genes as a novel means of nematode control (Atkinson et al. 2003; Bakhetia et al. 2005a; Lilley et al. 2005; Urwin et al. 2002). Since the nematode feeds exclusively and continually from one or a few plant cells during development this would provide a suitable approach to potentially enable constant targeted expression of dsRNA. RNAi has been widely used in plants as a tool for functional genomics as well as to bioengineer novel traits (Kusaba 2004; Mansoor et al. 2006). A number of commercial applications have been described (Byzova et al. 2004; Davuluri et al. 2005; Ogita et al. 2004) and RNAi has been used in plants to

confer resistance to viruses (Pooggin et al. 2003; Waterhouse et al. 1998) and the bacterial pathogen *Agrobacterium tumefaciens* (Escobar et al. 2001). A number of studies targeting both root knot and cyst nematodes have shown potential of using RNAi-mediated resistance for plant parasitic nematode control (Huang et al. 2006; Steeves et al. 2006; Yadav et al. 2006); reviewed by (Gheysen and Vanholme 2007). Three housekeeping genes and five parasitism genes expressed in the oesophageal glands have been successfully targeted using this approach and are discussed in the final chapter of this book.

10.3.4 Identification of Putative Orthologues for Key Genes Involved in the RNAi Pathway in C. elegans in Emerging Sequences of Parasitic Nematodes

In the absence of a transformation system for parasitic nematodes RNAi has shown potential for use as a functional analysis tool, particularly in plant-parasitic nematodes. However, although several cases of successful targeted knockdown of gene expression in parasitic nematodes have been reported there have been many difficulties applying this approach in these nematodes. Problems have arisen with the efficiency, specificity and reproducibility of this technology in parasitic nematodes. In order to exploit this tool for high-throughput functional genomics more optimisation will be necessary. It has been suggested that nematodes respond differently to dsRNA delivered by feeding and soaking from that administered by injection (Davis et al. 2008). Furthermore, the efficiency of the systemic spread of the RNAi signal may depend on the developmental stage of the nematode (Tomoyasu et al. 2008). Differences have also been found in the response of specific tissues to RNAi. For example, neuronal genes in *C. elegans* are refractory to RNAi (Kamath et al. 2003), whereas *flp* neuropeptides are susceptible to knockdown in *G. pallida* (Dalzell et al. 2010; Kimber et al. 2007). In addition, there are relatively few reports of successful RNAi studies in animal parasitic nematodes and questions about the susceptibility of many nematodes to exogenous dsRNA have arisen (Geldhof et al. 2007; Knox et al. 2007). Despite being from the same clade, studies in *H. contortus* and *O. ostertagi* have indicated that most of the genes targeted in these species are refractory to RNAi, whilst their putative orthologues are susceptible to RNAi in the larval stages of *C. elegans* (Geldhof et al. 2006). Although this may be due to issues regarding dsRNA delivery or the *in vitro* culture conditions there is growing evidence that there may be fundamental differences within the RNAi pathway of these target species. In further support of this, six out of eight *Caenorhabditis* species tested were resistant to RNAi via soaking or feeding, indicating that even nematodes within a single genus can vary in their potential to take up and respond to exogenous RNAi (Descotte and Montgomery 2003). Important genes in these RNAi-refractory species may be absent, poorly conserved or have modified functions in the RNAi pathway.

Analysis of available genome and EST sequences for the presence of homologues to components of the *C. elegans* RNAi pathway is starting to suggest that different mechanisms of RNAi may exist in various genera and species of nematode. BLAST (tBLASTx) searches were performed against the genome sequences for the root knot nematode *Meloidogyne incognita* and the Clade III filarial animal parasite *Brugia malayi* (Abad et al. 2008; Ghedin et al. 2007; Opperman et al. 2008) at http://www.ncbi.nlm.nih.gov/. BLAST (tBLASTx) searches were performed against the incomplete genome and EST sequences for the potato cyst nematode *Globodera pallida* and Clade V barber-pole worm *Haemonchus contortus* at http://www.sanger.ac.uk/ and http://www.nematode.net/. Putative orthologues of several genes involved in the RNAi pathway in *C. elegans* have been found in the sequences of *M. incognita*, *G. pallida*, *B. malayi* and *H. contortus* (as summarised in Table 10.3). However, homologues to a number of key proteins involved in the RNAi pathway have not been detected. The lack of homologues found in the genome sequence of *H. contortus* for the argonautes *rde-1*, SAGO-1, SAGO-2 and PPW-1 (which function in amplification) as well as *alg-1*, *alg-2* and ERGO-1 (which function in MiRNA or endo-RNAi pathways) may explain why RNAi targeted knockdown has been less successful in *H. contortus* and other Clade V nematodes (Geldhof et al. 2006). Alternatively, the inability to detect these orthologues may be more likely due to an incomplete genome sequence for *H. contortus*. However, no potential orthologues have been found in any of the sequences from parasitic nematodes for *sid-1*, *sid-2*, *rde-2*, *rde-4*, *rsd-2* or *rsd-6* (which function in uptake and processing of dsRNA).

The absence of both *sid-1* and *sid-2* in genome sequences of parasitic nematodes may account for the inability to deliver dsRNA to animal-parasitic nematodes by feeding but does not explain why ingestion of dsRNA is achievable in root knot and cyst nematodes. The ability to target *flp* neuropeptides of *G. pallida* with RNAi in the absence of stimulants suggests that other methods of dsRNA uptake may also be operating in these nematodes (Kimber et al. 2007). Interestingly, although BLAST searching in the genome sequence of the RNAi-refractory *C. briggsae* and *C. remanei* strongly suggests an orthologue for SID-2 the function of this protein is not conserved (Winston et al. 2007). The alteration of function to this protein is thought to be due to sequence divergence in the extracellular domain and effective RNAi is restored in *C. briggsae* when the functional SID-2 protein from *C. elegans* is expressed in this species. Protein products encoded by *rde-4* and *rsd-2* also play a crucial role in the RNAi pathway in *C. elegans* (Parrish and Fire 2001). *Rde-4* encodes a dsRNA-binding protein and is crucial for complex formation with dicer in *C. elegans*, whilst *rsd-2* is involved in transporting dsRNA between cells and to the germline (Parrish and Fire 2001; Tabara et al. 1999). In the absence of orthologues to all three proteins crucial in the RNAi pathway in *C. elegans*, RNAi would be predicted to be unachievable in these species. However, RNAi in plant-parasitic nematodes has proved to be functional, systemic and heritable by ingestion of dsRNA. It is therefore possible that plant-parasitic nematodes may have evolved alternative pathways for uptake and transport of dsRNA as suggested for Arthropods (Tomoyasu et al. 2008; Whangbo and Hunter 2008).

Table 10.3 Putative orthologues of *C. elegans* (*C.e*) genes were searched in the complete genome sequences of *Meloidogyne incognita* (*M.i*) and *Brugia malayi* (*B.m*) (Abad et al. 2008; Ghedin et al. 2007), available at http://www.ncbi.nlm.nih.gov/, and in the current genome and EST databases for *Haemonchus contortus* (*H.c*) and *Globodera pallida* (*G.p*) available at http://www.sanger.ac.uk/ and http://www.nematode.net/. The presence or absence of orthologues is indicated by Y and N respectively and n.d., not determined

Protein function	*C. elegans*	*B. m*	*H. c*	*M. i*	*G. p*
Exo-RNAi					
Helicase	Drh-1 drh-2	Y	Y	Y	Y
	Smg-2	Y	Y	Y	Y
RNA binding	Rde-2	N	n.d	N	n.d
	Rde-3	N	n.d	Y	n.d
	Rde-4	N	n.d	N	n.d
	Smg-5	N	n.d	N	n.d
Chromatin binding	Zfp-1	Y	n.d	N	n.d
	Mut-16	N	n.d	N	n.d
RNase	Mut-7	Y	Y	N	Y
Dicer	Dcr-1	Y	Y	Y	Y
Amplification					
RdRP	Rrf-3	Y	n.d	Y	Y
	Rrf-1	Y	Y	Y	Y
	Rrf-2	Y	Y	Y	Y
RdRP germ-line specific	Ego-1	Y	Y	Y	Y
Argonautes	Rde-1	Y	n.d	Y	Y
	SAGO-1	Y	n.d	Y	Y
	SAGO-2	Y	n.d	Y	Y
	PPW-1	Y	n.d	Y	Y
	PPW-2	Y	Y	Y	Y
Suppressor of RNAi					
Exonuclease	Eri-1	Y	n.d	Y	Y
	Eri-3	Y	n.d	N	n.d
	Eri-5	Y	n.d	Y	n.d
Potent suppressor	Gfl-1	Y	Y	Y	Y
Uptake-systemy					
Intestine uptake	Sid-2	N	n.d	N	n.d
Uptake and systemy	Sid-1	N	n.d	N	n.d
Spreading in germline	Rsd-2	N	n.d	N	n.d
Germline endocytosis	Rsd-3	Y	n.d	Y	Y
Vesicle trafficking	Rsd-6	N	n.d	N	n.d
MiRNA or endo-RNAi pathways					
Drosha, a dicer homolog	Drsh-1	Y	Y	Y	Y
Argonaute	Alg-1	Y	n.d	Y	Y
	Alg-2	Y	n.d	Y	Y
	PRG-1	Y	Y	Y	Y
	PRG-2	Y	Y	Y	Y
	ERGO-1	Y	n.d	Y	Y
RNA binding	Vig-1	Y	n.d	N	n.d
RNA binding tudor	Tsn-1	Y	Y	Y	Y

10.4 General Conclusion and Future Perspectives

Comparative analyses of the available genome and EST sequences have demonstrated the high potential of using *C. elegans* to assign function to genes in plant-parasitic nematodes. Where possible orthologues of predicted proteins in the genomes of *M. incognita* and *M. hapla* exist, those predicted in the genome sequence of *C. elegans* provide the highest identity, followed by predicted proteins from the genome and EST sequences of animal-parasitic nematodes (Abad et al. 2008; Opperman et al. 2008). Many of these genes play key roles in basic nematode biology and similarities between *C. elegans* and plant-parasitic nematodes have been found in neuronal biology, metabolism, cuticle structure and developmental pathways. The high correlation of gene conservation across eukaryotes and RNAi phenotype in *C. elegans* supports the application of RNAi in a model organism to predict gene function in target species (Britton and Murray 2006). Furthermore, unlike the situation in parasitic nematodes and six out of eight *Caenorhabditis* species tested (Descotte and Montgomery 2003), RNAi is highly efficient and effective in *C. elegans*. Further to a system in which to study target orthologues *C. elegans* also provides a suitable heterologous system for the expression of target genes.

Where divergences in genome sequences occur these are likely to reflect the different responses to specific environmental cues, with a reduction in genes associated with a free-living lifestyle, and enlargement of those associated with a parasitic lifestyle. For example, the G protein-coupled receptor family (GPCR), representing the single largest gene family in *C. elegans* with 1,280 members (Bargmann 2006; Robertson and Thomas 2006) is drastically reduced in *M. incognita* and *M. hapla* with only 108 and 147 genes respectively (Abad et al. 2008; Opperman et al. 2008). Furthermore, *C. briggsae*, *M. hapla* and *M. incognita* all lack the *daf-28* gene, necessary in *C. elegans* for dauer formation (Opperman et al. 2008). A similar reduction is also observed with respect to neuropeptide diversity and detoxification enzymes (Abad et al. 2008). This reduced gene count may represent gene loss observed during niche specialisation in the host plant, with the egg and J2 life stages outside the plant having restricted neuronal access to the environment. Alternatively, this discrepancy may reflect gene expansion in *C. elegans* and adaptation to a heterogeneous niche. Less expected is the reduction in the number of genes assumed to play key roles in basic nematode biology in *Meloidogyne* spp. For example, *M. incognita* and *M. hapla* encode only 122 and 81 collagen genes respectively, compared with 165 in *C. elegans*. Furthermore, the number of genes encoding nuclear steroid hormone receptors (NHRs) in the genome of *M. hapla* and *M. incognita* is highly reduced with 76 and 92 genes respectively, compared to 284 genes in *C. elegans*. Although *C. elegans* has a surprisingly high number of NHRs it lacks orthologues to those of physiological importance in other eukaryotes, including *B. malayi* (Bertrand et al. 2004). Among the 92 predicted NHRs in *M. incognita*, some clear orthologs were found in *C. elegans*, with fewer found in *B. malayi* (Abad et al. 2008).

The absence of orthologues in *C. elegans* to some genes in plant-parasitic nematodes may indicate a potential role in parasitism. Root knot and cyst nematodes display the most evolutionarily advanced modes of parasitism of the plant-parasitic nematodes. Sedentary plant parasites dramatically modify root cells of susceptible hosts into elaborate feeding cells by modulating complex changes in cell morphology, function and gene expression (Davis et al. 2000). The pharyngeal gland cells (one dorsal and two subventral) actively produce secretions involved in the induction of transcriptional changes in the parasitised plant cells. The isolation and analyses of plant-parasitic nematode genes specifically adapted for parasitism will not benefit from the use of model nematode systems. Studies concerning the establishment of parasitism rely on detailed analyses of the nematode life cycle once inside a host plant and by chemical characterisation of induced secretions from pre-parasitic stages. ESTs analysed from pharyngeal gland specific cDNA libraries of *M. incognita* and *H. glycines* include sequences with similarity to bacterial genes, and some sequences with no homology to any reported genes (Davis et al. 2000). Several studies have supported the hypothesis that these nematodes have acquired genes via horizontal gene transfer (Blaxter 2003; Scholl et al. 2003; Smant et al. 1998; Yan et al. 1998). Analyses of the genome sequence of *M. hapla* has found many of these candidates including genes encoding enzymes believed to be associated with parasite invasion and establishment of infection (Opperman et al. 2008). In the current absence of a suitable laboratory system for plant-parasitic nematodes *C. elegans* continues to provide the most suitable model in which to study basic and conserved nematode biology.

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Chapter 11
Parallels Between Plant and Animal Parasitic Nematodes

Aaron G. Maule and Rosane Curtis

11.1 Introduction

The ability of nematodes to inhabit and flourish in hugely diverse ecological niches underpins their success as the most abundant metazoans on earth, believed to account for at least 50% of the metazoan biomass. However, despite their pivotal role in food-webs and in nutrient recycling, it is accounts of the more sinister activities of nematode parasites that pervade the scientific literature. Even the most cursory glance at the prevalence of nematode infections of man, usually based on rationalized extrapolations of empirical datasets, indicates why nematode parasites are of so much interest (see Stoll 1947; Walsh 1984; Coombs and Crompton 1991; Chan et al. 1994; Hotez 2008; Brooker 2010). For example, they are the causative agents of six (ascariasis, trichuriasis, hookworm disease, lymphatic filariasis, onchocerciasis and dracunculiasis) of the 13 core neglected tropical diseases of humans (Payne and Fitchett 2010) impacting in the range of 3 billion people. They are a very significant burden on animal welfare and agricultural livestock productivity globally (see Waller 2003) and, they cause in the region of US$ 125 billion losses (12.3% annual yield) to plant crops each year (Sasser and Freckman 1987; Chitwood 2003). Understanding their biology and the drivers for their success are vital to managing their impact in the longer term.

Nematode success has been achieved in spite of an unusually simple and uniform body plan. Clearly, their simple body form has not impeded their success. Indeed, it serves to belie their molecular complexity which accumulating genomic and transcriptomic datasets are illuminating (Parkinson et al. 2004a, b; Mitreva et al. 2005; Abad et al. 2008; Opperman et al. 2008). Since nematode parasitism arose on multiple occasions (at least 4 times in animal parasitic nematodes and three times in plant parasitic nematodes; Blaxter et al. 1998), it is not surprising that there are numerous parallels between the animal and plant parasites. Naturally, many of these

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parallels are simply features that are common across nematodes, including parasitic and free-living forms. In contrast, others better define the distinct challenges imposed by the adoption of a parasitic life style. For example, the adoption of a parasitic habit commonly means that some life stages must accommodate a rapid switch from a free-living to a parasitic niche, with the ability to not only survive, but exploit sudden and often extreme changes in their external physico-chemical environment—a problem faced by many animal and plant parasites alike. Another key attribute many of the parasites share is their ability to arrest larval development in the absence of the appropriate host. Although this trait is common to parasites, it is not unique to this life-style and has parallels with the resting dauer stage of the free-living *Caenorhabditis elegans*. Further, upon host-infection both animal and plant parasitic nematodes enter a world that has co-evolved to counter their invasion and survival strategies and so both must be uniquely adapted to evade or survive in the face of an immune system onslaught (Dangl and Jones 2001; De Veer et al. 2007; Colditz 2008). Indeed, some authors have proposed that the competing pressures prevailing at the host-parasite interface combine to accelerate molecular evolutionary change (Mitreva et al. 2005).

Nematode parasite adaptability is further displayed in their ability to parasitize both animals (including humans) and plants. Indeed, as endoparasites they flourish in a wide variety of tissue types and organ systems, ranging from plant vascular tissues to muscle cells, from gastrointestinal tract to blood, from lymph duct to the eye. In all of these habitats there are parallels in the goals of each parasite in that they strive to acquire the nutrients needed to facilitate reproductive success and life-cycle completion.

Over the last decade and more, numerous nematode expressed sequence tags (ESTs; nucleotide sequence reads from random cDNA clones), representing temporal snapshots of the transcriptomes of diverse nematode species, have accumulated (see Chap. 9). In addition, and building on the *C. elegans* genome sequence and associated resources, several other nematode genome projects have been completed (Stein et al. 2003; Harris et al. 2004; Ghedin et al. 2007; Abad et al. 2008; Opperman et al. 2008) and others are in progress—amongst these are parasites of animals and plants (see Chaps. 6 and 10). While these are providing platforms for the first detailed comparisons of whole genome complements from free-living and parasitic nematodes, and between animal and plant parasites, it is worth remembering that these represent only a tiny sample of nematode genetic diversity. In the longer term, it would be hoped that multiple comparisons of this kind will illuminate the gene complements that define nematode parasitism, possibilities made more likely by such initiatives as the 1,000 (actually designated 959 to represent the number of somatic cells in *C. elegans*) Nematode Genomes project http://www.nematodes.org/nematodegenomes/index.php/Main_Page.

It is well beyond the scope of this chapter to cover all facets of nematode biology that could be compared between animal and plant parasitic nematodes. Here we present, what in our opinion are, some of the more notable and/or interesting parallels between these two groups of nematodes. In addition to the examples provided here, there are clear parallels to be drawn in terms of mechanisms and use of RNA interference in plant and animal parasites. These issues are tackled in Chap. 10.

11.2 Morphology

11.2.1 Body Form

One common feature of nematodes is their simple and uniform body plan (pseudocoelomate with a typical tube-within-tube body plan) and relatively small somatic cell number (between 900 and 1,000). Body form in animal and plant parasitic nematodes is most commonly vermiform or fusiform. Parallels between animal and plant parasitic nematodes are common, as indicated by older classification systems that housed many species from both groups within the Secernentea, nematodes that possess numerous caudal papillae and the presence of an excretory system with lateral canals (Chitwood and Chitwood 1933, 1937, 1958). Even with the advent of molecular based comparisons, grouping of worms within the Secernentea retains validity, unlike the Adenophorea which is paraphyletic.

Significant divergence from the fusiform body shape is seen in the adult female cyst or root knot nematodes of plants which adopt the typical saccate body form. Beyond this, the most obvious divergence in nematode parasite body form is seen in the anterior stomodeum and buccal regions which are often uniquely adapted to their particular parasitic lifestyle. The stylet (protrusible spear or stomostyle) of plant endoparasitic nematodes is noteworthy in that it facilitates entry to the plant root and subsequent migratory activities, the release of pathogenicity (or parasitism) gene products and the uptake of plant cell nutrients. Greater buccal cavity divergence is seen in animal parasitic nematodes, ranging from a simple sucking mouth in some filarial nematodes to a large asymmetrical buccal cavity with cutting plates and teeth as seen in hookworms such as *Ancylostoma*. In addition to these variations in body form, surface topography, associated with surface grooves (transverse and longitudinal) and lip formation (triradiate lips are most common) can be highly diverse and adaptive. For example the three-lip pattern and shallow transverse striations of the cuticle are common to many endoparasitic plant parasites such as *Pratylenchus* and to animal parasites such as *Ascaris*. Numerous species from both the animal and plant parasitic nematode groupings have cuticular projections or spines; sometimes these are associated with the lips and are believed to aid in feeding.

Morphological divergence in the tail region of animal parasitic male nematodes is common and centres on the development of complex copulatory bursae designed to facilitate reproduction through the transfer of sperm to the vulva or gonoduct during copulation.

11.2.2 Glands

The glands of the digestive system of nematodes fall in two categories: the oesophageal glands (also called pharyngeal glands) and rectal glands. The rectal glands vary

in number and are absent in some groups of nematodes but the six rectal glands of the genus *Meloidogyne* are responsible for the secretion of the gelatinous matrix (Bird and Bird 1991). An oesophageal gland cell is situated in the dorsal and in both subventral sectors of the posterior portion of the oesophagus of animal parasitic nematodes and these cells produce digestive secretions which are released through ducts to the lumen. The opening of the dorsal gland cell is situated in the anterior region of the nematode and often in the buccal cavity whilst the other openings are located in the posterior region of the nematodes. Some tricurid nematodes have a stichosome, a multicellular organ that consists of cellular stichocytes. It opens in the oesophageal lumen and functions as a secretory gland and storage organ. The oesophageal glands in tylenchid nematodes have evolved into three large secretory cells, like animal parasitic nematodes, one dorsal and two subventral glands. These are connected to the oesophageal lumen and stylet through complex valves and are both very active in the migratory stages whilst in the sedentary stages the dorsal gland cell becomes enlarged and the subventral dorsal gland cell becomes atrophied.

The secretory-excretory (SE) system of nematodes functions as an osmoregulatory, ion regulatory system and secretory organ and opens to the exterior thorough the SE pore (Bird et al. 1988). A tubular type of SE system is common among animal parasitic nematodes and it consists of a system of tubes and one or two gland cells which have a joint excretory duct. The lateral tubes run inside the lateral chord of the hypodermis and the subventral glands are connected to the SE tube in the anterior region of the nematode. The SE system of the microfilariae larvae has a single cell and the adult worms lack a SE system.

The SE products released by nematodes can interfere with every aspect of the host immunity (Hewitson et al. 2009) and the majority of candidate plant nematode parasitism genes identified to date encode polypeptides that are predicted to be secreted from the nematode oesophageal glands (Davis et al. 2009). Not surprisingly, recent analyses of the nematode EST datasets indicate that secreted protein novelty and diversity is more evident in nematode parasites than in free-living nematodes (Wasmuth et al. 2008). At this time, there is relatively little evidence of commonality amongst the secretory protein profiles of animal and plant nematodes other than antioxidant proteins present at the parasite surface (see Chap. 13).

11.2.3 Cuticle

Nematodes moult several times through their developmental cycle and each time change their cuticular and antigenic surface (Blaxter et al. 1992; Raleigh et al. 1996). The cuticle of most nematodes can be subdivided into three main zones (cortical, medium and basal) covered with an epicuticle and overlaid by an amorphous carbohydrate-rich surface coat or glycocalyx. A detailed review describing the structure of the cuticle and contrasting the cuticular features of a wide range of nematode species has been published previously (Bird and Bird 1991; Lee 2002).

The biochemical components of the cuticle can be divided into: structural components (insoluble); surface associated soluble proteins, which may have enzymatic activity; and surface associated low molecular weight components, including lipids. The structural components of the cuticle confer its rigidity and elasticity and contribute to determining body shape and locomotion. The most abundant and best characterised cuticle proteins are the collagens and the other non-collagenous components generically called 'cuticulins' (Kramer et al. 1982; Johnstone 1994; Page and Johnstone 2007). Collagens from a wide range of animal species have a conserved and characteristic triple helical tertiary structure formed from large Gly-X-Y repeat regions in which every third amino acid is a glycine residue and where Y is frequently proline, which forces the helical turn. Collagen genes are present in extremely large gene families in a wide range of nematode species: over 170 genes are present in *C. elegans* (Page and Johnstone 2007) and 122 are predicted from the genome of *Meloidogyne incognita* (Abad et al. 2008). Cuticulins contain cysteine rich regions and are extensively cross linked, particularly in the outer cortical layers (Page and Johnstone 2007) and it seems that they are important in formation of the lateral alae and other cuticular annulations and ridges (Sapio et al. 2005).

The cuticle itself is synthesized during a series of moults and whilst cuticle collagen and cuticulins are synthesised by and secreted from the hypodermis, non-structural proteins can be made in gland cells (SE system, oesophageal glands, amphids, phasmids) as well as in the hypodermis (Blaxter and Robertson 1998). Few proteins are present at the nematode surface and they are mostly glycoproteins. The major components of the cuticle surface are mucins, a set of O-glycosylated proteins which are highly expressed in the juveniles (Gems and Maizels 1996).

The cuticle comprises the primary interface between a nematode and its environment and has a dynamic nature with a continuous turn-over of surface-associated antigens (Blaxter et al. 1998). Following cuticle striping of *Necator brasiliensis* with CTAB, maximum recovery was achieved after 48 hours and the turnover of the cuticle surface of *Globodera pallida* was between 1 and 2 hours (Preston-Meek and Pritchard 1991; Fioretti et al. 2002). SE products of animal parasitic nematodes such as *Trichinella spiralis* and *Haemonchus contortus* share cross-reactive epitopes with SE products of plant-parasitic nematodes (Lopez et al. 1999).

11.2.4 Muscle System

Longitudinal muscle invests the inner surface of the body wall of nematodes. The muscle layer is well secured to the hypodermal layer by hemidesmosomes and displays variety in the numbers of muscle cell rows that occur in the quadrants bordered by the four hypodermal cords. These differences appear to be species specific and relate more to nematode size than their life style or the type of host they parasitize. Muscle cell organisation typically includes a spindle region that is closely apposed to the hypodermis and is invested with the contractile proteins and the bag region which extends into the pseudocoel and houses the nucleus and much of the

synthetic apparatus of the cell. Few, if any, differences in the muscle arrangement relate to the type of host infected by the parasite.

Specialised muscle groups invest the feeding organ or pharynx and the reproductive organs. Again, these are structurally variable and appear to be highly adapted to nematode life style or feeding habit. The pharynx is the most specialised muscle structure in nematodes operating as a complex muscular pump and a secretory organ in many species. Pharyngeal structure varies from a simple cylinder to a complex multi-part structure, commonly with a narrow anterior region (corpus) and a broader posterior region (postcorpus or bulb). Further divisions of the corpus most commonly include an anterior, non-muscular procorpus and a more muscular, posterior metacorpus. Divisions of the postcorpus into a narrow anterior isthmus and a posterior bulb give the typical three-part pharynx appearance. The structure of the pharynx reflects the feeding strategy of the nematode in question, with more muscle often emphasizing the need for mechanical destruction of food and limited muscle equating with food that needs little mechanical processing. While gastrointestinal nematodes commonly display cylindrical pharynges, endoparasitic plant nematodes often have pharynges with reduced muscle, often concentrated in the metacorpus which acts as a simple fluid-pump.

11.2.5 Nervous System

The brain of parasitic nematodes is a parallel processing unit, capable of receiving multiple sensory inputs and triggering a behavioural response. The bulk of knowledge of nematode nervous systems has been derived from *C. elegans* and stems from the transmission electron miscroscopic mapping of the entire nervous system, including the 302 nerve cells, ~5,000 chemical synapses and ~2,000 neuromuscular synapses of the adult hermaphrodite (White et al. 1976, 1986). Even today, the impact and detail of this work are astounding. Unfortunately, we do not have such detailed information for any other nematode. Significant data on nervous system structure are also available for *Ascaris suum*, the large gastrointestinal nematode of pigs (Goldschmidt 1908, 1909; Stretton et al. 1978; Angstadt et al. 1989). While there is some information for other animal parasitic nematodes and some plant parasitic nematodes, the detail is relatively scarce and prevents meaningful comparisons.

11.2.5.1 Sensory Structures

The variety of environments occupied by parasitic nematodes means that numerous different sense organs abound, including those involved in chemosensation, mechanosensation, osmosensation and thermosensation. Multiple reviews cover the structure and function of nematode sense organs (McLaren 1976; Wright 1980; Bargmann and Mori 1997; Driscoll and Kaplan 1997; Jones 2002) such that here we will only briefly consider those features that pertain to animal and plant parasitic forms.

Not surprisingly, external nematode sense organs are concentrated at the anterior end, regularly comprising two laterally situated amphids and a variable number of cephalic and labial sensory structures. Fewer sense organs are found in the posterior end of nematodes, but they are commonly associated with the male tail where they play key roles in mating. Internal sense organs usually facilitate mechanoreception and have been linked to feeding and the regulation of motor function.

Here we will focus on the amphids, the most highly developed nematode sense organs and central to orientation to and from attractants and repellents. These cup shaped recesses in the nematode head house dendritic endings from numerous sensory nerves, most of which play a role in chemosensation, critical to the host finding and within-host orientation activities of many parasitic nematodes. The amphidial neurons extend into a large receptor cavity formed by the so-called sheath cell. Within this cavity the dendritic processes of the amphidial neurons continue towards the exterior and some extend within the amphidial canal, formed by the sheath cell initially and then the socket cell. It is after passing through the socket cell that these dendrites are exposed to the external environment. Those dendritic processes that do not enter the amphidial canal can form a variety of projections, some of which enter the sheath cell. These projections or processes are variable in shape and have been designated finger-like and wing-like. Some projections do not enter the sheath cell and have been designated microvillar processes; several hundred of the latter have been reported in some plant parasitic nematodes (Baldwin and Hirschmann 1973). The plant parasite *Xiphinema americanum* displays some unusual features in the arrangement of the amphids, including multiple dendritic processes emanating from individual sensory neurons (Wright and Carter 1980). Some plant parasitic nematodes also display well developed accessory cilia of unknown function, although proposed functions include photoreception and electromagnetic field detection (Wergin and Endo 1976; Endo 1980; Robertson and Forrest 1989). Although detailed morphological data are only available for a few nematode species, some parallels can be drawn between animal and plant parasites.

While the overall structure of the amphids is similar between free-living and parasitic nematodes, and similarly between animal and plant parasitic forms, differences in the detail abound. For example, the potato cyst nematode *Globodera rostochiensis* has only seven dendritic processes (Jones et al. 1994) whereas thirteen amphidial neurons were identified in the infective larval stage of *Strongyloides stercoralis* (Ashton et al. 1995) and eight in the filarial nematode *Onchocerca volvulus* (Strote and Bonow 1993; Strote et al. 1996). It is not surprising that the host-finding activity of some animal parasitic nematodes such as *S. stercoralis* and the dog hookworm *Ancylostoma caninum* involves both chemosensory and thermosensory elements within the amphids (Ashton and Schad 1999). Other differences in animal parasites is the occurrence of fewer or no microvillar projections and dendritic branches entering the sheath cell commonly having their origins from dendrites proceeding to the amphidial canal.

Other interesting comparisons between animal and plant nematode amphids relate to their secretory activities—a common feature of sheath cells. For example, sheath cell secretions from migratory stages of *M. incognita* (second stage

juveniles, J2s, and adult males) have been found to include a 32 k Da glycoprotein of unknown function (Stewart et al. 1993). A role in sensory function is supported by the fact that an antiserum against this protein compromised normal chemotactic behaviour (Stewart et al. 1993). Such secretions may also play a role in the host-parasite interaction: *Heterodera glycines* is thought to secrete a feeding plug from its amphids (although the source of the feeding plug is a matter of some debate; see Chap. 4), thought to prevent leakage from the plant cell syncytium when the stylet is withdrawn during the feeding cycle (Endo 1978). Amphidial secretions from animal parasitic nematodes include anticoagulants from the feeding stages of *Necator americanus, Syngamus trachea* and *A. caninum* (Thorsen 1956; McLaren et al. 1974; see Jones 2002), believed to be adaptations to their blood meal. Amphidial secretions of some animal and plant parasitic nematodes include acetylcholinesterase. In animal parasites its release has been linked to attachment in the host gut, host membrane permeability and immune response amelioration (Lee 1969, 1970, 1996); its role in the amphidial secretions of the plant parasitic nematodes is unknown.

A recent study of the anterior sensory apparatus (excluding the amphids) of the mycophagus nematode *Aphelenchus avenae* noted strong similarities with *C. elegans* (Ragsdale et al. 2009).

11.3 Life Histories

11.3.1 Strategies

Transmission to the next host is one of the events common in the life cycles of all parasites which may occur more than once during a single life-cycle if the parasite develops in one or more intermediate hosts. The adult or mature parasite completes its development or reproduces in the final or definitive host, releasing eggs, larvae or infective stages that must be transmitted to another host. Animal parasites may inhabit widely differing environments at various stages in their life-cycle, and they are adapted for life in each of these environments. There is only one host in a direct life-cycle, but in indirect life-cycles passage through two hosts can be mandatory for completion of the life-cycle. The eggs or larvae may be eaten by the intermediate host (or vector), the definite host becomes infected either by eating the infected intermediate host or when the intermediate host (commonly an insect) feeds on host blood or tissues, infection is achieved when larvae cross the skin either by direct penetration or delivery by blood-feeding vector. The animal parasitic nematodes can be divided into four groups according to their feeding habits: feeders on the contents of their host's gut (e.g. *Ascaris*); epithelium feeders (e.g. *Ancylostoma*); tissue feeders involving tissue penetration (e.g. *Trichuris*) and fluid tissue feeders (e.g. *Wuchereria*). In contrast, most plant parasites invade specific parts of the plant directly and show little variation in their parasitic strategies. They can be divided

into ectoparasites, which feed from the outside of the plants, and endoparasites, some of which (the sedentary endoparasites) have a more complex relationship with their hosts, becoming sedentary and feeding and reproducing while inside the plants.

All parasitic nematodes show an extraordinary capacity, both morphologically and physiologically, to accommodate several quite dissimilar environments which can differ in ambient temperature, oxygen availability, salt, water contents, pH and food contents. Nematodes respond to a wide range of stimuli, including heat, light, gravity, electrical fields, various chemicals and mechanical events and these may play important roles in host location (Curtis et al. 2009). Environmental signals must also be responsible for initiating the switching mechanisms to allow the nematodes to adopt many forms and develop physiological attributes to a particular phase in the life cycle. Plant and some animal parasitic nematodes (hookworms such as *Ancylostoma* sp. and *Necator* sp.) possess active free-living larval stages that are concerned with locating a new host using their chemosensory organs. Newly hatched second stage juveniles of plant parasitic nematodes display random movement when no attractants are present but switch to oriented migration through a concentration gradient of root compounds towards the root (reviewed in Curtis et al. 2009). Members of the order Strongylida (*Ancylostomatoidea, Strongloidea, Trichostrongyloidea* and *Heligmosomatoidea*) are transmitted to the vertebrate host, usually a mammal, by actively penetrating the skin as a free-living larval stage. Initiation of penetration may come through a response to a thermal gradient. *Ancylostoma* and *Necator* tend to be inactive and are only stimulated into activity by the increased temperature due to the presence of humans or animals. Therefore, they possess behavioural patterns that are clearly adaptations for locating warm-blooded animals in a terrestrial environment. *Nippostrongylus* in contrast seems to search actively for its rat hosts by waving movements of the head and once a host is located, the larvae spiral rapidly down the hairs following a thermal gradient.

Both animal and parasitic nematodes use enzymes to degrade tissues, cells and (in the case of plant parasites) cell walls to facilitate host invasion and migration. Enzymes of parasite origin including metalloproteases and aspartic proteases have been implicated in skin penetration in a number of species such as *Necator americanus* (Brown et al. 1999; Brindley et al. 1995; Hotez et al. 1990). However, it is clear that penetration may also involve a sequence of other signals for the larva. Plant parasitic nematodes use their sharp anterior stylet to pierce the root cells during host penetration assisted by enzymes released through the stylet from the nematode oesophageal glands, these also play important roles during nematode migration inside root tissue.

Once nematodes have successfully reached their definitive host they must establish themselves, grow and mature to achieve reproductive success. Animal parasitic nematodes can establish themselves in the blood vessel, or the gastrointestinal system or host tissues. *Trichinella spiralis* is an intracellular nematode which alternates between intestinal and skeletal muscle cell infection. There is a strong parallel between *T. spiralis* and some plant parasitic nematodes as they seem to have a direct role in host cell remodelling during the initiation and chronic phase of infection by

manipulating host gene expression and cell cycle repositioning (Jasmer 1990, 2001; Jasmer et al. 2003). *T. spiralis* induces a nurse cell system in muscle cells which has some similarities to the plant host giant-cell. Nematode proteins, such as the glycoprotein p43 were detected in the cytoplasm of infected muscle cells between 10 and 14 days post infection (dpi). Other nuclear antigens have also been identified in the host cell nuclei in the chronic phase of infection (Despommier 1975; Despommier et al. 1975, 1990). During the initial events of infection the muscle cell undergoes considerable changes in its architecture with the disappearance of myofibrils and a shift of the nucleus to a central position in the cell. By day 4 post infection nuclei and nucleoli begin to undergo hypertrophy and the infected muscle cell re-enters the cell-cycle and becomes permanently blocked at G2/M (Lathrop et al. 1985).

Plant nematodes perforate the cell wall with their stylet and oesophageal gland secretions are injected into the cytoplasm of the cell, upon which modifications of the cytoplasm of the recipient and surrounding cells are observed (Wyss 2002). These processes are described in detail in Chaps. 4 and 5.

A cross reactive secreted cuticular proteic epitope was identified in the free-living stages and parasitic stages of *T. spiralis* and *M. incognita* (Lopez-Arellano and Curtis 2002). *In planta* this antigen was immunolocalised surrounding the cuticle of the adult females of *M. incognita* and in the plasma membrane of root cells of *Arabidopsis thaliana*, close to the feeding cell formed during infection with *M. incognita*. *In vivo* this antigen was localised on *T. spiralis* surface and as secreted droplets close to the collagen capsule surrounding the nematode nurse cell (Curtis 1996; Lopez-Arellano and Curtis 2002) (Fig. 11.1).

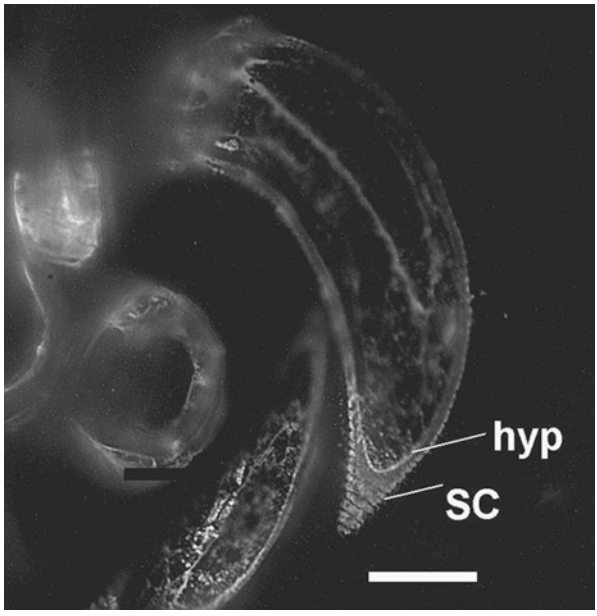

Fig. 11.1 Immunofluorescence labeling of the surface cuticle and hypodermis of *Trichinella spiralis* larval stage 1 (L1), inside the nurse cell using a monoclonal antibody raised to the plant-parasitic nematode *Heterodera avenae*. SC = surface cuticle; hyp = hypodermis. Scale bar = 10 µm

11.3.2 Adaptations to Parasitism

Dorris et al. (1999) suggest that parasitism arose independently at least four times in animal parasitic nematodes and on three occasions in plant parasitic nematodes. The first comprehensive study of the molecular evolution of helminths was a phylogenetic analysis of the small subunit ribosomal DNA sequences from 53 nematodes and suggested that parasitism of animals and plants arose independently (Blaxter et al. 1998). Work by Holterman et al. (2006) suggests that parasitic nematodes of plants arose from fungivorous ancestors. However, Blaxter (2003) considers that in addition, the origins of parasitism could also be traced through associative, more-or-less exploitative interactions and may include considerations of mutualistic symbiosis.

It is believed that the most evolutionary advanced adaptations for plant parasitism by nematodes are the products of parasitism genes expressed in the pharyngeal glands and secreted through their stylet into host tissue and cells (Hussey 1989; Gao et al. 2003; Dubreuil et al. 2007; Rosso et al. 2009; Davis et al. 2009). These specialised secreted proteins mediating parasitism have been coined the 'parasitome' and some likely mechanisms for acquisition of parasitome genes include: adaptation of pre-existing genes to fulfil new functions; a) gene duplication and divergence of paralogs; and, b) horizontal gene transfer (Scholl et al. 2003; Mitreva et al. 2009). Indeed, some organisms have enlisted symbionts to provide traits or functions found in other kingdoms (Schultz 2002).

Plant and animal parasitic nematodes produce an array of proteolytic enzymes which play important roles in the host-parasite interactions. Plant parasitic nematodes also have genes encoding secreted plant cell wall-degrading enzymes such as beta-1,4-endoglucanases (cellulases), polygalacturonases, xylanases and pectate lyases (see Chap. 12). These enzymes are absent from other metazoan species and have possibly originated from bacterial genes through horizontal gene transfer, a mechanism considered important for the origin of PPNs (Bird et al. 2009). Other parasitism genes isolated from sedentary plant parasitic nematodes identified as putative candidates for horizontal gene transfer from bacteria include chorismate mutase, glutamine and polyglutamate synthetases, L-threonine aldolase and Nod factors (Davis et al. 2000, 2009; Gao et al. 2002; Jones et al. 2003; Lambert et al. 2005). Recent genome-wide analysis of two root-knot nematodes provides a more complete picture of the expansion and diversification of horizontal gene transfer in these nematodes which allow inferences about the evolutionary history of these parasites (Abad et al. 2008; Opperman et al. 2008). The cellulases were considered an unique feature of the genome of plant parasitic nematodes but it has now been shown that the necromenic nematode *Pristionchus pacificus* also has candidate cellulases, an indication of pre-adaptation for parasitism of animals consistent with the intermediate evolutionary position of *P. pacificus* between the free-living nematode, *C. elegans* and animal parasitic nematodes (Dieterich et al. 2008).

Other parasitism genes have characteristics suggesting they have evolved by adaptive molecular mimicry rather than by gene transfer events. The dorsal gland polypeptide (HgCLE) reported from *H. glycines* has regions of amino acid similarity to a plant ligand involved in intercellular signalling (Huang et al. 2006). It is

feasible that this nematode pseudoligand is used for parasite modifications of the plant cells. The simplicity of features shared by plant (CLE) and nematode (Hg-CLE) sequences at the amino acid level suggests that convergent evolution rather than gene transfer from plant to nematode may explain the similarity between these polypeptides.

Animal parasitic nematodes have evolved a multiplicity of evasive strategies to survive in an immunologically competent host. *B. malayi* expresses homologues of the mammalian cytokine macrophage migration inhibitory factor (MIF) which might mimic host MIF and it was shown that it can synergise with host IL-4 to induce the development of suppressive macrophages *in vitro* (Cho et al. 2007; Pastrana et al. 1998; Vermeire et al. 2008).

The parasite's ability to exist for long periods of time in their host has been attributed to a rapid turnover of their cuticle surface, shedding of surface antigens and membrane rigidity, which are likely to render the parasite less susceptible to immune attack (Simpson et al. 1984; Kusel and Gordon 1989). Disguise of the parasite cuticle surface with the acquisition of host derived antigens (Smithers and Doenhoff 1982) and the action of parasite surface proteases that can cleave the Fc region of immunoglobulins (Auriault et al. 1981) are some other mechanisms that may also help parasites to evade the host immune response by inhibiting important cellular functions.

Evasion of host immunity by *Toxocara canis* infective larvae is mediated by the nematode surface coat (SC), as this nematode is able to shed the entire SC in response to binding antibodies or eosinophils, thus permitting parasites to physically escape immune attack (Maizels et al. 2001; Maizels and Loukas 2001). The major constituent of the SC of this nematode is the O-linked TES-120 (Toxocara excretory/secretory) glycoproteins series, which has a typical mucin domain and may explain a generally non-adhesive property of this parasite. Membrane associated mucins are closely concerned with the adhesion status of cells through electrostatic charge and due to steric effects of long chains protruding from the surface. TES-120 is secreted in internal excretory glands and ducted to the surface via the oesophagus and excretory pore and it is also released from the *Toxocara* surface. The overexpression of some membrane-associated mucins suggests a possible model for the role of SC in immune evasion of parasitic nematodes by changing the nematode surface cuticle adherence to defence cells, and/or by releasing soluble mucins that might interact with host cells and blocks defence responses (Gems and Maizels 1996). *Toxocara canis* also secretes large quantities of a C-type lectin thought to compete with host innate immune system receptors or mask worm carbohydrates from host immune cells (Loukas et al. 2000; Loukas and Maizels 2000). A proteinase inhibitor, member of the cystatin (cysteine protease inhibitor) family, located on the surface of both larval stage 3 (L3) and adult *B. malayi*, and secreted by these parasites *in vitro* blocks conventional cysteine proteases but also the asparaginyl endopeptidase involved in the Class II antigen processing pathway in human B cells (Maizels and Loukas 2001).

The ability of nematodes to adapt to nearly all ecosystems and the repeated independent evolution of parasitism is thought to require genomic signatures (Diet-

erich and Sommer 2009). Comparative analysis of the genomes of animal and plant parasitic nematodes might reveal events leading to the evolution of parasitism per se. Ogawa et al. (2009) have shown that a conserved endocrine signalling mechanisms controls the formation of dauer and the infective larvae in nematodes. This is the first link between these stages which suggests that the dauer stage is a pre-adaptation for parasitism.

11.4 Neuronal Signalling Systems

As with nervous system structure, the signalling molecules released by nerves for intercellular communication in nematodes are highly conserved. For the most part, these encompass a variety of classical signalling molecules such as acetylcholine (ACh), 5-hydroxytryptamine (5-HT; serotonin), γ-aminobutyric acid (GABA) and glutamate. The role of these small signalling molecules appears relatively consistent between animal and plant parasitic nematodes. However, there are some notable nuances associated with, for example, responses to the biogenic amine, octopamine. This molecule has inhibitory effects on the *C. elegans* pharynx (Horvitz et al. 1982; see Komuniecki et al. 2004). In contrast, octopamine stimulates pharyngeal pumping in plant parasitic nematodes with consequent inhibition of somatic body wall muscle activity, a fact that has resulted in its use as a pharyngeal stimulant for the uptake of double stranded (ds)RNA for the induction of gene silencing (see Urwin et al. 2002).

More obvious differences in neuronal signalling molecules associate with the neuropeptide signalling systems of nematodes. Neuropeptides form a major component of the intercellular signalling repertoire in nematodes with well over 50% of nerves containing neuropeptides. Most nematode neuropeptide data emanate from work on *C. elegans* and *A. suum*, where parallels have been drawn between the neuropeptides they employ. Nematode neuropeptides have been assigned to three categories, the insulin-like peptides (INSs), the FMRFamide-like peptides (FLPs) and the neuropeptide-like proteins (NLPs). Currently, there are no data on the INSs in the plant parasites such that no comparisons will be made here.

Current knowledge of nematode NLPs is based on a BLAST survey of the available EST data for nematodes (McVeigh et al. 2006) and a provisional analysis of the *M. incognita* genome (Abad et al. 2008). The NLPs are a collection of all the neuropeptides that do not fall within the INS or FLP designations and encompass multiple families of peptides with diverse functions. In *C. elegans* they include at least 11 distinct neuropeptide families (Li et al. 1999; Nathoo et al. 2001). Unfortunately, little is known about the functions of these peptides in animal and plant parasitic forms, suffice it to say that diverse NLPs occur in both groups of parasites. The available datasets suggest that peptide signatures encoded on multiple *nlp* genes are common to both animal and plant parasitic nematodes (see Table 11.1), although some *nlp* genes have been identified only in animal parasitic forms: *nlp-5, nlp-7, nlp-11* and *nlp-19* (see McVeigh et al. 2006) (see Table 11.1).

Table 11.1 Known neuropeptide commonalities across *Caenorhabditis elegans*, plant parasitic nematodes and animal parasitic nematodes. Neuropeptide gene names as designated for *C. elegans* or as in McVeigh et al. (2005, 2006) and Abad et al. (2008). *Nlp-24-33* omitted from table as putative antimicrobial peptides in *C. elegans*

Neuropeptide-like protein (*nlp*) genes			FMRFamide-like peptide (*flp*) genes		
C. elegans	Plant parasitic nematodes	Animal Parasitic Nematodes	*C. elegans*	Plant parasitic nematodes	Animal Parasitic Nematodes
nlp-1	*nlp*-1	*nlp*-1	*flp*-1	*flp*-1	*flp*-1
nlp-2	*nlp*-2	*nlp*-2	*flp*-2	–	*flp*-2
nlp-3	*nlp*-3	*nlp*-3	*flp*-3	*flp*-3	–
nlp-4	–	–	*flp*-4	–	*flp*-4
nlp-5	–	*nlp*-5	*flp*-5	*flp*-5	*flp*-5
nlp-6	*nlp*-6	*nlp*-6	*flp*-6	*flp*-6	*flp*-6
nlp-7	–	*nlp*-7	*flp*-7	*flp*-7	*flp*-7
nlp-8	*nlp*-8	*nlp*-8	*flp*-8	*flp*-8	*flp*-8
nlp-9	*nlp*-9	*nlp*-9	*flp*-9	–	*flp*-9
nlp-10	*nlp*-10	*nlp*-10	*flp*-10	*flp*-10	*flp*-10
nlp-11	–	*nlp*-11	*flp*-11	*flp*-11	*flp*-11
nlp-12	*nlp*-12	*nlp*-12	*flp*-12	*flp*-12	*flp*-12
nlp-13	*nlp*-13	*nlp*-13	*flp*-13	*flp*-13	*flp*-13
nlp-14	*nlp*-14	*nlp*-14	*flp*-14	*flp*-14	*flp*-14
nlp-15	*nlp*-15	*nlp*-15	*flp*-15	–	*flp*-15
nlp-16	–	–	*flp*-16	*flp*-16	*flp*-16
nlp-17	*nlp*-17	*nlp*-17	*flp*-17	*flp*-17	*flp*-17
nlp-18	*nlp*-18	*nlp*-18	*flp*-18	*flp*-18	*flp*-18
nlp-19	–	*nlp*-19	*flp*-19	*flp*-19	*flp*-19
nlp-20	*nlp*-20	*nlp*-20	*flp*-20	*flp*-20	*flp*-20
nlp-21	*nlp*-21	*nlp*-21	*flp*-21	*flp*-21	*flp*-21
nlp-22	*nlp*-22	*nlp*-22	*flp*-22	*flp*-22	*flp*-22
nlp-23	–	–	*flp*-23	–	*flp*-23
nlp-34	–	–	*flp*-24	–	*flp*-24
nlp-35	–	–	*flp*-25	*flp*-25	*flp*-25
nlp-36	*nlp*-36	–	*flp*-26		*flp*-26
nlp-37	*nlp*-37	*nlp*-37	*flp*-27	*flp*-27	*flp*-27
nlp-38	*nlp*-38	*nlp*-38	*flp*-28	*flp*-28	–
nlp-39	–	–	–	–	*flp*-29
nlp-40	*nlp*-40	–	–	*flp*-30	–
nlp-41	–	–	–	*flp*-31	–
nlp-42	*nlp*-42	*nlp*-42	*flp*-32	*flp*-32	*flp*-32
nlp-43	*nlp*-43	*nlp*-43	*flp*-33		
nlp-44	*nlp*-44	*nlp*-44	*flp*-34		
nlp-45	–	–			
nlp-46	*nlp*-46	*nlp*-46			

FLPs are one of the largest neuropeptide families known and in nematodes include a diverse collection of peptides that have a conserved C-terminal tetrapeptide signature; this signature comprises an aromatic amino acid residue, a variable residue (that can be any amino acid excluding cysteine), an arginyl residue and a C-terminal phenylalaninamide—sometimes, peptides with this signature are co-

encoded with peptides that lack the first aromatic amino acid and which are still, therefore, designated FLPs. While *C. elegans* expresses 31 *flp* genes that encode in the region of 70 distinct FLPs, fewer are expressed in the parasitic forms, arguably indicative of the more restricted repertoire of stimuli they are exposed to during their endoparasitic stages. Nevertheless, a BLAST survey of parasitic nematode FLP-encoding ESTs revealed that most *C. elegans* FLP signatures were expressed in animal and plant parasitic forms (McVeigh et al. 2005). The only published, genome-wide analysis for a parasitic nematode FLP complement is for *M. incognita* (Abad et al. 2008). Analysis of this dataset uncovered 17 (*flp*-1, 3, 5–7, 12–14, 16, 18–22, 25, 27 and 32) of the 29 *C. elegans flp* genes and two additional *flp* genes (*Mi-flp-30* and *Mi-flp-31*) that appear to be restricted to plant parasites and which may have functions specific to plant parasitism. Combining the genomic data for *M. incognita* and the EST survey data from McVeigh et al. (2005) reveal that while most *C. elegans* FLPs are common to both, more are represented within the animal parasitic nematode datasets (see Table 11.1). More genomic level comparisons are needed to establish if these differences are genuine or merely reflect imbalances in the available data.

A raft of worm tissue and whole animal studies have been carried out on *A. suum* and indicate that FLPs have diverse activities on behaviour and motor function, including the activities of somatic body wall muscle, pharyngeal muscle, ovijector muscle and nerves (for review see McVeigh et al. 2008). FLPs have also been shown to modify body wall muscle activity in the sheep parasite *H. contortus* (Marks et al. 1999). Although tissue-level and behavioural studies on FLP functions have not been carried out in the plant parasitic nematodes, an RNA interference (RNAi) study in which a series of *flp* genes from *G. pallida* were knocked down revealed aberrant behavioural phenotypes and migrational abilities (Kimber et al. 2007). These data are consistent with FLPs playing important roles in neuromuscular function in both animal and plant parasitic nematodes.

11.5 Endosymbionts

An endosymbiont is defined as an organism that lives within the body or cells of another organism. Numerous micro-organisms have been described as cytoplasmic symbionts of eukaryotes, including parasitic nematodes. Molecular phylogenetic studies examining endosymbiont species revealed that many of the endosymbioses between bacteria species and their invertebrate hosts were the result of ancient infections followed by vertical, within host lineage, transmission (Moran and Baumann 2000). *Wolbachia* species are among the most abundant symbiotic microbes on earth, estimated to occur in 66% of all arthropod and nematode species examined (McNulty et al. 2010). The first reports of endosymbiont species of bacteria, such as *Wolbachia* associated with nematode species appeared following electron microscopy studies on nematodes in the 1970s. Of the endosymbiont bacteria identified to date, the genus *Wolbachia* is the most studied (Taylor 2003; Foster et al. 2005). *Wolbachia* of filarial nematodes are obligate mutualists; they are believed to

provide essential cofactors such as riboflavin and heme to their nematode hosts (see Brownlie and O'Neill 2005).

Wolbachia are associated with medically important parasitic filarial nematodes such as *B. malayi* and *W. bancrofti*, the causative agents of lymphatic filariasis which is transmitted by mosquito vectors. *Wolbachia* species of bacteria have also been identified in *Onchocerca volvulus* (Spiruida: Onchocercidae) which causes onchocerciasis or river blindness and is transmitted by the Black fly (Taylor 2003; Saint André et al. 2002). Recent studies suggest that it is actually the *Wolbachia* bacteria themselves that induce the acute inflammation indicative of filariasis as well as the corneal inflammatory response associated with onchocerciasis. A variety of molecular studies on *Wolbachia* endosymbionts revealed that they can be assigned to one of at least seven supergroups, designated A-F and H, on the basis of 16S rRNA, *Wolbachia* surface protein and *ftsZ* gene phylogenetics (O'Neill et al. 1992; Sironi et al. 1995; Bandi et al. 1997, 2001; Casiraghi et al. 2001a, b; Werren 1997; Vandekerckhove et al. 1999; Bazzocchi et al. 2000; Lo et al. 2002; Crainey et al. 2010). Whilst *Wolbachia* species isolated from arthropods are distributed throughout four of these supergroups, nematode derived *Wolbachia* from species such as *O. volvulus* and *Dirofilaria immitis* are confined to supergroup C, while supergroup D *Wolbachia* occur in *B. malayi, W. bancrofti* and *Litomosoides sigmodontis* (Bandi et al. 2001; Lo et al. 2002); some lineages of filarial worms appear to have lost symbiotic bacteria during evolution (Casiraghi et al. 2004). It has been suggested that bacterial endosymbionts such as *Wolbachia*, may not only be of medical and veterinary importance, but may also represent a putative novel target used for the delivery of both anti-insecticidal and anti-parasitic products to recipient hosts (Johnston and Taylor 2007).

Multiple endosymbiont bacteria species have also been reported in plant parasitic nematodes where their association appears to be mutualistic. For example, nematodes belonging to the genera *Steinernema* and *Heterorhabditis* have an association with endosymbiotic bacteria of the genus *Xenorhabdus* in which the bacterium is transmitted to the insect haemocoel by the nematode vector. Within the insect haemolymph, the bacterium multiplies and kills the insect host (Kaya 1993). Several verrucomicrobial species of *Candidatus Xiphinematobacter* associate with plant parasitic nematodes of the genus *Xiphinema* (Dorylaimida: Longidoridae) (Vandekerckhove et al. 2000) and species similar to '*Candidatus Cardinium hertigi*i', associate with the cyst-forming *G. rostochiensis*, *H. glycines* and *Heterodera goettingiana* (pea cyst nematode) (see Noel and Atibalentja 2006). Whilst there is little evidence of *Wolbachia* in plant parasitic nematodes, a recent study in the burrowing nematode *Radopholus similis* identified sequences from reproductive tissues that were designated as belonging to a bacterial species distantly related to the known *Wolbachia* supergroups (Haegeman et al. 2009).

Whilst some animal and plant parasitic nematodes can harbour endosymbiotic bacteria, these are species dependent and do not appear to be illustrative of a particular life style. Nevertheless, where bacteria occur as endosymbionts in animal or plant parasitic nematodes, they are seen to have potential as novel targets for their control.

11.6 Host-Parasite Interactions

11.6.1 Host Immunity

Parasitic nematodes inhabit man, his livestock and his crops and they cause serious damage. All hosts have developed defence mechanisms for protection against invasion and establishment by metazoan parasites. Defence mechanisms of vertebrates against invading organisms are divided into specific and non-specific responses. To enter the body parasitic nematodes have first to negotiate the first layer of non-specific defence; these are physical and chemical barriers that the body has developed to allow maximal protection from invasion without compromising their function (Chappell 1980; de Veer et al. 2007). The skin provides a formidable physical barrier ranging from hardened scales (reptiles, fish) to several layers of epidermal cells with an outside layer of dead, keratinised cells that are continuously sloughed off (mammals). The mucosal surfaces consist of a single layer of epidermal cells and provide little protection against the mechanical and enzymatic entry of large parasites. Not surprisingly, most parasites enter the body through the mucosal surfaces of the gastrointestinal tract (de Veer et al. 2007). However, mucosal membranes have peristalsis which can help to limit infection and some have an active layer of mucus containing anti-microbial peptide (AMP) families such as defensins. These AMPs are not directly toxic to nematodes but their additional roles as chemotatic and paracrine signalling molecules may facilitate the innate inflammatory response during parasite infections (Bartholomay et al. 2003; Bevins 2006). Chemical and biochemical barriers also play an important role in protecting the body against infection, the low pH and an array of hydrolytic enzymes in the stomach provide a hostile environment to most parasites, although several nematodes have developed a mechanism to parasitise this organ, e.g. *Haemonchus, Teladorsagia, Ostertagia* in sheep and cattle (de Veer et al. 2007).

Once animal nematode parasites breach the mucosal and epithelial barriers they still face a second line of defence in the subepithelial tissues made up of sentinel cells and mediators of innate immunity (see below Sect. 11.6.2). These non-specific responses tend to have a cellular basis and involve the action of phagocytic cells whose function is to engulf the invading organism and digest them lysosomally; this action can be enhanced by the action of antibodies and the complement system which are important effectors of the innate and adaptive immune responses.

Adaptive immunity is unique to vertebrates and these specific immune responses are associated with immunological memory leading to the formation of specific antibodies stimulated by the presence of foreign material, nematode antigens. Antibodies react with antigens and effect lysis, agglutination, precipitation or enhanced phagocytosis of the cells producing the antigens (Chappell 1980).

Most animal parasitic nematodes stimulate the host to produce IgE antibodies induced by antigens with allergenic features, these antibodies do not appear to be exclusively responsible for limiting infection, however, a relationship between IgE

production, eosinophilia and synthesis and secretion of histamine from masts cells at the site of invasion or presence of helminths has been shown. Self-cure can occur in *H. contortus* infections of lambs and *N. brasiliensis* infections of rats and may be due to a common expulsive mechanism for gut dwelling nematodes related to the release of histamine in the gut. Acquired immunity for *T. spiralis* can develop following a primary infection, so the invading larvae of the challenged infection become eliminated due to local inflammatory responses. In trichinosis, protective immunity is directed essentially against invading larvae rather than established worms.

Plants are also very capable of defending themselves against the dangers of the environment using pre-existing constitutive a) physical barriers, such as thick cuticle, rigid cell walls, thorns, needles, as well as using b) chemical barriers by exuding toxic or repellent compounds. These can alone deter many micro-organisms and herbivores. Nevertheless, plants have also evolved sophisticated mechanisms to defend themselves with an array of inducible defence responses against microbial pathogens. In response to attack, plants produce highly attacker-specific hormonal blends. This contributes to the specificity of the plants defence and regulates different defence mechanisms effective against a variety of different pathogens (see Sect. 11.6.2).

Since life on earth depends on plant production, it is believed that selection has shaped how microbes and animals exploit plants and how plants resist. This has in turn structured most ecological systems, the evolution of plants, herbivores and microbes and the development of much of earth's diversity (Ehrlich and Raven 1964). It is increasingly evident that plants and animals have shared mechanisms which they use to respond to their biotic and abiotic environment. They share elements of fatty acids, proteins, steroids, neurotransmitters, reactive oxygen species, nitric oxide and fundamental sensing; plants and animals possess parallel analogue fatty acid signalling systems and growth hormone signalling systems. When they do not have a particular system they may acquire it from other organisms, for example symbiotic bacteria (reviewed in Schultz 2002).

11.6.2 Plant and Animal Innate Immune Response

Both plants and animals have a complex innate mechanism with striking similarities which they use to recognise and respond to attack by pathogenic organisms, this mechanism has ancient origins (Nurenberger et al. 2004; Ausubel 2005). Some plant-animal parasitic nematode similarities probably involve homology (shared ancestral traits) while others represent adaptive convergence on solutions for similar situations or threats (Schultz 2002). Innate immunity relies on a set of defined receptors referred to as pathogen or pattern-recognition receptors (PRRs) that recognise common features referred to as pathogen or microbe-associated molecular patterns (PAMPs or MAMPs). These chemical signatures are unique to microbes, are not produced by (potential) hosts, and appear to be indispensable for microbial

fitness (Nurenberger et al. 2004). After recognition by PRRs a wide range of mitogen-activated protein kinase (MAPK) dependent signalling events occur, ultimately leading to PAMP triggered immunity (PTI). In addition to biologically induced resistance, chemicals can enhance the defence responses in plants and the amino-acid B-aminobutyric acid is well known to induce broad-spectrum pathogen resistance in a wide variety of plant species (Jakab et al. 2001; Cohen 2002).

In insects, mammals and plants a family of conserved transmembrane Toll-like receptors (TLRs) function directly or indirectly as PRRs for MAMPs. TLRs are characterised by an extracellular leucine rich-repeat (LRR) domain which is found in a variety of receptors in plants and animals. The intracellular cytoplasmatic domain of Toll is the Toll-interleukin 1 receptor referred to as TIR (Ausubel 2005).

Like insects and vertebrates, plants respond to a wide variety of pathogen molecules and the best described nematode-associated molecular patterns (NAMPs) for animal parasitic nematodes are glycan moieties present in the nematode surface coat or SE nematode products. A fraction of *Necator brasiliensis* SE fluid consisting of large glycoproteins is capable of eliciting type II immunity when injected into mice (Balic et al. 2004). NAMPS have not yet been identified for plant parasitic nematodes.

Some of the components of intracellular signal transduction induced in innate immunity are common to animal and plants cells. In plants, the production of reactive oxygen species (ROS) is one of the earliest responses after pathogen recognition and is also an important component of innate immunity in animals (Nurenberger et al. 2004). Plants reinforce the cell wall at the site of pathogen attack by lignifications, suberisation and callose deposition; the synthesis of antimicrobial compounds such as pathogenesis related (PR) proteins and phytoalexins and the transcription activation of other defence-related genes, all contribute to MAMP triggered immunity (MTI), acting at different stages of the infection (Heath 2000; Agrawal and Fishbein 2006).

A variety of *Arabidopsis* genes involved in innate immunity have been identified and confirmed to be involved in conferring pathogen resistance (Glazebrook 2001). Mutant analysis has shown that several low-molecular weight signalling molecules, including salicylic acid, jasmonic acid (JA), ethylene and nitric oxide are key in the regulation of plant innate immune pathways (de Vos et al. 2006 Glazebrook et al. 2003; van Loon et al. 2006). This response is complex, involving several parallel defence responses with cross-talk between signalling pathways at key regulatory steps (Glazebrook 2001; Kim et al. 2005; Pieterse and van Loon 2004; Thomma et al. 1998). The genes identified in these signalling pathways do not have any mammalian homologue. Nevertheless, there are various common features of innate immunity in vertebrates, invertebrate animal and plants which include defined PRRs, conserved MAPK signalling cascades and the production of antimicrobial peptides, production of active oxygen nitrogen species, calcium fluxes, activation of transcription factors and the inducible expression of immune effectors (Ausubel 2005). According to Ausubel (2005) this similarity seems to be a reflection of the overall conservation of the components of the MAPK

signalling cascades which might have appeared very early in evolution before the emergence of multicellularity.

Plants have co-evolved resistance mechanisms (resistance gene-mediated resistance) to enable them to respond quickly to pathogen determinants that suppress plant innate immunity (McDowell and Woffenden 2003; Jones and Dangl 2006). The recognition of these nematode effectors (also called avirulence factors) results in R-gene mediated resistance that often leads to localised cell death (hypersensitivity response) which stops nematode infection.

11.6.2.1 Host Immunisation Against Parasitic Nematodes

More than 2 billion people are infected with helminth parasites which are responsible for enormous levels of morbidity and mortality with delay of physical development in children and loss of work force productivity (Brindley et al. 2009) and a variety of different approaches has been adopted to produce vaccines to protect humans and animals from animal parasitic nematodes. These include artificial treatments using attenuated parasites, homogenised parasite tissue, dead parasites, soluble parasite antigens, heterologous protection or controlled live infections and gene-therapy like approaches (Chappell 1980; Brindley et al. 2009).

Animal and plant parasitic nematodes offer a unique challenge to their host system as they actively migrate through the tissues exposing a large cuticular surface that cannot be phagocytised and which changes with each moult. Molecules from the nematode surface coat together with their amphidial and oesophageal secretions are the first molecules to interface with the host's immune system and therefore must play important roles in the host-parasite interaction. Surface and secreted antigens have been the focus for vaccine development and knowledge of the functions of these molecules is an important prerequisite for their effective use in vaccine research. The *Onchocerca* spp. secreted larval protein (Wu et al. 2004) and the venom allergen homologues (VAH), a large family of extracellular proteins, have host protective potential and the latter have been found to be present in the SE products of animal and plant parasitic nematodes (Jasmer et al. 2003). These VAH are expressed in the subventral glands of plant nematodes during the early events of infection, invasion and feeding site induction (Gao et al. 2003) and have been shown to be secreted from *Ascaris caninum* during the transition to parasitism (Hawdon et al. 1996); in *H. contortus* this protein is also present in the SE products and considered to be a putative protective agent (Schallig et al. 1994). Since VAH proteins are found in fungi, plants, invertebrates and mammals and seem to occur in all nematodes, they may be important in the host parasite interaction of plant and animal parasitic nematodes (Jasmer et al. 2003).

Several investigators have reported that immunisation with certain fractions/products of the parasite facilitate parasite survival by immunosupression of the host while other products of the parasite facilitate immunostimulation and inflammatory pathology (O'Connor et al. 2003; Sahoo et al. 2009). Many modern parasite vaccines are designed to induce a Th1 response for optimal efficiency. Indeed, im-

munisation with inflammatory proteins of *B. malaya*, corresponding to 4 cytokine-stimulating SDS-PAGE resolved fractions, induces a Th1/Th2-immune response and confers protection against filarial infection (Sahoo et al. 2009).

Induced disease resistance or 'plant immunisation' is an attractive form of plant protection which is based on the activation or priming of existent resistance mechanisms in the plant leading to enhanced defence responses and improved protection as they acquire the ability to respond, or respond more strongly, to pathogen attack.

Priming for disease resistance has been described for several plant species and can be induced by various stimuli and a number of chemical treatments have been shown to 'switch' on primed defence responses against nematodes. It has been demonstrated that JA is an elicitor of plant defence responses (Devoto and Turner 2003). Although plant nematodes are able to escape plant host defences in susceptible interactions, when the production of these defence compounds was boosted by foliar application of JA the induced systemic defence response was able to reduce root-knot nematode infection in susceptible tomato plants (Cooper et al. 2005). It has been shown that exogenous application of jasmonate stimulates production of 20-hydroxyecdysone in spinach and nicotine in tobacco, compounds which are toxic to nematodes *in vitro* (Cooper et al. 2005). Other plant products such as brassinolide, jasmonates, salicylic acid and ethylene have also been used to prime defence responses to nematodes (Van Peer et al. 1991; Wei et al. 1991). Arbuscular mycorrhizal fungi, non-pathogenic rhizobacteria (Haski-Gunther et al. 1998; Santhi and Sivakumar 1997; Kempster et al. 2001; Li et al. 2006), bacterial components (Reitz et al. 2002; Van Peer and Schippers 1992) and chemicals such as the amino acid β-aminobutyric acid (Oka et al. 1999; Oka and Cohen 2001) can also enhance the defence capacity of plants against nematodes. Nurenberger et al. (2004) report preliminary research showing that pre-treatment (immunisation) with lipopolysaccharide rendered *Arabidopsis* plants more resistant against subsequent infections with virulent strains of *Erwinia carotovora* when compared with control plants treated with buffer. Reitz et al. (2002) showed the importance of rhizobial lipopolysaccharide for the induction of systemic resistance to *G. pallida*. These findings indicate that single PAMPs induced a physiological resistance state in plants.

Genome data will provide the basis for a comprehensive understanding of the molecular mechanisms involved in nematode nutrition and metabolism, host-dependent development and maturation, immune evasion and evolution. They are likely to predict new potential vaccine candidates and control targets.

11.6.2.2 Host Modulation/Signalling

Animal and plant parasitic nematodes are masterful immunoregulators, a trait which underpins their longevity in their hosts. Typically Th1/17 host immunity is blocked and productive effector responses are suppressed by animal nematodes. Molecules at the surface of plant and animal parasitic nematodes with anti-oxidant properties may play a role in suppressing or modulating the effects of host defences and striking parallels have been found between the proteins used by plant and animal

parasites to suppress defence signalling. For example, surface-associated superoxide dismutase and glutathione peroxidase are thought to neutralise oxyradical attack by their host and these enzymes are present at the surface of plant and animal parasitic nematodes (Waetzig et al. 1999; Robertson et al. 2000; Jones et al. 2004; reviewed in Hewitson et al. 2009 and Hogenhout et al. 2009). Secretions of adult stage *B. malayi* are recognised to have immunomodulatory potential and some of these secretions mimic the immunomodulatory effects of actual infection (Hewitson et al. 2009). Interestingly, the glycolytic enzyme triose phosphate isomerase (TPI) which is one of the most abundant proteins secreted by adult *Brugia* is also secreted by *M. incognita* (Bellafiore et al. 2008).

Nematode effector proteins might contribute to their virulence either by redirecting plant cellular processes to obtain more nutrients from their host or by suppressing host defences (see Chaps. 13 and 14). Numerous parasite-derived proteins including cytokine homologues, protease inhibitors, glycoconjugates, and small lipid proteins have been discovered or hypothesised to be involved in immune interference by influencing cytokine network and signal transduction pathways or inhibiting essential enzymes (Hartman et al. 1997; Gomez-Escobar et al. 1998; Harnett et al. 2004; Hewitson et al. 2009).

11.7 Concluding Remarks

Although the parallels between animal and plant parasitic nematodes discussed here are restricted by space limitations, they provide strong evidence that they have much in common. Unfortunately, at time of writing we have not yet witnessed detailed whole-genome level comparisons between animal and plant parasites and free-living forms such as *C. elegans* that could yield insights into the molecular basis of parasitism and commonalities that define the parasite lifestyle, independent of host. As next generation sequencing technologies drive the deep-mining of more and more nematode genomes and transcriptomes, we are likely to see the rapid evolution of our understanding of nematode parasites. Further, with the development of reverse genetic tools in parasites, our ability to probe and compare their biology and so drive the development of novel control strategies is likely to be enhanced dramatically. Ultimately, the exploitation of these datasets for parasite control will be the yardstick by which their usefulness is measured.

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Part III
Molecular Genetics and Cell Biology of Plant-Nematode Interactions

Chapter 12
Degradation of the Plant Cell Wall by Nematodes

Eric L. Davis, Annelies Haegeman and Taisei Kikuchi

12.1 Introduction

The plant cell wall represents a formidable barrier to ectoparasitic nematodes that feed from outside of plant tissues and to endoparasitic nematodes that must penetrate, migrate and feed within host plant tissues. The plant cell wall is a thick, rigid structure formed from an extensive network of cellulose microfibrils, hemicelluloses, pectins, and proteins that all undergo regulated changes in architecture during normal plant growth and development (Carpita and Gibeaut 1993). Two types of cell walls can be distinguished in plants—the primary wall, which surrounds actively growing cells, and the secondary wall, which is deposited as the plant cell matures. Primary walls are deposited during cell growth and need to be both mechanically stable and sufficiently extensible to permit cell expansion while avoiding the rupture of cells under turgor pressure. Primary cell walls consist mainly of polysaccharides that can be broadly classified as cellulose, the cellulose-binding hemicelluloses, and pectins. Secondary cell walls are deposited after the cessation of cell growth and confer mechanical stability upon specialized cell types such as xylem elements and sclerenchyma cells. These walls represent composites of cellulose and hemicelluloses, and are often impregnated with lignins. In addition to polysaccharides, plant cell walls contain hundreds of different proteins. Many of these proteins are considered to be 'structural' proteins (Cassab 1998), whereas others participate in cell wall remodeling and turnover (Darley et al. 2001).

The sclerotized, protrusible stylet of phytoparasitic nematodes provides a tool to mechanically breach the host plant cell wall. Such stylet activity can be readily observed for nematodes grown in monoxenic plant root culture and has been documented for both ectoparasitic and endoparasitic nematodes in video microscopy (Wyss 1987; Wyss and Zunke 1986; Wyss et al. 1992). An early body of evidence suggested that nematodes also secrete hydrolytic cell wall-degrading enzymes to

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assist in this process (reviewed in Deubert and Rohde 1971). Protein extracts and exudates from a number of phytoparasitic and fungal-feeding nematode species contained cellulolytic, amylolytic, chitinolytic, and pectolytic enzyme activity, suggesting the potential for endogenous production and secretion of cell wall-degrading enzymes from nematodes. Since the nematodes that were analyzed for this enzyme activity were reared on plant tissues and sometimes recovered non-aseptically, the true origins of the cell wall-degrading enzymes remained in question.

As studies of the proteins synthesized in the oesophageal gland cells and secreted through the stylet of tylenchid phytoparasitic nematodes (Hussey 1989) began to incorporate the developing tools of molecular biology, the first confirmation of cell wall-degrading enzymes of nematode origin emerged (Smant et al. 1998). Amino acid sequence of cyst nematode proteins that were affinity-purified using a gland-specific monoclonal antibody (De Boer et al. 1996) was used to derive the first endogenous coding sequence of endo-1,4-β-glucanases genes isolated from any animal (Smant et al. 1998). Even more remarkable was the similarity of the cyst nematode endoglucanases to bacterial members of glycosyl hydrolase (GH) family 5 (Henrissat and Bairoch 1996), providing some of the first evidence of potential horizontal gene transfer (HGT) from prokaryotes to eukaryotes (Davis et al. 2000; Keen and Roberts 1998; Smant et al. 1998; Yan et al. 1998). Complementary studies on the developmental expression patterns of the isolated cyst nematode endoglucanase genes associated endoglucanase expression with the migratory life stages of cyst nematodes (De Boer et al. 1999; Goellner et al. 2000), including redeployment of endoglucanase expression specifically within cyst nematode males that regain motility later in development. Immunolocalization of secreted cyst nematode endoglucanases along the migratory path of cyst nematode juveniles within plant root sections provided the first definitive evidence of *in planta* secretion of proteins derived from the oesophageal gland cells of phytonematodes (Wang et al. 1999).

Endoglucanase genes in a number of other phytoparasitic nematode species have subsequently been identified (see Davis et al. 2009; Kyndt et al. 2008; Mitchum et al. 2007) since the 1998 Smant et al report. The carbohydrate-binding module (CBM) present in some phytoparasitic nematode endoglucanases also exists in the absence of an endoglucanase active site, leading to the isolation of a number of non-enzymatic cell wall-modifying proteins from nematodes (see Davis et al. 2009; Kikuchi et al. 2009; Kyndt et al. 2008; Mitchum et al. 2007). Candidate gene approaches and differential gene expression analyses in phytoparasitic nematodes have also identified genes encoding a number of different pectolytic and hemicellulolytic enzymes (see Davis et al. 2009; Mitchum et al. 2007) that would provide the ability to degrade other complex carbohydrate components of the plant cell middle lamella and wall. In phytoparasitic nematodes and related species that have adapted to feed on fungi, genes encoding enzymes with the potential ability to modify the plant and fungal cell wall have also been identified (Jones et al. 2005, 2008; Kikuchi et al. 2004, 2005, 2007). The growing number of genes encoding cell wall-modifying proteins that have been isolated from phytoparasitic nematodes, fungal

feeders, and even non-parasitic nematode species has provided a framework for discussions on their evolutionary mechanisms and origins (Baldwin et al. 2004; Davis et al. 2000; Dieterich and Sommer 2009; Haegeman et al. 2010; Hotopp et al. 2007; Jones et al. 2005; Kyndt et al. 2008; Ledger et al. 2006; Mitreva et al. 2009; Scholl et al. 2003; Wasmuth et al. 2008).

12.2 Enzymatic Degradation of Plant Cell Walls

12.2.1 Cellulose

The key structural component of the plant cell wall is cellulose, the most abundant biopolymer in the world. Cellulose is composed of successive glucose residues which are inverted 180°, forming a flat ribbon with cellobiose as the repeating unit (Taylor 2008). These (1,4)-β-linked glucan chains are able to form extensive hydrogen bounds to adjacent glucan chains (Somerville 2006). Approximately 36 of these crystalline chains are arranged in parallel in 3 nm thick microfibrils forming insoluble cable-like structures. Cellulose microfibrils are among the longest molecules known in nature, since they are believed to consist of 8000 (primary cell wall) to 15,000 (secondary cell wall) glucose molecules (Somerville 2006).

Glycoside hydrolases are enzymes that catalyze the hydrolysis of the glycosidic bonds in sugar polymers. These glycosyl hydrolases are classified into different families according to their sequence similarity (Henrissat and Bairoch 1996). Cellulases or endo-1,4-β-glucanases for example are capable of degrading cellulose by hydrolyzing the (1,4)-β bonds. Several endoglucanases (or cellulases, EC 3.2.1.4) belonging to different glycosyl hydrolase families have been found in nematodes, facilitating the penetration and migration of the nematode through the plant cell wall.

Most of the identified endoglucanases in nematodes belong to glycosyl hydrolase family 5 (GHF5). GHF5 endoglucanases were found in several nematodes belonging to the superfamily Tylenchoidea (order Rhabditida, suborder Tylenchina, infraorder Tylenchomorpha) (De Ley and Blaxter 2002). The majority belong to the well studied sedentary nematode genera *Heterodera*, *Globodera* and *Meloidogyne* (Abad et al. 2008; Bera-Maillet et al. 2000; Gao et al. 2002a, 2004b; Goellner et al. 2000; Ledger et al. 2006; Rehman et al. 2009; Rosso et al. 1999; Smant et al. 1998; Yan et al. 2001). Besides these sedentary nematodes, GHF5 endoglucanases have also been identified in the migratory nematodes *Radopholus similis*, *Ditylenchus africanus* and *Pratylenchus* species (Haegeman et al. 2008; Kyndt et al. 2008; Uehara et al. 2001). The GHF5 endoglucanases consist of several domains. They all have a signal peptide, which is required to secrete the protein and a catalytic domain with the actual enzyme activity. Some endoglucanases have an additional carbohydrate binding module (CBM) at the C-terminal end of the protein, which is thought to aid the enzyme in the binding to its substrate (Boraston et al. 2004).

Interestingly, the endoglucanases present in *Bursaphelenchus xylophilus* (Aphelenchoidea superfamily) are GHF45 endoglucanases (Kikuchi et al. 2004). The presence of a different glycosyl hydrolase family suggests a different origin for endoglucanases in the Aphelenchoidea. Nevertheless, within the Aphelenchoidea a GHF5 endoglucanase has also been identified in the fungivorous nematode *Aphelenchus avenae* (Karim et al. 2009). It should however be noted that the taxonomic position of this nematode is being questioned and recent evolutionary trees suggest that it is more related to the Tylenchoidea instead of the Aphelenchoidea (van Megen et al. 2009).

Little is known about the cell wall degrading enzymes produced by phytoparasitic nematodes which belong to the Dorylaimida and Triplonchida orders. Only one dorylaimid nematode, *Xiphinema index*, has been shown to posses a GHF12 endoglucanases (Jones et al. 2005). The fact that yet another glycosyl hydrolase family is involved, confirms the independent origin of plant parasitism within nematodes (Blaxter et al. 1998).

12.2.2 Hemicellulose

Cellulose microfibrils are embedded in a matrix of complex polysaccharides, which are divided into two classes: pectins and hemicellulose. Hemicelluloses are cellulose binding polysaccharides that form a strong but resilient network together with the cellulose. More specifically, they are defined as carbohydrate polymers of either xylose, glucose, mannose or mannose and glucose joined mainly by (1,4)-β and (1,3)-β glycosidic bonds (Bacic et al. 1988). The major component of hemicellulose is xylan, the second most abundant polysaccharide in nature. Xylan is composed of (1,4)-β-linked xylopyranose units and can have various substituents and variable structures according to the plant species (Collins et al. 2005). Other components of hemicellulose, such as lichenan, contain both (1,3)-β and (1,4)-β linkages (Bacic et al. 1988).

Nematode GHF5 endoglucanases can show activity against hemicelluloses, but the activity seems limited to (1,4)-β linked polysaccharides (Gao et al. 2004a). The GHF45 endoglucanases of *B. xylophilus* are also active against glucomannan (Shibuya and Kikuchi 2008). Limited activity of some endoglucanases against lichenan has also been observed (Bera-Maillet et al. 2000; Gao et al. 2004b; Shibuya and Kikuchi 2008). Some endoglucanases are able to hydrolyze xylan, as demonstrated for endoglucanases of *Heterodera glycines* (Gao et al. 2004b). In addition, specific endoxylanase enzymes have been identified in a small number of nematodes: in *Meloidogyne* species and in *Radopholus similis* (Abad et al. 2008; Haegeman et al. 2009a; Mitreva-Dautova et al. 2006; Opperman et al. 2008). These xylanases belong to a glycosyl hydrolase family situated between GHF5 and GHF30 and have a domain structure similar to the endoglucanases consisting of a signal peptide, a catalytic domain and in some cases a C-terminal CBM (Haegeman et al. 2009a).

12.2.3 Pectin

Pectin is a major structural component of the plant cell wall, along with cellulose and hemicellulose. Pectin is located mainly in the middle lamella and primary cell wall and functions as a matrix anchoring the cellulose and hemicellulose fibres (Carpita and Gibeaut 1993). The breakdown of pectin leads to the maceration of plant tissues, the characteristic symptom of soft-rot diseases (Lietzke et al. 1994). Pectin degradation requires the combined action of several enzymes. These can be divided into two groups: pectin esterases, which remove the methoxyl groups from pectin and depolymerases (hydrolases and lyases) which cleave the backbone chain (Tamaru and Doi 2001). Phytoparasitic nematodes possess two types of depolymerase for pectin degradation: pectate lyase and polygalacturonase. No pectin esterase has been reported from phytoparasitic nematodes.

12.2.3.1 Pectate Lyase

Pectate lyase (pectate transeliminase, EC 4.2.2.2), which catalyzes the cleavage of the internal α-1,4-linkages of unesterified polygalacturonate (pectate) by ß-elimination, plays a critical role in pectin degradation (Barras et al. 1994). Pectate lyases are distributed widely among bacterial and fungal plant pathogens and have been the focus of several studies that have examined their function as virulence factors (Barras et al. 1994). They are used by plant pathogens to degrade host cell walls to allow penetration and colonisation.

Genes encoding pectate lyases have been cloned from several sedentary phytoparasitic nematodes, including species of *Heterodera*, *Globodera*, and *Meloidogyne* (Popeijus et al. 2000; de Boer et al. 2002; Doyle and Lambert 2002; Huang et al. 2005; Kudla et al. 2007; Vanholme et al. 2007), and the migratory phytoparasitic nematode *B. xylophilus* (Kikuchi et al. 2006). These pectate lyases are produced in the oesophageal gland cells and are secreted from the stylet of the nematode. Together with cellulases and hemicellulases, the pectate lyases are thought to soften the plant cell wall to facilitate migration within the plant. Knock-down of a pectate lyase gene by RNAi in *H. schachtii* J2 resulted in fewer infections (Vanholme et al. 2007), and transient expression of a *G. rostochiensis* pectate lyase in *Nicotiana benthamiana* leaves resulted in severe malformations of the infiltrated tissues (Kudla et al. 2007), indicating an important role of pectate lyase in infection and parasitism of plants.

The presence of pectate lyase genes in the non-pathogenic nematode *B. mucronatus* (Kikuchi et al. 2006) and in the fungivorous nematode *A. avenae* (Karim et al. 2009) suggested that pectate lyase is more widely distributed among nematodes that have any association with plants than was previously thought. Recent phylogenetic analyses divided nematode sequences into three different clades, indicating different origins (Kikuchi et al. 2006; Kudla et al. 2007; Vanholme et al. 2007). However, the phylogenetic distribution of nematode pectate lyases based on available data does not match the expected phylogeny of nematode families (Kudla et al. 2007). This could imply that pectate lyases have several independent origins within plant-

parasitic nematodes. However, it could also be a consequence of the fact that the number of homologues available is too small to yield robust phylogenies. The origin and evolution of the pectate lyases within nematodes will probably be clarified in the future when more sequence data becomes available.

12.2.3.2 Polygalacturonase

Polygalacturonases catalyze the hydrolysis of pectic polygalacturonic acid and release oligogalacturonides. These are classified into two classes according to their mode of action: endo-polygalacturonases and exo-polygalacturonases (Jaubert et al. 2002). A nematode polygalacturonase identified from *Meloidogyne incognita* was predicted to be an exo-type belonging to GHF28 based on its amino acid sequence (Jaubert et al. 2002). Polygalacturonase-like sequences have been identified in expressed sequence tag (EST) data for other *Meloidogyne* species, including *M. javanica*, *M. arenaria*, *M. hapla*, *M.* and *M. chitwoodi* but not in other nematode ESTs, indicating a limited distribution of the gene in nematodes. Like pectate lyases, the polygalacturonases are likely to be produced in the oesophageal gland cells and secreted from the stylet of the nematode into plant tissue to facilitate the penetration and intercellular migration of the nematode (Jaubert et al. 2002).

12.2.3.3 Arabinogalactan Endo-1,4-β-Galactosidase and Arabinase

A new type of cell wall degrading enzyme was recently identified in cyst nematodes, namely a putative arabinogalactan endo-1,4-β-galactosidase belonging to GHF53 (EC 3.2.1.89) (Vanholme et al. 2009). Although activity assays on this enzyme are lacking, it is thought to hydrolyse β-1,4-galactan in the branch regions of pectin. This could make the pectin backbone more accessible to the pectate lyases. Searches through nematode ESTs and the *Meloidogyne* genomes have failed to identify similar enzymes in nematode species other than cyst nematodes. In the genome of *M. incognita* however, two predicted proteins show homology to GHF43 arabinases (Abad et al. 2008). These proteins possibly hydrolyse the α-1,5-linkages of arabinan polysaccharides, which are present as side chains of pectin. This suggests that the arabinases could have a similar role for root-knot nematodes as the galactosidase enzymes have for cyst nematodes (Vanholme et al. 2009).

12.3 Non-Enzymatic Modification of Plant Cell Walls

A number of the known phytoparasitic nematode genes that encode cell wall-modifying proteins with non-hydrolytic activity have a CBM predicted to bind to cellulose (see Davis et al. 2009; Kyndt et al. 2008; Mitchum et al. 2007). The cellulose-binding domain was a unique feature of some of the first phytoparasitic nematode endoglucanases to be isolated (Smant et al. 1998) but soon afterwards expressed

gene analyses of *M. incognita* isolated a transcript encoding a protein with a cellulose binding domain (CBP), a linker domain found in the endoglucanases, and a novel upstream peptide sequence without a predicted glycosyl hydrolase active site (Ding et al. 1998). The Mi-CBP was expressed exclusively within the nematode oesophageal gland cells, was detected in nematode stylet secretions, and was confirmed to bind to a cellulose substrate (Ding et al. 1998). CBPs have since been identified from several phytoparasitic nematode species with the common features of a cellulose-binding domain and a signal peptide for secretion, but with variation in peptide sequence upstream of the cellulose binding domain (see Davis et al. 2009; Kikuchi et al. 2009; Kyndt et al. 2008; Mitchum et al. 2007). The predicted peptide sequence upstream of a cellulose-binding domain isolated from *G. rostochiensis* had similarity to the non-hydrolytic expansin proteins found in plants (Qin et al. 2004). Plant expansins soften cell walls by disrupting noncovalent bonds between cell wall fibrils, allowing the sliding of fibrils past each other to promote enzyme access and cell wall growth and flexibility (Cosgrove 2000). Functional assays of the Gr-EXP1 protein in plant tissues confirmed its expansin-like activity (Qin et al. 2004), representing the first report of an expansin-like protein outside of the plant kingdom. More recently, EST analyses have identified putative expansin-like sequences in several phytoparasitic nematode species including both sedentary and migratory endoparasites (Abad et al. 2008; Haegeman et al. 2009b, 2010; Kikuchi et al. 2007, 2009; Opperman et al. 2008; Roze et al. 2008). The presence of expansin domains in the absence of a CBM has been reported in *B. xylophilus* and other phytoparasitic nematode species, providing evidence for hypotheses on the evolution of the expansin gene family in nematodes (Kikuchi et al. 2009).

Similar to the function of expansins, overexpression of a bacterial CBP in plants promoted an increase in plant cell elongation (Shpigel et al. 1998). For hydrolytic proteins and expansins, the cellulose-binding domain appears to facilitate accumulation of the active sites of the proteins on the surface of the insoluble complex carbohydrates of the plant cell wall. Yeast two-hybrid analysis of an *H. schachtii* CBP with an *Arabidopsis* root library identified a plant pectin methyl esterase (PME3) as a specific interacting plant protein (Hewezi et al. 2008). The nematode CBP and plant PME were confirmed to interact within the plant host, and expression of Hs-CBP in *Arabidopsis* concomitantly increased expression of PME3. Overexpression of either Hs-CBP or PME3 in *Arabidopsis* caused an increase in root growth and increased infection by *H. schachtii*, whereas the opposite effects were observed in PME3 mutants of *Arabidopsis*. The results suggested that secreted Hs-CBP directly interacts with plant PME to facilitate parasitic establishment of the nematode within the root, potentially through targeted alteration of plant cell walls within infection sites (Hewezi et al. 2008).

12.4 Degradation of Fungal Cell Walls

Some reports have suggested that plant parasitism may have evolved, in part, from fungal feeding nematodes (Jones et al. 2005). In fact, some nematodes including *B. xylophilus* have the ability to feed on both plants and fungi, whereas others use

either plants or fungi exclusively. Identifying genes specific to these nematodes and others shared between them will provide clues to help identify parasitism genes and to understand the evolution of plant parasitism in nematodes. Here, we describe fungal cell wall degrading enzymes in phytoparasitic nematodes. The fungal cell wall contains various polysaccharides, but its main components are beta-1,3-glucan and chitin (Peberdy 1990).

12.4.1 ß-1,3-Glucan

ß-1,3-glucanases are widely distributed among bacteria, fungi, and higher plants. ß-1,3-glucanases catalyse the hydrolysis of ß-1,3-D-glucosidic linkages in ß-1,3-D-glucan. This polymer is a major component of fungal cell walls (Peberdy 1990). Nematode ß-1,3-glucanases have been identified from *B. xylophilus* and *B. mucronatus* (Kikuchi et al. 2005). Most *Bursaphelenchus* species feed solely on fungi, and all species rely on fungi as a food source at some stage in their life cycle. Only a few species, including *B. xylophilus*, have the ability to parasitize plants. The *Bursaphelenchus* ß-1,3-glucanases showed high similarity to GHF16 proteins from bacteria. Similar sequences were also identified from the fungivorous nematode *A. avenae* (Karim et al. 2009), whereas no sequences similar to ß-1,3-glucanases have been found in other phytoparasitic nematodes. The *B. xylophilus* genes are expressed solely in the oesophageal gland cells of the nematode and the protein is present in the secretions of the nematode. Therefore, the enzyme likely weakens fungal cell walls to facilitate nematode feeding (Kikuchi et al. 2005).

12.4.2 Chitin

Chitinases cleave the beta-1,4-glycosidic bonds of chitin, a beta-1,4-linked polymer of N-acetylglucosamine. Chitin is the main component of the fungal cell wall (Peberdy 1990). *Bursaphelenchus xylophilus* has several chitinase genes in its genome. Some of these belong to GH18 and have enzymatic activity. They are expressed in the oesophageal glands and are likely to be secreted from the stylet of the nematode (T. Kikuchi unpublished). The EST dataset of the fungivorous nematode *A. avenae* includes chitinase-like sequences. In these fungivorous nematodes, chitinases may facilitate the penetration of the fungal cell wall, enabling the nematode to feed on the cell contents.

Obligate phytoparasitic nematodes, which do not feed on fungi, also have chitinase genes. Hg-CHI-1 from *H. glycines* is expressed specifically in the subventral glands and is likely to be secreted from the stylet (Gao et al. 2002b). Furthermore, expression was detected only in parasitic stages, implying that it has a function in the parasitic process (Gao et al. 2002b). However, plants do not contain chitin and the role of the chitinase in *H. glycines* therefore remains to be determined (Gao et al. 2002b).

It is possible that chitinases in nematodes may serve as antifungal defences for free-living species or be involved in eggshell degradation as well as being effectors

for fungivorous nematodes. The genome sequence of *M. incognita* revealed a radical reduction in chitinases and chitin-binding proteins in the species. *M. incognita* possessed only 15 enzymes that were potentially involved in chitin degradation and binding, whereas *Caenorhabditis elegans* has 96 such enzymes (Abad et al. 2008). The suggested reason for this reduction is that, as obligate phytoparasitic nematodes including *M. incognita* spend most of their life cycles within the host plants, they may benefit from plant barriers and protection against fungi (Abad et al. 2008).

12.5 Evolutionary Aspects of Cell Wall Modifying Proteins

12.5.1 Introduction

Plant parasitism has originated at least three times independently during nematode evolution: in the Tylenchomorpha, Triplonchida and Dorylaimida (Blaxter et al. 1998). Remarkably, it was recently proposed that endoparasitism within the Tylenchomorpha has evolved at least ten times from ectoparasitism: resulting in migratory endoparasitism on six occasions and in sedentary endoparasitism on four occasions (Holterman et al. 2009). Sedentary endoparasitism appears to have evolved directly from migratory endoparasitism in the case of *Meloidogyne* spp only (Bert et al. 2008; Holterman et al. 2009). During evolution, plant-parasitic nematodes have adapted well to the challenges posed by invading and parasitising their host by acquiring plant cell wall modifying proteins. The apparent separate evolutionary paths of different nematode species suggest that differences in the arsenal of parasitism genes probably exist between different nematodes with comparable lifestyles. An example of this difference is the presence of an arabinogalactan endo-1,4-β-galactosidase in cyst nematodes of the genus *Heterodera*, while the *Meloidogyne* genomes lack this enzyme (Vanholme et al. 2009). In contrast to root knot nematodes, no endoxylanases were found in cyst nematodes to date. However, endoglucanases in cyst nematodes were found to exhibit activity towards xylan, which could circumvent the apparent absence of xylanases (Gao et al. 2004b). A similar trend was observed in the genomes of plant pathogenic bacteria, where strikingly different numbers and combinations of genes encoding cell wall degrading enzymes occur (Van Sluys et al. 2002). These genus- or species-specific adaptations towards plant parasitism are essential to understand the evolution of plant parasitism.

12.5.2 Evolutionary Mechanisms

Most cell wall-modifying enzymes exist in multigene families that originated through extensive gene duplication. Each copy of the gene can subsequently undergo functional specialization and evolve a specific expression pattern. For example, the expression patterns of the endoglucanases of *H. glycines* and *Radopholus similis*

revealed that some gene copies are expressed during early stages, while others are expressed exclusively in later stages (Gao et al. 2004b; Haegeman et al. 2008). Due to the relatively high amount of nematode sequence data for GHF5 endoglucanases, several hypotheses on the evolution of these genes have been put forward. A model proposed by Ledger et al. (2006) was adapted and extended by Kyndt et al. (2008). The evolution of the endoglucanases seems to reflect the species evolution: similar relationships between species have been found comparing rRNA genes. This apparent parallel gene and species evolution suggests that the ancestral endoglucanase emerged early in the evolution of Tylenchomorpha and that this ancestral endoglucanase must have included a CBM. The gene family clearly became extended at an early stage during evolution, since some endoglucanases have an aberrant gene structure as a result of an early duplication event (Kyndt et al. 2008). Later during evolution, additional duplication events occurred and some gene copies have lost their CBM. The constantly ongoing evolution of endoglucanases is confirmed by the finding of several endoglucanase pseudogenes in *Ditylenchus africanus* (Haegeman et al. 2010).

In other genes, such as endoxylanase, a similar evolutionary pattern can be expected. Although this gene family is less extensive (6 copies in the *M. incognita* genome, compared to 21 copies for endoglucanase; Abad et al. 2008), duplication events have also expanded the gene family. Since the introns of *M. incognita* and *R. similis* endoxylanases are in the same position, both most probably originated from a common ancestral endoxylanase (Haegeman et al. 2009a).

It also seems that genes can acquire domains from other genes through domain shuffling. Expansin-like genes of *D. africanus* and *G. rostochiensis* for example possess a CBM that could have been derived from endoglucanases by domain shuffling (Haegeman et al. 2010; Kudla et al. 2005). Moreover, cellulose binding proteins (CBPs) are similar to CBMs, and could have originated from endoglucanases by the loss of the catalytic domain (Ledger et al. 2006). These CBPs were shown to activate a plant pectin methylesterase, and are therefore thought to make the plant cell wall more accessible to cell wall degrading enzymes (Hewezi et al. 2008). This illustrates the plasticity of the genes to adapt to the nematode's needs, and reflects the complexity of the evolution of these gene families.

12.5.3 Origin Through Horizontal Gene Transfer

One of the most remarkable findings concerning cell wall modifying proteins from phytoparasitic nematodes is that some of these are absent from all other nematodes and most other animals studied to date (Jones et al. 2005). Many of the corresponding genes resemble bacterial sequences, suggesting that these genes could have been acquired from bacterial plant pathogens through horizontal gene transfer (HGT). For example the nematode endo-1,4-β-glucanases from the Tylenchomorpha, which belong to GHF5, show very little similarity to eukaryote endoglucanases but are similar to bacterial sequences. This has led to the conclusion that these enzymes

were at some point in evolution acquired from bacteria, and that later extensive gene duplication resulted in gene families. In animal-parasitic nematodes and insects, it was proven that genes derived from their bacterial symbiont *Wolbachia* are present and transcriptionally active in the genome of the nematode or insect (Dunning-Hotopp et al. 2007). Since a *Wolbachia*-like symbiont was recently discovered in a phytoparasitic nematode species as well (Haegeman et al. 2009c), ancestors of these symbionts could be the origin of some of the cell wall modifying proteins.

Remarkably, the GHF45 endoglucanases from *B. xylophilus* show the highest homology to fungal sequences. Since *B. xylophilus* is a facultative fungal feeder, it makes sense that this gene was acquired from fungi (Kikuchi et al. 2004). Moreover, an endo-1,3-β-glucanase (GHF16) is present in *B. xylophilus* and the fungal feeder *B. mucronatus* as well as in *A. avenae* with sequence characteristics that suggest it was acquired by HGT from bacteria (Karim et al. 2009; Kikuchi et al. 2005). It is possible that these nematodes acquired endo-1,3-β-glucanase genes from bacteria to obtain a fungal feeding ability, and a subgroup subsequently acquired cellulase genes from fungi, which permitted them to parasitize plants (Jones et al. 2005).

However, the horizontal gene transfer hypothesis should be handled cautiously. The problem is that there are no strict objective rules about how to test whether a given gene was acquired from another non-related organism via HGT. The most commonly used—and in many cases the only—reason to claim that genes are of HGT origin is that no homologous genes can be found in other eukaryotes, only in bacteria or fungi (Mitreva et al. 2009). It will probably become more clear in the future when more sequence data will become available, although endogenous GHF5 and GHF45 endoglucanases have also been found in insects and molluscs (Girard and Jouanin 1999; Lee et al. 2004; Sugimura et al. 2003; Xu et al. 2001). Moreover, a putative GHF5 endoglucanase was recently discovered in the free-living nematode *Pristionchus pacificus*, and was proposed to be a pre-adaptation towards plant parasitism (Dieterich et al. 2008). It should be noted that this putative GHF5 endoglucanase has a very low similarity to GHF5 endoglucanases from plant-parasitic nematodes, and specific functional tests for this putative enzyme are lacking. Therefore, no preliminary conclusions should be made about the possible presence of endoglucanases in free-living nematodes. Nevertheless, to determine if HGT actually is the case, a combination of methods should be proposed that together may lift the weaknesses of the individual approaches. For example, a combination of phylogenetic methods, analysis of the distribution pattern, and habitat overlap between inferred donor and recipient could be applied (Mitreva et al. 2009).

12.5.4 Cell Wall Modifying Proteins and Plant Defense

Although cell wall modifying enzymes are of vital importance for the successful penetration and migration of nematodes through the plant cell wall, only a small part of the nematode transcriptome consists of cell wall modifying enzymes. This could be the result of a long co-evolution between parasite and host. During evolution, it

is likely that plants have learned to recognize nematode produced cell wall modifying proteins to trigger early plant defense responses. In oomycetes, CBMs may also act as an elicitor of defense responses in plants (Dumas et al. 2008). It is not yet clear if this is a general phenomenon of CBMs released by plant pathogens and to what extent nematode CBMs induce, either directly or indirectly through cell wall modifications, defense responses. Bacterial endoxylanases can likewise trigger plant defense systems (Belien et al. 2006). Moreover, nematode expansin-like proteins show significant similarity to putative avirulence proteins and pathogenicity factors, suggesting that expansin-like proteins may also be recognized by the plant. In the case of bacteria and fungi, the resulting defense responses can include the production of plant inhibitors of fungal and bacterial cell wall modifying enzymes, such as a polygalacturonase inhibiting protein or a xylanase inhibiting protein (Juge 2006). Whether or not these or similar inhibitors are active against nematode cell wall modifying enzymes remains to be elucidated, but the high similarity of nematode enzymes to bacterial enzymes is an important indication that the nematode enzymes may also be targeted by the plant inhibitors. The delicate balance between nematode secreted proteins and plant defense responses has probably led to a careful and economic selection of secreted cell wall modifying proteins by the nematode during evolution.

12.6 Concluding Remarks

The identification of endogenous genes encoding multiple types of cell wall-degrading enzymes in phytoparasitic nematodes has confirmed early physiological evidence for their expression and potential roles in plant parasitism (reviewed in Deubert and Rohde 1971). Since the initial identification of endoglucanase genes in cyst nematodes (Smant et al. 1998), both a candidate gene approach and extensive EST analyses have been the primary means of gene identification. The relatively recent release of the genome sequence of two root-knot nematode species (Abad et al. 2008; Opperman et al. 2008) and other genome projects currently in progress are providing a more global view of the extent and organization of gene families encoding endogenous cell wall-modifying proteins in phytoparasitic nematodes. The genome sequences of both *M. incognita* and *M. hapla* have not only confirmed the presence of multiple cell wall-modifying genes that were found in expressed sequence analyses, but they have revealed how unexpectedly large some of these gene families are, most notably the genes encoding pectolytic enzymes and expansin-like proteins (Abad et al. 2008; Opperman et al. 2008). The genomic sequence of *M. incognita* (Abad et al. 2008) has identified some previously undiscovered glycohydrolase genes (GHF32, GHF43) in root-knot nematodes (or any metazoan), but it remains unclear if these genes play a role in plant parasitism or other physiological process within nematodes. The genomic organization of pectate lyase genes in *M. hapla* (Opperman et al. 2008) suggests a clustering and potential local expansion of this gene family within the genome, consistent with earlier reports of apparent

endoglucanase gene duplication and inversion in the genome of cyst nematodes (Yan et al. 2001).

The existence of gene families that encode cell wall-modifying enzymes in nematodes presents the potential for functional redundancy, although the biological significance of this potential remains unclear. The partial reductions in nematode parasitic success observed using RNAi targeted to transcripts encoding nematode cell wall-degrading enzymes (Chen et al. 2005; Haegeman et al. 2009a; Rosso et al. 2005; Vanholme et al. 2007) suggest that such functional redundancy may be real, although the level of sequence complementarity and expression required for complete target gene silencing combined with technical challenges of the RNAi assays presents inherent difficulties in interpretation of the results. The reductions in parasitism observed in the RNAi experiments (Chen et al. 2005; Rosso et al. 2005), however, present convincing evidence that the secretion of cell wall-degrading enzymes plays a functional role in successful plant parasitism by nematodes. Expression of cell wall-degrading enzymes in sedentary females of *M. incognita* (Rosso et al. 1999), perhaps to loosen plant tissues for egg-laying, and potential other functional roles of nematode cell wall-modifying enzymes may be realized upon further analyses. To date, however, the expression of nematode cell wall-modifying enzymes is almost exclusively localized within the oesophageal gland secretory cells and developmentally consistent with the putative functional role of these secretions in migratory life stages of phytoparasitic nematodes.

One of the most significant themes arising from all of the current analyses of cell wall-modifying genes in phytoparasitic nematodes is the mounting evidence to support the potential role of horizontal gene transfer from microbes in the evolution of plant parasitism by nematodes (Davis et al. 2000; Dieterich and Sommer 2009; Jones et al. 2005; Kyndt et al. 2008; Mitreva et al. 2009; Scholl et al. 2003; Wasmuth et al. 2008). An interesting observation is the apparent association of type of endogenous nematode cell wall-modifying enzyme with the microbial community present in a given nematode ecological niche. The abundance of GHF5 enzymes with similarity to genes present in bacteria found within the environment of soil-dwelling nematodes versus the presence of GHF45 enzymes in *B. xylophilus* that are similar to those of the fungi found in its niche appears to have evolutionary relevance. Although the potential physical mechanisms of such proposed gene transfer remain elusive and difficult to definitively confirm, evidence for potential avenues of horizontal gene transfer of genes encoding cell wall-modifying enzymes is emerging. The recent discovery of a *Wolbachia* endosymbiont in *R. similis* (Haegeman et al. 2009c) presents such a potential for prokaryotic gene transfer and is supported by evidence from *Wolbachia* symbionts of other nematode and metazoan species (Hotopp et al. 2007). The presence and expression of endogenous GHF5 enzymes in the genome of *P. pacificus* (Dieterich and Sommer 2009; Dieterich et al. 2008) is fascinating and presents a potential model of an ancient ancestor to phytoparasitic nematodes that may have acquired this gene from endosymbiotic or environmental microbial sources. Caution not to overinterpret the role of HGT in evolution of parasitism is given by the monophyletic nature and apparent ancient eukaryotic origin of GHF9 enzymes in a wide spectrum of metazoan taxa (Davison

and Blaxter 2005). Interestingly, GHF9 enzymes were not identified within the Nematoda through this study (Davison and Blaxter 2005), further suggesting an alternative, microbial origin of genes encoding cell wall-modifying enzymes in the evolution of plant parasitism by nematodes.

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Chapter 13
Suppression of Plant Defences by Nematodes

Geert Smant and John Jones

13.1 Plant Parasitic Nematodes as Biotrophic Pathogens

Plant parasitic nematodes (PPN) parasitise plants using a wide range of feeding strategies. Some PPNs, including many ectoparasites and migratory endoparasites, are herbivores that simply feed on plant tissues as they are encountered (see Chap. 1). However, some of the most economically damaging PPNs, including the root-knot and cyst forming nematodes are biotrophic and induce complex feeding structures within their hosts. These nematodes rely on a single feeding site for all the nutrients required for development to the adult stage and these structures therefore have to be kept alive for a period of up to six weeks. This period of biotrophy is comparable to that of the "classical" biotrophic fungi and oomycetes such as rusts and powdery mildews and is considerably longer than the biotrophic phases of the life cycles of many other plant pathogens.

All biotrophic pathogens must suppress host defences (see below) but the importance/relevance of this aspect of plant nematology has, until recently, been underestimated. However, the ability to induce a feeding site without the host detecting the nematode or the process of induction is a key moment in the nematode life cycle. A nematode juvenile is an individual as opposed to being a rapidly dividing microbial coloniser and it has only one attempt at inducing the feeding structure. Failure of this process is catastrophic for the genotype. Once the structure has been induced it needs to be protected for the duration of the life cycle. Nematodes will therefore have been under strong selection pressure for the ability to effectively suppress host defences.

In addition to suppressing host defences that target the feeding site, endoparasitic nematodes have also evolved a range of mechanisms that protect the nematode from direct attack by the host. In this Chapter we review the current state of knowledge of both of these processes.

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13.2 Protection of the Feeding Site (Biotrophy)

The dependency of biotrophic nematodes on fully functional feeding structures is a vulnerability that is clearly demonstrated when nematodes fail to suppress innate immunity in resistant host plants. In crop cultivars resistant to nematodes a feeding structure often turns into a battlefield where the plant, which is not reluctant to use chemical warfare or other weapons of mass destruction, kills its own cells to first isolate and subsequently exterminate the invader. This quick and deadly response—referred to as a hypersensitive response (HR)—is highly specific to certain genetic lineages of the intruder species, with the code for this recognition specificity locked in the resistance genes of the host plant (see also Chap. 22; Mur et al. 2008). In this chapter we will only address active defence responses in host plants which, as opposed to constitutive physical and chemical barriers in plant tissues, only occur following specific recognition of a nematode.

The specific recognition of an intruder is of key importance to plant innate immunity, as unintended triggering of defence responses leads to costly sacrifice of the plant's own cells and tissues. Most of our current understanding of recognition specificity in plant innate immunity derives from insights of plant responses to pathogenic bacteria. However, we are beginning to gather experimental evidence indicating that at least some of the same principles apply to nematode-plant interactions.

Plant pathogens betray their presence to plants with molecules present on their exterior. These molecular signatures are generally referred to as pathogen-associated molecular patterns (PAMPs; Chisholm et al. 2006). Plants use cell surface receptors—the so-called pattern recognition receptors—to survey the apoplast for PAMPs and, when these are detected, to trigger PAMP-triggered immunity (PTI; Zipfel 2009 and Fig. 13.1). The literature on bacterial and fungal plant pathogens suggests that PAMPs have a number of characteristic features (Bent and Mackey 2008). First, PAMPs are mostly parts of structural molecules that are essential for infectivity, reproduction and/or survival of the pathogen. Secondly, because of their importance for pathogen fitness, molecules carrying PAMPs are not easily lost or changed by mutations, which explains why PAMP-triggered immunity is relatively durable. Thirdly, as direct consequence of their evolutionary stability PAMPs are often conserved across taxa, and PAMP-tiggered immunity is therefore often effective against a range of distantly related pathogens.

A classical example of a PAMP is the 22 amino acid epitope in flagellin (flg22), a structural protein in bacterial flagella (Zipfel et al. 2004). Flg22 from both plant and animal pathogenic bacteria can activate PAMP-triggered immunity in plants. Similarly, several structural components of fungal cell walls have been shown to elicit PAMP-triggered immunity (Shibuya and Minami 2001). To date little is known of nematode molecules acting as PAMPs in plants. A recent paper reported a PAMP-triggered immune response to derivatives of chitins and it suggested that cuticular chitins of plant-parasitic nematodes may act as PAMPs (Libault et al. 2007). Although chitins are present in nematode eggshells, the cuticle of plant-parasitic

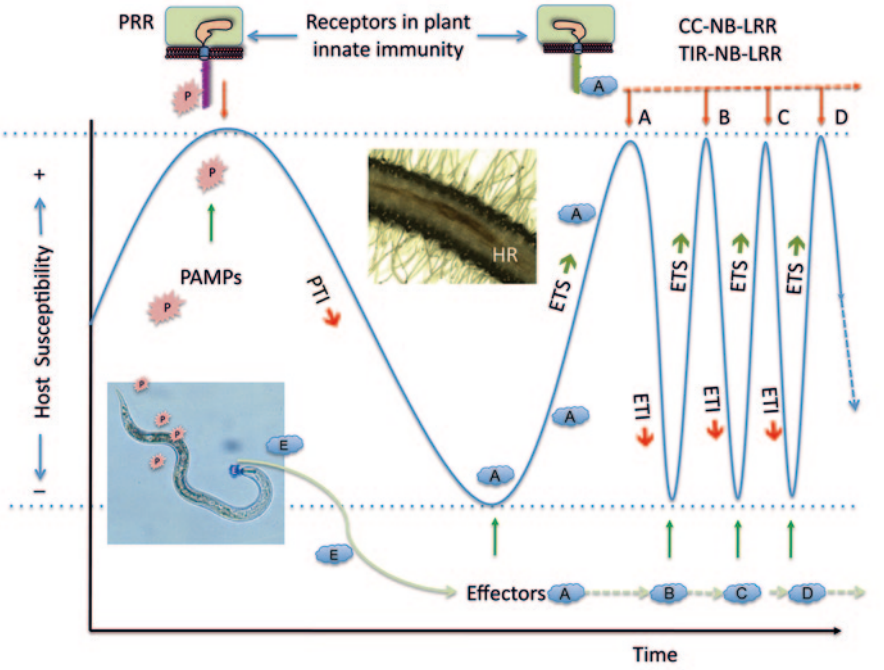

Fig. 13.1 Zig-zag-zig model as applied to plant-nematode interactions. *PTI*—pathogen (or PAMP) triggered immunity; *ETI*—effector triggered immunity; *PRR*—pattern recognition receptor; *ETS*—effector triggered susceptibility; *HR*—hypersensitive response. (Jones and Dangl 2006)

nematodes is not thought to contain chitins. However, chitin may be present in the stylet of plant parasitic nematodes and plants may respond to insertion of the stylet by producing callose in the region of stylet insertion (Golinowski et al. 1997). Local callose deposition is a hallmark feature of PTI in response to bacterial and fungal pathogens where it may help strengthen cell walls and isolate the pathogen. Further investigations into this phenomenon are required in order to shed light on these and other conserved, structural components of nematodes acting as PAMPs and comparative studies with animal parasites may be useful in this respect (see Sect. 13.3 below).

The second category of molecules capable of activating plant innate immunity are degradation products of plant cells that are produced as a result of the activity of invading pathogens (Lotze et al. 2007). These damage-associated molecular patterns (DAMPs) are important elicitors of defence responses to pathogens in metazoans. The induction of defence responses following the exogenous application of breakdown products of pectic polysaccharides from plant cell walls is one of a few examples of the possible role of DAMPs in plant innate immunity (Denoux et al. 2008). Biotrophic nematodes secrete a wide repertoire of cell wall degrading enzymes during host invasion, which are likely to generate abundant cell wall fragments that could act as danger signals to the plant (see Chap. 12). Earlier work on the

host tissue damage caused by invading biotrophic nematodes shows that invading nematodes do induce a response in plants, which leads to elevated levels of fluorescent polyphenolic compounds (Grundler et al. 1997). It is not clear whether this response in cells along the migratory tract of the nematode genuinely represents a type of DAMP-triggered immunity, nor is it clear whether DAMP-triggered immunity is a significant factor in nematode susceptibility. It is possible that invading nematodes which move through the host plant are simply able to outpace DAMP-triggered immunity. It is remarkable, and perhaps significant with regard to the release of possible DAMPs, to see the abrupt change in behaviour that occurs in biotrophic nematodes as they change from migration to feeding site initiation. During migration cyst nematodes move brutally through cell walls but shift behaviour to show a subtle probing of cells and careful perforation of cell walls that become part of the feeding structure (see Chap. 4). As the parasite undergoes the transition from migratory to sedentary lifestyle and becomes completely dependent on a single feeding structure, it seems that it can no longer afford to ignore possible release of DAMPs.

Bacterial and fungal pathogens have evolved a wide range of effectors including molecules that they release into the apoplast or deliver into the cytoplasm of plant cells to suppress PTI. In contrast to PAMPs/DAMPs, these effectors are deliberately secreted by the pathogen to render the plant susceptible to infection. Not surprisingly, the evolution of specific suppressors of PTI in pathogens has led to novel immune receptors in plants capable of activating effector-triggered immunity (ETI). Most of these receptor proteins have a nucleotide-binding domain and a leucin-rich repeat domain (NB-LRR; Fig. 13.1). While PTI affects diverse species displaying a conserved PAMP, ETI is highly specific to certain strains of a pathogen species secreting a unique effector. However, effector-triggered immunity is also vulnerable to manipulation as pathogens evolved ‘next-generation’ effectors that suppress ETI. There is now ample evidence of repeated cycles of complementary innovations in plant immune receptors and in effectors of pathogens, which has lead to the conceptual zig-zag-zig model describing the amplitude of host susceptibility in relation to a multilayered innate immunity in plants (Fig. 13.1; Jones and Dangl 2006).

Does the zig-zag-zig model apply to nematode-plant interactions? So far, the experimental evidence to validate the model in nematode-plant interactions is scarce because the importance of plant innate immunity has long been undervalued in our field. PAMP-triggered immunity, as a first layer of defence of plant innate immunity, is largely *terra incognita* for the plant nematology community. Salicylic acid (SA) is an important defence signalling molecule and mutants of *A. thaliana*, deficient in various parts of the SA production or perception and signalling pathways support higher numbers of cyst nematodes (Wubben et al. 2008). Analysis of nematode infection of these mutants suggested that nematodes may suppress local SA signalling in roots. In addition, ultrastructural responses of *A. thaliana* to invasion of nematodes for which it is not a host include many responses typical of PTI including callose production, cell wall thickening and production of active oxygen species (Waetzig et al. 1999). These data suggest that PTI may indeed be important in plant nematode interactions.

By contrast, the importance of ETI as a key factor in nematode resistance in cultivated plants has been clear for some decades. Several major resistance genes encoding ETI receptors with recognition specificity to populations of biotrophic nematodes have been found in a wide range of crops and many of these have been mapped or cloned (see Chap. 22). However, the identification of the matching nematode effectors (the avirulence genes) has proven to be more challenging. At present three nematode effectors have been linked experimentally to ETI. The first example is the root-knot nematode gene *Mi-Cg1* which is required for the *Mi-1.2* resistance gene to confer resistance in tomato to *Meloidogyne incognita* (Gleason et al. 2008). The nature of *Mi-Cg1* is somewhat puzzling as knocking-down the *Mi-Cg1* gene by RNA interference renders avirulent individuals virulent, suggesting that its product is indeed an effector recognised by the *Mi-1.2* receptor protein. However, the *Mi-Cg1* transcript does not seem to encode a secretory protein capable of interacting with an immune receptor. It is possible that this transcript is involved in regulation of an effector that is recognised by *Mi-1.2*. It has also been suggested that a protein secreted from the amphids of *M. incognita* is an avirulence gene recognised by *Mi-1.2* (Semblat et al. 2001). cDNA-AFLP analysis was used to compare near isogenic lines of *M. incognita* virulent and avirulent against *Mi-1.2* and a novel gene (*Map-1*) was identified encoding a secreted protein. This protein may be a member of a gene family and expression analysis suggested that variants of the protein containing fewer repeat sequences are expressed only in avirulent nematode species/lines. However, no experimental analysis demonstrating the generation of a hypersensitive response in plants with the *Mi-1.2* gene in response to the nematode protein has been forthcoming. A more complete example of a nematode effector linked to ETI is the *Gp-RBP-1* SPRYSEC gene from *Globodera pallida* (Sacco et al. 2009). Transient co-expression of *Gp-RBP-1* and the nematode resistance gene *Gpa-2,* which encodes a CC-NB-LRR type immune receptor, in leaf tissues induces a specific HR, making this effector the likely cause of avirulence in nematode populations. Interestingly, *Gp-RBP-1* variants capable of inducing a *Gpa-2* dependent HR have been found in virulent nematode populations, suggesting that these virulent nematodes have evolved other effectors that suppress *Gpa-2* mediated ETI. Resolving the identity of such nematode suppressors of innate immunity in plants is one of the key objectives of several research programmes at the moment. Although further details of these suppressors are required, these examples suggest that at least part of the zig-zag-zig model is relevant to innate immunity to nematodes in plants.

If protection of the feeding structure against PTI and ETI responses is indeed critical for long-lived biotrophic nematodes, how do they suppress plant innate immunity? In other words, what are the kinds of activities we may expect to find in nematode effectors suppressing PTI and ETI in feeding structures? To address these questions the scientific literature on bacterial and fungal/oomycete plant pathogens is a source of inspiration (Hein et al. 2009; Ellis et al. 2009; Göhre and Robatzek 2008). These pathogens have evolved effectors that intercept PTI/ETI signalling at essentially all crucial stages in order to suppress innate immune responses.

One pathogen effector that operates at the very top level of the signalling cascade by intercepting the PAMP signals that trigger PTI is the Ecp6 effector of *Clado-*

sporium fulvum. This protein includes a LysM domain capable of binding chitin oligosaccharides (De Jonge and Thomma 2009). Fungal colonization of plants is often associated with a release of chitin oligosaccharides and these chitin oligosaccharides are perceived as PAMPs by cell surface receptors containing LysM. It is thought that the LysM domain in Ecp6 outcompetes the LysM domains in PTI receptors for binding of chitin derivatives, preventing recognition of the PAMP by its receptor, and avoiding activation of PTI.

One step further down the PTI signalling cascade, the AvrPtoB effector of *Pseudomonas syringae* pv. *tomato* DC3000 acts on the turnover rate of the PAMP receptor FLS2 in *A. thaliana* (Göhre et al. 2008). FLS2 is a cell surface receptor that recognizes the flg22 epitope of bacterial flagellin, and upon detection of the flg22 it activates PTI after being internalised into the host cell. AvrPtoB is a modular protein with a substrate-binding domain at its N-terminus, while its C-terminus exhibits E3 ubiquitin ligase activity. Plant E3 ligases covalently attach ubiquitin monomers to lysine residues in target molecules, thereby directing these targets to the protein degradation machinery and thus controlling the lifetime of these molecules (Craig et al. 2009). AvroPtoB therefore hijacks the host's own protein turnover system as binding of AvrPtoB to FLS2 targets this receptor, as well as other receptor proteins, to the plant cell ubiquitin-dependent proteasomal degradation pathway. AvrPtoB may thus reduce the levels of cell surface receptors involved in PTI signalling, and so suppress PTI (Spallek et al. 2009). The ability to exploit the ubiquitination pathway in order to control levels of host proteins is particularly remarkable for *P. syringae*, given that bacteria do not possess the ubiquitination system within their own cells.

One of the best characterised examples of the zig-zag model in operation comes from the *P. infestans*—potato pathosystem. *Phytophthora infestans* secretes a 10KDa sterol binding protein, INF1, into plants during infection. This protein is recognised as a PAMP in various plants including *N. benthamiana* (Kamoun et al. 2003), which responds to the presence of the protein with features typical of PTI including callose deposition as well as a strong non-host cell death response (Hann and Rathjen 2007). Many *P. infestans* effectors have been identified through EST and genome sequencing projects (Hass et al. 2009) including a large gene family identified by the presence of a conserved motif which mediates translocation into host cells (Whisson et al. 2007). Several Avr genes have been identified from this gene family, including Avr3a the product of which is recognised by the R3a resistance protein (Armstrong et al. 2005). When expressed in plants Avr3a suppresses the cell death response generated by exposure to INF1 (Bos et al. 2006). Avr3a is therefore an effector that suppresses PTI and which is itself recognised by a resistance gene and acts as a trigger for ETI. *Phytophthora infestans* has, of course, responded to the pressure imposed by the presence of the R3a resistance gene. Two alleles of Avr3a are present in *P. infestans* populations and only one of these, known as Avr3a KI, is recognised by R3a. The other allele, Avr3aEM, is identical in sequence expect for two amino acid changes (KI to EM at positions 80 and 103) but evades recognition by R3a. The Avr3aEM protein is also impaired in the ability to suppress the response to INF1, suggesting functional redundancy in terms of the suppression of INF1 induced PTI.

Several bacterial and fungal suppressors of ETI signalling also work on recognition complexes built of immune receptors and effectors (Hein et al. 2009; Ellis et al. 2009; Göhre et al. 2008). To understand the mechanisms of these suppressors it is important to realize that most ETI receptors (i.e. resistance or R proteins) do not recognise matching effectors by physically interacting with them as in a ligand-receptor model (Dangl and Jones 2001). Despite significant efforts, experimental support of a direct interaction with pathogen effectors has been found for only a few ETI receptors, suggesting that a direct interaction is rather an exception (Jia et al. 2000; Kim et al. 2002). It is therefore believed that most of the ETI receptors indirectly detect pathogen effectors by the perturbations these effectors bring about to other plant proteins. Although the identities and the biological functions of host proteins targeted by bacterial and fungal effectors are mostly still elusive, it is assumed that these pathogens target them to enhance their virulence. ETI receptors are therefore likely to act as guards of key nodes in disease signalling pathways, whose positive or negative regulation contributes to the virulence or aggressiveness of the pathogen (i.e. the guard model; Dangl and Jones 2001).

One of the best-characterized interactions to illustrate the guard model involves *P. syringae* effectors and the RPM1-interacting protein 4 (RIN4) in *A. thaliana*. *Arabidopsis thaliana* overexpressing RIN4 respond less to both flg22 exposure and challenges with *P. syringae,* while *rin4* loss-of-function mutants show enhanced basal defence responses (Kim et al. 2005). Based on these findings, RIN4 is believed to be a negative regulator of plant innate immunity. Two effectors, AvrRPM1 (from *P. syringae* pv. *maculicola*) and AvrB (from *P. syringae* pv. *glycinae*), have been shown to physically interact with RIN4, which leads to hyperphosphorylation of the RIN4 protein. Plants carrying the RPM1 immune receptor respond AvrRPM1 and AvrB-induced modifications to RIN4 by activating ETI against *P. syringae* carrying these effectors (Mackey et al. 2002). AvrRpt2 (from *P. syringae* pv. *tomato*), which cleaves RIN4, is capable of suppressing the RPM1-induced hypersensitive response as a next generation effector (Kim et al. 2005b). However, *A. thaliana* has evolved yet another immune receptor named RPS2 which monitors AvrRpt2-induced cleavage of RIN4. And so, the upper hand in the fight for survival repeatedly changes from plant to pathogen, and vice versa.

Do plant-parasitic nematodes secrete suppressors of PTI/ETI signalling? As mentioned above, some lines of *G. pallida* expressing effectors that induce a *Gpa2* resistance gene dependent HR are virulent on potato plants carrying the *Gpa2* resistance gene (Sacco et al. 2009). This finding suggests that these nematodes do indeed secrete suppressors of ETI. A candidate ETI suppressor is SPRYSEC19 of *G. rostochiensis*, which physically binds to the LRR domain of SW5F—a CC-NB-LRR receptor protein of tomato (Rehman et al. 2009). Transient co-expression of SW5F and SPRYSEC19 in *N. benthamiana* does not induce a HR and tomato genotypes harbouring the *SW5F* gene are not resistant to nematode expressing the SPRYSEC19 protein. Thus binding of SPRYSEC19 to SW5F does not evoke a classical defence response. One of the alternative hypotheses currently being tested is that SPRYSEC19 interacts with SW5F and possibly other CC-NB-LRR receptor proteins to suppress the activation of ETI by these proteins.

There are two remarkable extensions of the guard model that are currently under investigation. First, it seems that one immune receptor may simultaneously guard different host proteins that are targeted by pathogen effectors. For example, in pepper AvrBs3 from *Xanthomonas campestris* pv. *vesicatoria* regulates transcription of plant genes harbouring a *upa*-box (Kay and Bonas 2009). AvrBs3 is a transcription-activator like effector that causes cellular hypertrophy via the master regulator of cell size *upa20* carrying the *upa*-box in its promoter sequence (Kay et al. 2007). The pepper resistance gene *Bs3* also includes an *upa*-box in its promoter which specifically regulates the expression of Bs3 in response to AvrBs3 from *X. campestris*. Secondly, effectors from diverse and unrelated pathogens may have the same host protein as operational virulence target. In this case the plant's deployment of a single receptor is made even more effective, by focussing its surveillance on signalling proteins essential for the virulence of many different pathogens. Several examples of this have been characterised in the literature. The resistance gene *Mi-1.2* confers resistance against a wide range of pathogens including *M. incognita*, the potato aphid *Macrosiphum euphorbiae,* the tomato psyllid *Bactericerca cockerelli* and the white fly *Bemisia tabaci* (Vos et al. 1998; Nombela et al. 2003; Casteel et al. 2006). Although little is known about the pathogen avirulence genes in any of these interactions it is likely that *Mi-1.2* guards a plant factor which is targeted by all three pathogens. One example of multiple pathogens targeting the same host virulence target is provided by RCR3. RCR3 is a tomato cysteine protease which is required for successful function of the resistance gene *Cf2* against the fungal pathogen *Cladosporium fulvum* (Dixon et al. 2000). *C. fulvum* secretes an effector, Avr2, which is a proteinase inhibitor that suppresses normal activity of RCR3 (Rooney et al. 2005). A recent study has shown that an unrelated pathogen, the oomycete *P. infestans,* also secretes proteinase inhibitors (EPIC1 and EPIC2B) targeting RCR3 (Song et al. 2009). Plants may therefore have evolved a highly efficient system for ETI which uses a limited set of immune receptors (about 150 in *A. thaliana*) to guard a number of key operational virulence targets, which may, in turn, be targeted by a range of unrelated pathogens.

Although we do not fully comprehend ETI activation and suppression in nematode-plant interactions, the final outcome of ETI signalling—a hypersensitive response—nonetheless dramatically affects the quality of the feeding structure (Rice et al. 1985; Paulson and Webster 1972). The hypersensitive response is the end stage, and perhaps a side product, of the chemical warfare deployed by plants as part of their innate immunity (Mur et al. 2008). Minutes after ETI signalling is elicited, plant cells respond with the generation of reactive oxygen species, lipid peroxidation, changes in intracellular redox states and calcium levels. Shortly after this initial response, transcription of a group of genes collectively referred to as defence genes (e.g. pathogenesis-related proteins, phytoalexin-synthesizing proteins, and key enzymes of the secondary metabolic pathways) is switched on. And finally the hypersensitive response emerges as a cellular phenomenon as a type of programmed cell death largely following a fixed sequence of steps resulting in disintegration of subcellular organization and the collapse of the plant protoplast. While ETI to bacterial and fungal plant pathogens ends in a hypersensitive response in the cells directly in contact with the invader, ETI to biotrophic nematode often leads to

cell death in cells just outside the feeding structure. This creates a ring/line of dead cells effectively isolating or disconnecting the feeding structure from nearby vascular tissue. For a pathogen that depends to such an extent on the feeding structure the end result is the same.

13.3 Protection of the Nematode

Induction of host defences leads to changes that are designed to isolate the nematode and its feeding structure. In addition, plants will also respond to nematode invasion using a variety of generic defence strategies that are targeted at the pathogen itself. The host may synthesise and/or release a large number of naturally occurring compounds that have broad spectrum anti-pathogen effects and that may be nematicidal (Jasmer et al. 2003; Wuyts et al. 2006, 2007). These include phytoalexins such as isoflavonoids and terpenoids but little is known about how such compounds are inactivated by PPNs. However, a wide range of PPNs are known to produce Glutathione-S-transferase (GST) and one GST of *M. incognita* is expressed in the oesophageal gland cells from where it is presumably secreted into the host (Dubreuil et al. 2007)**.** Knockout of the transcript encoding this gene led to a decrease in parasitism suggesting it plays an important role in the nematode life cycle. In many animal parasitic organisms, including nematodes, GSTs detoxify endogenous and xenobiotic compounds using a range of biochemical mechanisms (e.g. Campbell et al. 2001). Although definitive functional (i.e. biochemical) studies on PPN GSTs are lacking it seems feasible that GSTs may have a similar role in the interactions between nematodes and plants, although evidence that GST is important in other plant-pathogen pathosystems is limited (e.g. Prins et al. 2001).

The other major defence compounds produced by plants that target pathogens are active oxygen species and these compounds are produced in response to nematode invasion (Waetzig et al. 1999). Active oxygen species target the pathogen directly and also strengthen cell walls and activate other signalling pathways. PPNs seem to be well equipped to metabolise any active oxygen species that are produced by the host. *G. rostochiensis* expresses a secreted glutathione peroxidase in the hypodermis and biochemical tests showed that the recombinant protein metabolises hydrogen peroxide (Jones et al. 2004). A peroxiredoxin is present on the surface of the same nematode that also metabolises hydrogen peroxide. This protein has become adapted to target hydrogen peroxide specifically as, unlike peroxiredoxins from other organisms, it does not metabolise larger hydroperoxides such as cumene or t-butyl hydroperoxide (Robertson et al. 2000). There are other proteins present at the nematode surface that may help the nematode avoid host defence responses. A secreted lipid binding protein, Gp-FAR-1 is present in the surface coat of *G. pallida* that binds a wide range of fatty acids including linoleic and linolenic acid (Prior et al. 2001). These fatty acids are metabolised by lipoxygenase as part of the signalling pathway leading to the production of jasmonic acid and Gp-FAR-1 was shown to inhibit this process *in vitro*.

These studies have revealed a remarkable degree of convergence between the mechanisms used by (unrelated) plant and animal parasitic nematodes to detoxify host derived defence compounds and active oxygen species (reviewed by Jasmer et al. 2003). For example, peroxiredoxins are found on the surface of *Dirofilaria immitis* (Klimowski et al. 1997), *Onchocerca volvulus* (Lu et al. 1998) and *Brugia malayi* (Ghosh et al. 1998) and glutathione peroxidase is one of the major surface proteins of a range of animal parasitic nematodes (reviewed in Henkle-Duhrsen and Kampkotter 2001). In both cases these proteins have been shown to metabolise host derived active oxygen species produced as part of the immune response. In addition, lipid binding proteins are found on the surface of a wide range of animal parasitic nematodes and may provide protection from the host immune system or suppress defence signalling pathways (Garofalo et al. 2003a) but these proteins have different expression patterns in *C. elegans* and are thought to have a different role in this non-parasitic nematode (Garofalo et al. 2003b).

There may be other parallels between animal and plant parasitic nematodes in terms of how host defences are activated or suppressed. The first stage of innate immunity—PTI—is highly conserved across animals and plants. Fungal and bacterial PAMPs such as chitin and flagellin activate PTI in both plants and animals. Signalling pathways, including Toll-like receptors of PAMPs, are also conserved (reviewed by Nurnberger et al. 2004). Activation of innate immunity by nematode parasites of animals is known to condition the subsequent immune response and Toll-like receptors have been implicated in this phenomenon (De Veer et al. 2007) but little is known about PTI signalling between nematodes and plants (see above). Glycans present in nematode secreted proteins may act as PAMPs in animal pathosystems (Balic et al. 2004) but nematode PAMPs (also referred to as Nematode Associated Molecular Patterns—NAMPs) remain to be characterised in detail in animal or plant parasites.

13.4 Conclusions and Future Prospects

Obligate biotrophic nematodes exploit the resources of their host plants in a fundamentally different way to other plant microbes. The induction of unique nematode feeding structures involves radical changes in host gene expression leading to profound molecular and cellular changes in plant tissues. Apart from these exogenously induced aberrances to plant tissues, the endoparasite exists as a foreign body inside the host and will inevitably release or cause the release of danger signals that are the principal triggers of plant innate immunity. Still, individual biotrophic PPN species can be very successful parasites of thousands of different plants, while being seemingly unaffected by the potentially lethal immune responses of these host plants. Although this may be partly explained by how well they are equipped with detoxifying enzymes to neutralize antimicrobial compounds of the plant, nematodes seem to have mastered host immune signalling to protect both their feeding structure and the essential processes inside their own body.

In spite of the importance of suppression of host defences by biotropic nematodes we know little about the details of this process. No nematode PAMP has been identified to date and the signalling pathways triggered by nematode PAMPs are unknown. Bizarrely, current efforts in functional analysis of nematode effectors mean that we are likely to identify PPN proteins that suppress host defences before we know anything about the host defence pathways that they target. The recent identification of the first nematode avirulence gene (Sacco et al. 2009) represents a major advance and, coupled to ongoing genome projects, should provide the first steps on the route to understanding the signalling between host and pathogen that helps determine susceptibility or resistance. Biotrophic nematodes are unique with regard to their feeding behaviour and future research will show whether nematodes also have evolved their own strategies to suppress plant innate immunity.

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Chapter 14
Other Nematode Effectors and Evolutionary Constraints

Marie-Noëlle Rosso and Eric Grenier

14.1 A Wide Range of Effectors are Secreted During Parasitism

Much work has been put into the characterisation of nematode effectors in recent years. Studies have shown similarities and specificities in the cell wall degrading enzymes used by migratory and sedentary nematodes for invasion of the host tissues (see Chap. 12). However, most studies have been focused on sedentary nematodes. For sedentary root-knot and cyst nematodes the success of the interaction relies on the ability of the nematode to (1) locate its host and invade root tissues, (2) induce and maintain the formation of feeding cells, (3) modulate the defence response of the plant throughout parasitism. The effectors of parasitism are the compounds that are secreted by the parasite into the host tissues to alter host physiology and assist the infection process (Hogenhout et al. 2009). As such, nematode effectors secreted at the interface with the host or injected into plant cells have key roles in parasitism. Several different nematode tissues produce molecules that are secreted outside the nematode body. The three most important sources are the cuticle, the amphids and the oesophageal glands (Fig. 14.1).

The surface coat of the cuticle is composed of proteins, carbohydrates and lipids. A characteristic of the cuticle surface is the continuous turn-over of surface associated antigens that may be essential for evasion of host defences (Curtis 2007), as has been demonstrated for animal parasites. The amphids are bilateral chemosensory organs located on the nematode's head and that open to the exterior via a prominent pore. Amphids secrete proteins and carbohydrates that are thought to have a primary role in the perception of chemotactic environmental stimuli (Curtis 2007; Perry 1996). In addition, amphidial secretions are proposed to participate in the formation of the feeding plug that forms a seal to anchor the stylet of the feeding nematode at the plant cell wall (Endo 1978), although this role is not fully ascertained (Jones

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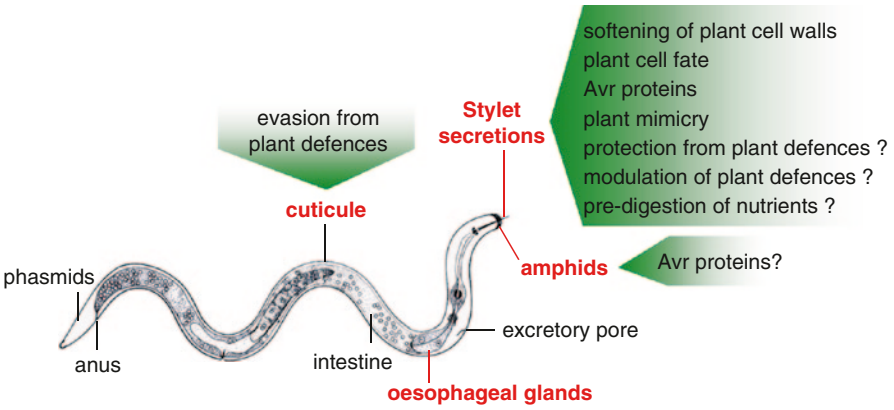

Fig. 14.1 Several organs can be the source of effectors secreted in the plant tissue during the interaction. Roles have been suggested or demonstrated for effectors secreted from the cuticule, the amphids and the stylet. Stylet secretions are synthesized in three oesophageal gland cells and are key effectors for which diverse roles have been demonstrated

et al. 1994; Sobczak et al. 1999). Interestingly, amphidial secretions may act as signalling molecules during the interaction and may elicit defence responses from the plant (Semblat et al. 2001). However, most efforts have focused on proteins secreted from the oesophageal glands. In Tylenchida, three enlarged unicellular glands (two subventral and one dorsal) are connected to the oesophagus into which they release the content of secretory vesicles by exocytosis. The oesophageal secretions, also called stylet secretions, are subsequently released into plant tissues via the stylet. Differences in the vesicular content of the glands suggest that the subventral and dorsal glands are predominantly active during migratory and sedentary stages respectively (Bird 1968, 1969). During migration it has been shown that gland cell secretions, including cell wall degrading enzymes, are released from the stylet into the host apoplasm (for root knot nematodes; Doyle and Lambert 2002; Vieira et al. 2010), or directly into the cells through which cyst nematodes migrate (Wang et al. 1999). However, after migration root-knot and cyst nematodes perforate the cell wall of selected plant cells with their stylet until the stylet orifice makes contact with the plasma membrane (Hussey 1989; Sobczak et al. 1999). At this stage the stylet acts as a syringe for the delivery of nematode effectors into host cells and oesophageal secretions could possibly be directly injected into the plant cell like bacterial effectors delivered via the type three secretion system (Mueller et al. 2008; Wang et al. 2010).

Studies aimed at the identification of nematode effectors have mainly used transcriptomics and proteomics. Transcriptomic studies have focused on nematode genes regulated during parasitism with special emphasis on genes expressed in the oesophageal glands (Elling et al. 2009; Ithal et al. 2007; reviewed in Rosso et al. 2009). The identified coding sequences were filtered through datamining pipelines to select genes that encode proteins possibly secreted by exocytosis (see Chap. 7). Such proteins have a predicted N-terminal secretion signal peptide with no additional predicted transmembrane domain. Similar selection for effector candidates

has been performed on nematode EST datasets (Haegeman et al. 2009; Jones et al. 2009; Jacob et al. 2007; Kikuchi et al. 2007; Nagaraj et al. 2008; Ranganathan et al. 2009; Roze et al. 2008; Vanholme et al. 2006). These studies have increased our knowledge on nematode effectors enormously. However, only part of the full proteome can be predicted through genome or transcriptome scanning (Antelmann et al. 2001). In addition, mRNA expression does not always correlate with the actual production of proteins (Gygi et al. 1999). Proteomics therefore offer a complementary approach that can directly identify proteins that are secreted by the nematode. Antibodies have been raised against nematode secreted products (reviewed in Davis et al. 2000) and subsequently used to identify genes encoding proteins of interest (e.g. Smant et al. 1998). Proteins present in the secretome of the nematode have also been analysed by mass spectrometry. Infective juveniles grown in vitro were stimulated to activate stylet secretion and the purified proteins were identified by comparing peptide sequences to sequence databanks (Bellafiore et al. 2008; Jaubert et al. 2002; De Meutter et al. 2001). For many proteins the expression in secretory tissues was confirmed. As observed in other eukaryotic organisms, a consistent number of proteins found in the secretome did not have a predicted signal peptide (e.g. Bellafiore et al. 2008; Fioretti et al. 2001; Jaubert et al. 2002; Robertson et al. 2000), suggesting that alternative secretory pathways independent from the classical endoplasmic reticulum-Golgi secretory pathway might contribute to the secretory activity of the nematode (reviewed in Agrawal et al. 2010).

Most nematode effectors isolated to date are proteinaceous products. Nevertheless, it is thought that nematodes might also secrete non-proteinaceous products into plant tissues during infection. Root-knot and cyst nematodes have been shown to produce auxin conjugates and cytokinins that may interfere with the hormone balance of the plant cell (De Meutter et al. 2005). Whether these compounds have a biological significance in the infection process is not known. In addition, a Nod-like effector, is supposedly secreted by *M. hapla* and induces changes in plants similar to the early legume response to nodulating bacteria (Weerasinghe et al. 2005).

Several strategies are used in order to assign a functional role to nematode effectors. The inability to transform plant parasitic nematodes has led to the development of RNAi (RNA interference) procedures adapted to the parasite. RNAi is the suppression of gene expression induced by double-stranded RNA molecules homologous to the targeted transcript (see Chap. 10). dsRNA molecules synthesized in vitro can be delivered to the nematodes by soaking or by artificial stimulation of uptake through the stylet. In addition, nematodes can ingest RNAi triggers when feeding on transgenic plants that produce dsRNA. RNAi has allowed the functional analysis of several genes involved in parasitism (reviewed by Rosso et al. 2009). The loss of ability of the nematodes to infest host plants and to establish a feeding site or impaired nematode development after RNAi is an indication that the targeted gene is important for the interaction.

A role for nematode effectors can be assigned from phenotypes induced by the over-expression of effectors in plant tissues. Interestingly, in a few cases nematode effectors were shown to complement *Arabidopsis thaliana* mutants, providing evidence for functional similarity with plant proteins and for the ability of nematode

effectors to mimic plant functions (Lu et al. 2009; Patel et al. 2010; Wang et al. 2005). Understanding the role of nematode effectors can also be addressed by identifying the plant targets able to interact with the effectors. Recent successful efforts to identify plant targets using yeast-two hybrid systems or bimolecular fluorescence complementation (BiFC) have provided new information on effector functions (Hewezi et al. 2008, 2010; Huang et al. 2006b; Lee et al. 2010; Patel et al. 2010; Rehman et al. 2009a; Sacco et al. 2009).

The proteinaceous effectors for which a function can be predicted showed that diverse cellular processes are targeted by the nematode for successful manipulation of the host response. Plant targets of nematode effectors have been identified that are involved in various cellular processes such as cell wall structure (Hewezi et al. 2008), gene expression (Huang et al. 2006b), cell growth (Lee et al. 2010), stress responses (Patel et al. 2010) and immunity (Rehman et al. 2009a). Plant cell wall modifications (see Chaps. 12 and 19) and modulation of plant defences (see Chap. 13) are key functions for nematode effectors and are discussed elsewhere in this volume. However, this chapter focuses on other functions of nematode effectors such as manipulating plant cell fate, protein synthesis and degradation and signalling pathways.

14.2 Signalling and Protection at the Plant-Nematode Interface

The amphids and the cuticle are in direct contact with the environment of the nematode. The products they secrete are located within the apoplasm of the host where they may trigger plant defences via non-self perception or modulate the response of the plant during infection. Few proteins secreted from the amphids have been characterized (Curtis 2007). However, one candidate avirulence protein, MAP-1 has been identified in the amphidial secretions of *Meloidogyne incognita* (Semblat et al. 2001). MAP-1 is a modular protein of unknown function with conserved repeats of 13 and 58 amino acids. Strikingly, variation in the MAP-1 modular structure correlated with nematode (a)virulence on *Mi-1*-resistant tomato plants (Castagnone-Sereno et al. 2009). The localisation of MAP-1 in J2 amphidial secretions, suggested that this protein may be involved in the early steps of recognition between (resistant) plants and (avirulent) nematodes (Vieira et al. 2010).

Secretory compounds synthesized in the hypodermis are delivered through secretion vesicles or diffusion across the cuticle at the external surface of the nematode (Curtis 2007). These secretions build up as a surface coat that undergoes continuous turn-over and it is thought that this turnover may hide the nematode from detection by the host throughout the interaction (Sharon et al. 2002). Plants respond to nematode attack by the production of reactive oxygen species (ROS) that are toxic to the pathogen, contribute to strengthening the cell wall physical barrier and trigger the activation of other defence responses (Melillo et al. 2006; Simonetti et al. 2009). Plant parasitic nematodes secrete ROS scavenging enzymes that can degrade ROS and ROS-derived toxic radicals. In *G. rostochiensis*, a peroxiredoxin (Gr-TPX) and

a glutathione peroxidase (Gr-GPX-1) are secreted at the surface of infective and parasitic J2 suggesting a role at the host-parasite interface in the protection of the parasite from H_2O_2 produced by the plant (Jones et al. 2004; Robertson et al. 2000). *M. incognita* also secretes putative superoxide dismutases (SOD), although the tissue localization of these SODs is still to be determined (Bellafiore et al. 2008). Surprisingly, a glutathione S-Transferase (MI-GST-1) from *M. incognita* was shown to be synthesised in the oesophageal glands of infective juveniles, secreted via the stylet and up-regulated during parasitism (Dubreuil et al. 2007). The secreted MI-GST-1 could possibly participate in the detoxification of hydroperoxides generated by the oxidative response of the plant. Alternatively, MI-GST-1 could intervene in the local regulation of the host defences and modulate ROS-mediated defence signalling (Baker and Orlandi 1995).

Interestingly, some secreted proteins can have diverse tissue origin in cyst and root-knot nematodes. For example, like many animal parasites, *G. pallida* has a fatty acid and retinol binding protein (Gp-FAR-1) present on its cuticle surface. A role for Gp-FAR-1 in the evasion of host defences has been suggested from its ability to bind lipid precursors of plant defence compounds and prevent their metabolism (Prior et al. 2001). Strikingly in *M. incognita*, a protein similar to Gp-FAR-1 is expressed in the subventral oesophageal glands of infective J2 (Bellafiore et al. 2008). It is tempting to suggest that a FAR protein secreted through the stylet into the tissues surrounding the nematode's head could locally modulate plant defence signalling. Another example is provided by proteins of yet unknown function, from the SXP-RAL2 family, that are secreted from the epidermis and the amphidial sheath cells in *G. rostochiensis* (Jones et al. 2000) whereas in *M. incognita* Mi-SXP-1 was expressed exclusively in the subventral oesophageal glands of the nematode (Tytgat et al. 2005). However, a substantial gene family of SXP-RAL2 proteins is present in many nematodes and it is possible that the studies described above have been conducted on different gene family members. Annexins may provide a further example: in *G. pallida* second stage juveniles the annexin *Gp-nex* was immunolocalised in the amphids, genital primordium and in the constraining muscles above and below the metacorpus pump chamber (Fioretti et al. 2001). However, in *H. schachtii* an annexin was identified that is expressed in the dorsal oesophageal gland. This protein binds to a plant putative 2OG-Fe(II) oxygenase known to be necessary for susceptibility to oomycete pathogens (Patel et al. 2010).

14.3 Stylet Secretions are Major Parasitism Effectors

14.3.1 Stylet Secretions can Play Roles in Different Cellular Compartments of Plant Cells

Stylet secretions have been studied in depth and are involved in both the migratory and the sedentary phases of parasitism. Stylet secretions can be directed to the

apoplasm of the host. During penetration of, and migration through, the root tissue the nematodes secrete a battery of cell wall degrading and cell wall modifying enzymes that are synthesized in the subventral oesophageal glands (see Chap. 12). Endoglucanases and pectate lyases have been localised in front of the nematode's head and along the migratory path of *H. glycines*, *M. javanica* and *M. incognita* juveniles (Doyle and Lambert 2002; Vieira et al. 2010; Wang et al. 1999). In addition, stylet secretions from cyst and root-knot nematodes contain effectors active during the sedentary phase of parasitism. A chorismate mutase from *M. javanica*, Mj-CM1, was visualized in cells neighbouring the nematode head before giant cells are formed, although the precise localisation of the protein could not be determined. It was suggested that this protein could modulate auxin levels or salicylic acid mediated defences of the host (Doyle and Lambert 2003). The first report for secretion of a nematode effector into a differentiated feeding site showed the accumulation of a calreticulin from *M. incognita* (Mi-CRT) at the cell wall of giant cells in the vicinity of the stylet tip (Jaubert et al. 2005).

On the other hand, scarce studies visualising nematode secretions in the cytoplasm of feeding cells suggested that stylet secretions could be directly injected into the plant cell cytoplasm (e.g. Sobczak et al. 1999). Observing nematode secretions in the plant cell cytoplasm is technically challenging due to the low amount of secreted proteins introduced by nematodes into plants and also due to the lack of a transformation method that makes impossible the use of reporter or tag fusions to trace nematode secretions *in vivo*. However, recent studies have demonstrated that one nematode protein is injected into the cytoplasm of the syncytium and is subsequently transported to the apoplasm from here. The secretory peptide Hg-4G12 from *H. glycines* and Gr-CLE proteins from *G. rostochiensis* share conserved motifs with plant CLE proteins (Lu et al. 2009). The nematode CLE preprotein was shown to be injected into the cytoplasm with the CLE domain subsequently transported to the apoplasm. A variable region of the nematode protein immediately upstream of the CLE domain(s) was shown to play a role in the trafficking of the CLE peptides to the apoplasm (Wang et al. 2010).

Some putative effectors encode proteins with nuclear localization signals that are functional in plant cells (Elling et al. 2007; Tytgat et al. 2004). If injected into the cytoplasm, these nematode effectors might be able to reach the nucleus of host cells. An investigation into a large family of cyst nematode effectors, the SPRYSECs, has shown that some of the gene family members are localised to the cytoplasm while others target the plant nucleus and nucleolus (Jones et al. 2009). Future investigations will help unravel the role of nematode secretions in the different subcellular compartments of host cells.

14.3.2 Stylet Secretions Mimic the Function of Plant Proteins

Some secretory proteins from the oesophageal glands show striking similarities with plant proteins. Proteins from the CLE-like family (see above) have not

been identified outside the plant kingdom excepted in plant parasitic nematodes. These peptides control the proliferation/differentiation balance of stem cells from the shoot and apical meristems and from the vascular bundle. In *H. glycines* and *G. rostochiensis*, proteins with conserved CLE motifs are expressed in the dorsal oesophageal gland of parasitic stages and have a predicted signal peptide for secretion, suggesting that they are secreted through the stylet during infection (Lu et al. 2009; Wang et al. 2005). Overexpression of *Hg-4G12* or Gr-CLE proteins in *Arabidopsis* caused premature termination of the shoot apical meristem similar to the phenotype observed after overexpression of the plant CLAVATA3 peptide or mutation of the WUS regulator of the CLAVATA signalling pathway. In addition, the nematode CLE-like proteins were able to rescue the phenotype of *Arabidopsis clv3* mutants suggesting that nematode and plant CLE peptides share functional similarity. A small peptide, named 16D10, has been identified in root-knot nematodes that is expressed in the subventral oesophageal glands of parasitic juveniles and has a predicted signal peptide for secretion. The knock-out of 16D10 expression by RNA interference showed that it is important for successful development of the nematode (Huang et al. 2006a). In addition, 16D10 overexpressed in tobacco or *Arabidopsis* roots stimulated root growth. Although 16D10 shares sequence similarity to CLE peptides it is likely that the mode of action of 16D10 is different from the cyst nematode CLE-like proteins. The activity of 16D10 is likely to be in the cytoplasm of plant cells, possibly via binding to a SCARECROW-like transcription factor (Huang et al. 2006b).

Other short peptides (<3 kDa) were isolated from stylet secretions of *G. rostochiensis* juveniles that are able to stimulate plant protoplast proliferation through a general signal transduction mechanism. The nature of these mitogenic peptides still remains to be determined (Goverse et al. 1999).

14.3.3 Stylet Secretions can Regulate Host Protein Synthesis and Degradation

Like microbial plant pathogens and animal parasitic nematodes (reviewed by Shindo and Van der Hoorn 2007; Williamson et al. 2003), cyst and root-knot nematodes are thought to control the degradation of host proteins. In *M. incognita*, a protein homologous to aspartyl proteases, produced in the subventral oesophageal glands of infective J2, is secreted into the plant tissues during migration and accumulates at the wall of the feeding cells during the sedentary phase of parasitism (Vieira et al. 2010). In addition, the *M. incognita* secretome contains metallopeptidases, aminopeptidases and a cysteine proteinase (Bellafiore et al. 2008; Huang et al. 2003) and cyst nematodes have been shown to secrete metalloproteases (Curtis 2007; Robertson et al. 1999). Secreted proteases could help soften or destroy plant cell walls during migration, participate in the pre-digestion of nutrients in sedentary stages or inactivate plant defence proteins, although degree of specificity would be required for this to be the case. It is suspected that cyst and root-knot nematodes may also

manipulate the proteasome pathway of host cells. A SKP1 homolog was identified that is expressed in the dorsal gland of *H. glycines* parasitic juveniles and has a predicted signal peptide for secretion (Gao et al. 2003). A SKP1 homolog was also identified in the secretome of *M. incognita* infective juveniles (Bellafiore et al. 2008). S-phase kinase-associated protein 1 (SKP1) is a key component of the cellular protein complex SCF (Skp, Cullin, F-box containing complex) that catalyzes ubiquitination of proteins destined for proteosomal degradation. SKP1 regulates a variety of signal transduction pathways, including cell cycle progression. The plant targets for SKP-like proteins secreted by the nematode are unknown. Further examples of how other pathogens exploit the ubiquitination pathway to modulate host defences are given in Chap. 13.

14.3.4 Stylet Secretions Could Regulate Host Signalling Pathways

A 14-3-3 protein expressed in the dorsal gland of infective J2 has been identified as being present in the secretome of *M. incognita* (Bellafiore et al. 2008; Jaubert et al. 2002; Jaubert et al. 2004). 14-3-3 proteins are adaptor proteins that guide protein-protein interactions and have essential roles in hormonal signal transduction processes in plants. For example, plant targets for 14-3-3 proteins include the receptors BAK1 and CLAVATA 1, kinases and transcription factors involved in hormonal signalling and/or biotic/abiotic stresses (Oecking and Jaspert 2009). In addition, plant 14-3-3 proteins regulate the plasma membrane H+-ATPase in response to fungal infection and during nodule development (Wienkoop and Saalbach 2003). Functional analysis of the secreted nematode 14-3-3 is required to fully understand its role in the infection process. As described above, accumulation of a calreticulin, Mi-CRT, secreted from the dorsal gland of *M. incognita* was observed at the cell wall of giant cells (Jaubert et al. 2005). Calreticulins secreted by animal parasitic nematodes and by *Trypanosoma* bind to primary receptors of the immune system to inhibit macrophage or dendritic-mediated host defences (Naresha et al. 2009; Oladiran and Belosevic 2009; Rzepecka et al. 2009). More calreticulin binding partners have been identified at the surface of animal cells that regulate calcium signalling and cell cycle (Borisjuk et al. 1998; Ghiran et al. 2003). Mi-CRT could intervene directly in calcium signalling in the apoplasm of giant cells via its calcium binding activity. Nevertheless a function for the nematode calreticulin in the modulation of the plant responses is still to be determined. Annexins are phospholipid binding proteins involved in the regulation of ion transport across membranes. The annexin Hs4F01 is expressed in the dorsal oesophageal gland of *H. schachtii* infective juveniles. When overexpressed in *Arabidopsis* AnnAt mutants, the nematode annexin was able to rescue the mutant's sensitivity to salt stress. Furthermore, a yeast two hybrid screen identified a plant oxidoreductase member of the 2OG-Fe(II) oxygenase family as potential target for Hs4F01 (Patel et al. 2010). Because 2OG-Fe(II) oxygenases are involved in plant susceptibility to oomycete pathogens, it was suggested that the nematode annexin may interact with the plasma membrane of the

syncytia to promote parasitism, possibly via modulation of the plant cell response to calcium changes. Auxin accumulation is important for the proper development of nematode feeding sites (Grunewald et al. 2009). Exciting recent findings have demonstrated the ability of a nematode effector to bind to an auxin transporter, possibly modulating auxin influx in syncytia (Lee et al. 2010)

14.3.5 Stylet Secretions of Unknown Function

Many proteins have been identified that have a predicted signal peptide for secretion, are specifically expressed in the oesophageal glands of the nematode and are therefore potential effectors. Two ubiquitin extension proteins, Hs-UBI1 and Hs-UBI2, were isolated from *H. schachtii* parasitic stages. Hs-UBI1 was immuno-localized in the secretory vesicules of the dorsal oesophageal gland of the nematode (Tytgat et al. 2004). In addition to a ubiquitin domain, the protein has a short C-terminal domain able to drive the targeting of a fused GFP reporter protein to the nucleolus of tobacco cultured cells. It was hypothesized that Hs-UBI1 may be cleaved in the cytoplasm of infected plant cells, and the C-terminal domain may be targeted to the nucleolus. The biological function of this domain in plant cells remains to be determined. Interestingly, an ubiquitin-extension protein is also expressed in the dorsal oesophageal gland cell of *H. glycines* and *G. pallida* (Gao et al. 2003; Jones et al. 2009). A triosephosphate isomerase (TPI)-homolog from *M. incognita* is expressed in the subventral oesophageal glands and abundantly secreted by infective J2 (Bellafiore et al. 2008). No function for this protein in the host-parasite interaction has been determined. Similarly, transthyretin-like proteins, galectin, SXP-RAL2 proteins and venom allergen homologs (Wang et al. 2007) have been shown to be expressed in gland cells but have no known function in the parasitic process (Bellafiore et al. 2008; Dubreuil et al. 2007; Gao et al. 2001; Jacob et al. 2007; Tytgat et al. 2005). Even more challenging is trying to determine of the role of candidate effector proteins that have no homologues in protein sequence databases (Gao et al. 2003; Huang et al. 2003). Whether these ‘pioneer’ genes result from evolutionary processes related to the finely tuned interaction of sedentary nematodes with their hosts is one of the exciting challenges of molecular nematology.

14.4 Nematode Effectors can Trigger Plant Resistance

Gene-for-gene resistance in plants is defined by the direct or indirect interaction between products of dominant plant resistance (*R*) genes and corresponding avirulence (*Avr*) genes that are often specific to a particular set of pathogen isolates. In the absence of recognition by a cognate host R protein, Avr proteins play a role in pathogen virulence, often by subverting host defence mechanisms and are referred to as pathogen effector proteins (Chisholm et al. 2006). Identification of pathogen-encoded Avr proteins from bacterial, viral, fungal and oomycete plant-pathogens

has yielded a remarkable list of proteins that elicit plant resistance. Identification of *Avr* genes from metazoan parasites, however, represents a major challenge owing to the complexity of their genomes and life cycles and a lack of tractable model organisms. This is particularly apparent for organisms like plant parasitic nematodes.

Progress has been made in recent years on the isolation of *Avr* genes from root-knot and cyst nematodes. One powerful approach has been rigorous differential molecular analyses of virulent and avirulent lines. This has allowed the identification of two putative *Avr* genes. One of these, *map-1.2,* is a gene that encodes a secreted protein from *M. incognita* which belongs to a small gene family. Members of this family have modular repetitive regions and some show specific expression in lines avirulent on *Mi-1* resistant tomato plants (Castagnone-Sereno et al. 2009; Semblat et al. 2001). Another, *Hg-cm-1*, encodes a chorismate mutase in which sequence polymorphisms correlate with virulence against soybean cultivars in *H. glycines* (Bekal et al. 2003; Lambert et al. 2005). However, the plant targets for these *Avr* candidates and their function in (a)virulence remain so far unknown. More recently, another *M. incognita* gene, *Cg-1,* was described as an *Avr* gene against *Mi-1* (Gleason et al. 2008). *Cg-1* could only encode short polypeptides (32 amino acids in length for the longest ORF), none of which include a signal peptide for secretion, suggesting that the transcript may be a regulatory molecule rather than the elicitor of the *Mi-1* resistance reaction. This also indicates that the ultimate outcome of an *R-Avr* interaction can be influenced by additional factors determined by the pathogen, including factors which can interfere with the function of plant *R* genes. In 2009, two genes from the same gene family, the SPRYSEC family, were shown to be involved in incompatible plant/nematode interaction (Rehman et al. 2009a). SPRYSEC-19 was isolated from the potato cyst nematode *G. rostochiensis* and was shown in yeast–two-hybrid analysis to interact with the LRR region of SW5-F, a novel CC-NBS-LRR protein which is highly similar to members of the SW5 tomato resistance gene cluster. However, as transient co-expression of SW5-F and SPRYSEC-19 in tobacco leaves did not trigger a hypersensitive response, no evidence was found that the interaction between SW5-F and SPRYSEC-19 conditioned resistance to the nematode in tomato. However, it is still possible that other *R* genes similar to SW5-F or other alleles of SPRYSEC-19 may be able to elicit a resistance or hypersensitive response. *Gp-Rbp-1* is another SPRYSEC member isolated from *G. pallida* (Blanchard et al. 2005) and this gene was shown to elicit a hypersensitive reaction when co-expressed with the *Gpa2* potato resistance gene (Sacco et al. 2009). Interestingly, all Gp-RBP-1 protein variants from an avirulent population were recognized by Gpa2, whereas in virulent populations, some Gp-RBP-1 protein variants were recognised by Gpa2 and others were not (Sacco et al. 2009).

14.5 Evolution of Nematode Effectors

A large number of candidate effectors have been identified from nematodes. For example, approximately one hundred proteins are secreted by *M. incognita* during infection (Bellafiore et al. 2008; Huang et al. 2003; Jaubert et al. 2002). Interestingly,

many of these candidate effectors are 'pioneers' with no homology in the sequence databases available to date, suggesting that they could have been acquired during evolution for specific adaptations to a plant parasitic life style. How these pioneer genes evolved in sedentary plant parasitic nematodes is unknown.

Hosts and pathogens are engaged in a struggle, with the hosts evolving to escape pathogen infection and pathogens evolving to escape host defences. Technological and methodological progress has allowed the detection of selection footprints on genes and testing of whether they are targets of selection. When selection has occurred, it is possible to study the evolutionary forces at play (balancing selection as in the "trench warfare" process or selective sweeps as in the "arms race" process) and whether selection has been acting in the more or less recent past (Aguileta et al. 2009). These dynamics of adaptative evolution and the ability of nematodes to respond to environmental challenges are influenced by the number of genes underlying traits, their standing variation and the ability to generate or recruit novel variants through mutations or hybridizations. Conceptually, several mechanisms affecting the evolution of parasitism can be envisioned. These include among others adaptation of pre-existing genes to encode new functions, domain shuffling, alternative splicing, gene duplication and acquisition of genes from other species by lateral gene transfer.

14.5.1 Lateral Gene Transfer

One of the most remarkable findings that has emerged from genomic studies is the presence of genes within plant-parasitic nematodes that are absent from all other nematodes and from most other animals studied to date. There is a strong argument that these genes have been acquired by lateral gene transfer (LGT). Claims for LGT were first based on incongruencies between a particular gene phylogenetic tree and the assumed underlying species phylogeny. Obviously, because full genomes are not available for all plant and animal species we are not able to make definitive statements, but with two *Meloidogyne* genomes among six nematode genomes currently completed (*M. incognita, M. hapla, C. elegans, C. briggsae, Brugia malayi and P. pacificus*) and four completed insect genomes it is possible to examine in a more accurate way the evolution of effectors and the role of LGT.

At least 14 LGT candidates have been identified through bioinformatic screens in plant parasitic nematodes and even an entire metabolic pathway for de novo biosynthesis of the active form of vitamin B6 was suggested to have been (re)obtained from bacteria in *H. glycines* (Craig et al. 2008; Scholl et al. 2003). A large number of plant cell wall modifying enzymes (cellulases, pectate lyases, polygalacturonases, xylanases, arabinanases, expansin-like proteins) are present in nematodes and the sequence of these enzymes resemble bacterial sequences suggesting that they have been acquired from bacteria (see Chap. 12). Systematic phylogenetic analyses have confirmed that acquisition of these enzymes was most probably due to LGT from different bacterial sources and that transfer was frequently followed by

independent lineage specific duplications (Abad et al. 2008; Danchin et al. 2010; Kyndt et al. 2008; Ledger et al. 2006). Root knot nematodes contain additional genes that appear to have been acquired by LGT and which are not present in cyst nematodes. These include a gene similar to nodL from *Rhizobium* (Scholl et al. 2003), genes encoding chorismate mutases (Jones et al. 2003; Lambert et al. 1999) and a gene similar to bacterial polyglutamate synthetase (Veronico et al. 2001). Rhizobacteria appear to be the predominant group of "donor" organism for HGT (Abad et al. 2008). This is significant for two reasons. First sedentary plant parasitic nematodes and rhizobacteria occupy similar ecological niches, second these organisms establish intimate developmental interactions with host plants and share some infection mechanisms (Koltai et al. 2001). It is therefore tempting to speculate that plant parasitism has been facilitated by acquisition of novel genes from soil bacteria through LGT.

14.5.2 Mutations and Positive Selection

Mutation is the fuel of evolution and contributes to adaptation. The role of point mutations that change the amino acid sequence of a protein is therefore important when studying the evolution of nematode effectors. For example in the bacteria *Escherichia coli* a single amino acid change in the protein *Fim*H involved in adhesion leads to loss of activity of the protein and altered host specificity (Pouttu et al. 1999). Molecular analyses of nematode phylogeny using both rDNA and mtDNA genes have been published (Blaxter et al. 1998; Holterman et al. 2006; Petit and Valette 2008; Picard et al. 2007; van Megen et al. 2009). The two features that emerge from these analyses are (1) the high level of sequence divergence between nematode species and between nematode populations inside a species and (2) the heterogeneity in evolutionary rate between different nematode lineages. A rate of 1.78 substitutions per synonymous site, more than twice that in mouse/human, has been observed in *C. briggsae/C. elegans* (Stein et al. 2003), but when calculated in terms of generations instead of years, the evolutionary rate of *C. elegans* is actually quite similar to that observed in *Drosophila* (2.7×10^{-9} vs 3.5×10^{-9} substitutions per site per generation) (Denver et al. 2009).

Little is known about the evolutionary rate of nematode effectors but the investigations conducted to date have confirmed that many nematode genes show huge diversity in sequence and structure. This statement should however be moderated since (1) the levels of within-species sequence divergence is strongly influenced by the different reproduction modes that exist in plant parasitic nematodes and (2) the diversity observed within the domains of a given effector may be different as already reported for cellulases that show more variation within the cellulose binding module (CBM) compared to the catalytic domain (Haegeman et al. 2008). Nonetheless, a striking observation was that in several cases a high proportion of the observed mutations corresponded to non synonymous substitutions (leading to changes in amino acid sequence). This was reported for the *Globodera Ia7* and *IVg9*

effectors where nearly 90% of the mutations are non synonymous (Blanchard et al. 2007) and for the *H. schachtii* pectate lyase *pel-2,* which has nearly 80% of non synonymous mutations compared to only 55% non synonymous mutations observed in the house-keeping elongation factor gene (De Verdal et al. 2009). It is therefore tempting to speculate that some of these effectors have evolved under strong selective pressure.

Obviously, the molecular interactions that occur between a pathogen and its host or soil environment offer excellent opportunities for positive selection to operate (Garofalo et al. 2003). Recently, Him et al. (2009) have addressed this question in an attempt to test whether *hsp-90* genes had undergone adaptative evolution in the phylum Nematoda and whether this evolution influenced binding to geldanamycin, a naturally occuring Hsp-90 inhibitor. The authors identified residues with significant evidence for positive selection, supporting rapid adaptative evolution along at least three separate lineages of nematodes. However they were unable to demonstrate that the observed selection was correlated with failure to bind geldanamycin. Positive selection will most easily be detected at the interspecific level, once advantageous mutations have been fixed, rather than when they are in a polymorphic state. Hopefully when selection varies spatially it leads to detectable molecular signatures. The evolutionary constraints that act on the SPRYSEC *Gp-rbp-1* have been investigated in indigenous and imported *G. pallida* populations (Sacco et al. 2009). In this case the PAML models of positive selection were significantly ($p < 0.001$) better adapted to the sequence data set (PAML assigns a likelihood score to models for selection implemented in the software: a likelihood score for a model incorporating positive selection that is higher than that for a null model without positive selection is evidence for positive selection). Six sites under positive selection were identified but only one was still strongly supported by other methods implemented in the HyPhy program. In this case, it was possible to show that the selection observed was correlated with the ability of Gp-RBP-1 variants to elicit a hypersensitive response in presence of the *Gpa2* resistance gene. Signs of positive selection were also detected in a paralogous SPRYSEC sequence dataset (Rehman et al. 2009a), suggesting that more members of this gene family that remain to be identified seem to be under similar evolutionary constraints.

14.5.3 Alternative Splicing

Alternative splicing of precursor messenger RNA was first described almost 30 years ago and is one of the main sources of proteomic diversity in multicellular eukaryotes (Nilsen and Graveley 2010). Alternative splicing involves the differential use of splice sites to create protein diversity. Nearly all instances of alternative splicing result from the use of one or more of four basic mechanisms: alternative choice of the 5' splice site, alternative choice of the 3' splice site, exon skipping and intron retention. Because point mutations in either exons or introns can create

or destroy splicing control elements it is easy to envisage that splicing patterns are constantly evolving. In addition, some alternative splicing patterns have been shown to be modulated in response to external stimuli (Shin and Manley 2004). It is therefore tempting to speculate that alternative splicing can be a way for nematodes to respond to environmental variation and in particular plant host variation. Alternative splicing is well documented in *C. elegans* (Zahler 2005) but few cases have been reported in plant parasitic nematodes. Pre-mRNA of the *G. rostochiensis* chorismate mutase gene was shown to be subjected to alternative splicing through intron 2 retention (Lu et al. 2008). When translated, the splice variant (*Gr-cm-1-IRII*) results in a truncated protein that lacks the chorismate mutase domain but is able to interact with the protein issued from the fully spliced transcript *Gr-cm-1*. These two alternatively spliced mature mRNA have different levels of expression throughout the nematode life cycle with *Gr-cm-1-IRII* transcript level reaching 16–29% of that of *Gr-cm-1*. It is tempting to speculate that in this case alternative splicing has a role in regulating *Gr-cm-1* expression rather than in diversifying the functions of the gene. It was also suggested that some *Gp*-RBP-1 isoforms are generated from alternative splicing and result in proteins of varying length (Sacco et al. 2009). These splice variants were still able to trigger *Gpa2* recognition although the kinetics and intensity of the hypersensitive reaction were different compared to agroinfiltrations made using the fully spliced cDNA.

14.5.4 Gene Duplication and Domain Shuffling

Most of the nematode effectors identified to date belong to gene families. Large gene duplications within gene families are often regarded as an important source of evolutionary novelties. The evolutionary fate of gene copies after a duplication event can vary: in most cases, one gene copy would be rapidly eliminated, whereas long-term conservation of both gene copies seems to be rare (Lynch and Conery 2000). The temporary redundancy between two paralogous copies is expected to result in a mutation rate acceleration and rapid divergence of one or both copies that could result in the acquisition of a slightly different or entirely new function for one gene copy. Evidence for both recent and ancient globin gene duplications have been reported in nematodes (Hunt et al. 2009). The function of globins remains enigmatic in invertebrates, but several investigations have suggested that these proteins may be involved in many diverse functions rather than a single one, which may explain the multiple gene duplications observed in this gene family. Duplication events can also be intragenic and affect only a particular motif or domain of a given protein. Such proteins that contain tandem arrangements of repeated motifs are often considered as a source of variability with the gain or loss of repeats leading to functional differences. Several different *G. rostochiensis* CLE genes have been identified that contain either single or multiple CLE motifs (Lu et al. 2009). Though CLE proteins with either a single or multiple CLE motifs function simi-

larly to plant CLE proteins, the evolution of multiple CLE motifs may be an important mechanism for generating as yet unknown functional divergence in nematode CLE proteins. Similar observations were made on the *M. incognita* candidate Avr protein MAP-1 which shows a variation in the number and arrangement of highly conserved repeats of 13 and 58 amino acids (Castagnone-Sereno et al. 2009). This difference in repeat motif number is thought to be responsible for the discrimination of avirulent and virulent nematodes by the *Mi-1* resistance gene (Semblat et al. 2001).

As previously mentioned it is hypothesized that GHF5 cellulases were acquired by nematodes through a single LGT event. An evolutionary scheme was first proposed (Ledger et al. 2006; Kyndt et al. 2008) in which the ancestral GHF5 gene carrying a catalytic domain, a linker and a CBM was inherited as a whole cassette and subsequently duplicated several times in nematode genomes with occasional losses of the linker and CBM domain. An alternative hypothesis may be that GHF5 and CBM domains were inherited separately and then assembled together through domain shuffling. Evidence for such domain shuffling events has been detected across Tylenchida (Danchin et al. 2010; Rehman et al. 2009b) which suggest that cellulases and expansins could have arisen through reshuffling of a GHF5 or expansin domain with ancillary CBM domains. Nematode effector evolution appears therefore as the result of a mixture of different mechanisms that have affected plant parasitic nematode lineages in a heterogeneous manner.

Considerable progress has been made to identify the wide range of compounds delivered by endoparasitic nematodes into the plant but several challenges are still remaining. Recent findings have provided evidence of functional roles for several effectors in the establishment of sedentary nematodes. These exciting findings boost current research on nematode effectors which appear as potential targets for the development of new control strategies as their alteration in crops may affect disease development. As the inactivation of host targets required for nematode development may lead to the generation of plants fully or partially resistant, substantial efforts are dedicated to the identification of the plant targets of these effectors and their molecular function in the plant response. In addition, a few effectors involved in the recognition of the nematode by the plant have been identified. Such nematode effectors are likely to be under strong selection pressures in agroecosystems where humans control the genetics of the crop and incorporate resistance genes that the pathogens have to cope with. One of our main challenges with the availability now of complete nematode genomes will be to link ecological traits of pathogen species with the molecular evolution pattern of their effectors. Ongoing studies will provide an accurate view on the degree of conservation of these effectors among plant parasitic nematodes and on the adaptive potential of nematode populations to the selective force driven by plant resistances.

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Chapter 15
Disease Resistance-Genes and Defense Responses During Incompatible Interactions

Isgouhi Kaloshian, Olivia J. Desmond and Hagop S. Atamian

15.1 Introduction

The basis of incompatible interactions is host recognition of specific pathogen or pest signals, known as effectors, followed by successful activation of defenses. In recent years, this interaction has come to be known as effector-triggered immunity (ETI) (Jones and Dangl 2006; Martin et al. 2003; also see Chap. 13 this volume). Most of the existing information about incompatible host-nematode interactions is based on the characterization of resistant plants during their interactions with root-knot or cyst nematodes. Therefore, this chapter will describe our current understanding of incompatible interactions between host plants and these two groups of sedentary endoparasitic nematodes.

Root-knot and cyst nematodes initiate two distinct types of feeding structures, with root-knot nematodes inciting formation of giant cells and cyst nematodes forming syncytia (Chaps. 4 and 5). Successful proliferation of sedentary endoparasitic nematodes requires an intimate association with the host, facilitated by molecular signal exchanges from one organism to the other (Chaps. 13 and 14).

In the evolutionary war between plant resistance and sedentary endoparasitic nematode virulence, incompatible interactions may be considered a battle won by the plant. In order to suppress plant defense responses, initiate a feeding site and commandeer host metabolism, nematodes inject a cocktail of proteins and other metabolites, known as effectors, into the extracellular space and targeted cells of the host. Incompatible interactions occur when plants, which have all of the components needed to act as a nematode host, are able to recognize effectors delivered into their cells and effectively respond to prevent the damaging manipulation of their resources by the intruder. Appropriate activation of host defense responses during incompatible interactions is mediated by disease resistance (*R*)-genes that have a dual function of directly or indirectly recognizing specific nematode effectors, also

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known as avirulence (Avr) factors, and subsequently switching on host signal transduction pathways that lead to physiological changes making conditions unfavorable for nematode survival. The establishment of such gene-for-gene resistance mechanisms (Flor 1971) applies evolutionary pressures on avirulent nematode populations to evolve strategies to evade host detection. If successful, plants are rendered susceptible, as they are unable to efficiently activate the arsenal of defense mechanisms they have in place. In this fashion the evolutionary war continues, with nematode effector recognition being the key determinant of which side wins each battle.

15.2 Nematode Resistance Genes

Root-knot and cyst nematodes parasitize a wide range of commercially important crops. Although sources of nematode resistance have been identified and nematode resistant crop varieties have been developed, only eight nematode *R*-genes have been cloned and demonstrated to confer resistance to root-knot or cyst nematodes to date (Fig. 15.1). Others have been mapped to defined single loci likely to represent monogenic resistance traits (Williamson and Kumar 2006). Five of the eight cloned nematode *R*-genes encode proteins belonging to the largest class of R-proteins with

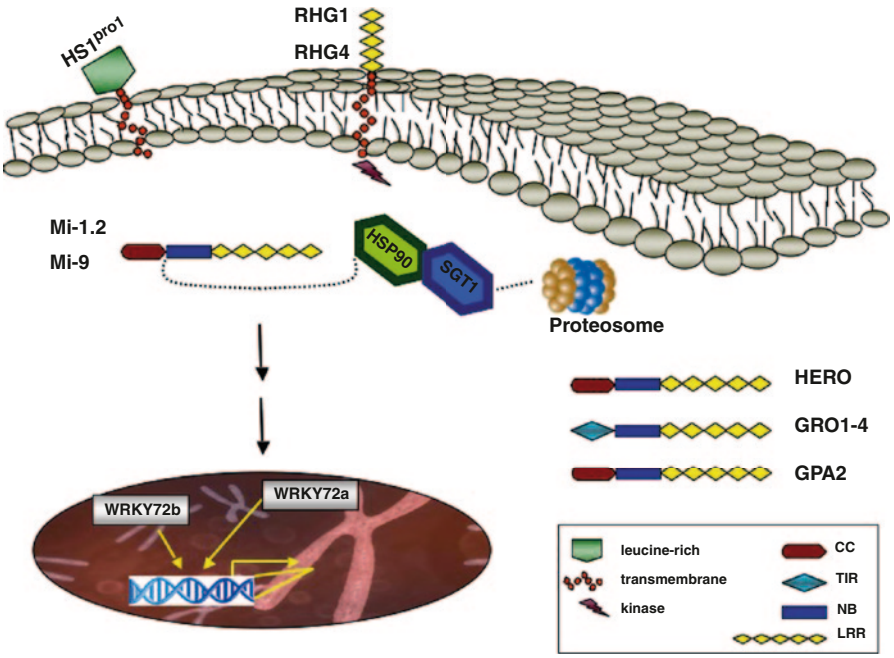

Fig. 15.1 Structures and localization of nematode resistance proteins and components of the Mi-1.2 signaling pathway. *Abbreviations*: CC, coiled-coil; TIR, toll-interleukin receptor-like; NB, nucleotide-binding; LRR, leucine-rich repeat

central nucleotide-binding (NB) and C-terminal leucine-rich repeat (LRR) domains (Fig. 15.1). These include *Mi-1.2*, *Mi-9*, *Hero, Gpa2* and *Gro1-4*, all of which are from Solanaceous plant species (Ernst et al. 2002; Jablonska et al. 2007; Milligan et al. 1998; Paal et al. 2004; van der Vossen et al. 2000; Desmond and Kaloshian unpublished results). Among these, *Gro1-4* encodes a NB-LRR protein with an N-terminal Toll-interleukin 1 receptor (TIR) domain, while the others encode N-terminal coiled-coil (CC) domain-containing NB-LRR proteins. The remaining three *R*-genes, $Hs1^{pro\text{-}1}$ from sugar beet and *Rhg1* and *Rhg4* from soybean, do not encode canonical NB-LRR proteins. $Hs1^{pro\text{-}1}$, the first cloned nematode *R*-gene, encodes a leucine-rich protein with a transmembrane domain, that has little similarity to other R-proteins, while the *Rhg1* and *Rhg4* encode proteins with extracellular LRRs, a transmembrane domain and a cytosolic serine-threonine kinase domain (Cai et al. 1997; Hauge et al. 2001; Lightfoot and Meksem 2002).

Mi-1.2 confers resistance to three species of root-knot nematodes, *M. arenaria, M. incognita,* and *M. javanica* (Dropkin 1969b; Roberts and Thomason 1986) while *Mi-9* confers resistance to *M. incognita* and *M. javanica* (Veremis et al. 1999). The resistance mediated by *Mi-1.2* in tomato is inactive at soil temperatures above 28°C, whereas *Mi-9*-mediated resistance in *Solanum arcanum* L. is functional at soil tempertaures as high as 32°C (Ammati et al. 1986; Dropkin 1969a; Veremis et al. 1999). The tomato gene *Hero A* confers resistance to *Globodera rostochiensis* populations and partial resistance to *Globodera pallida* pathotype $Pa_{2/3}$, while the potato *Gro1-4* confers resistance to *G. rostochiensis* pathotype Ro1. The sugar beet gene $Hs1^{pro\text{-}1}$ confers resistance to *Heterodera schachtii*. The soybean gene *Rhg1* requires an unlinked modifier allele to confer resistance to *Heterodera glycines* race 3 and race 14 across many soybean genotypes, while *Rhg4* confers resistance to *H. glycines* type 0 across a few soybean genotypes indicating the requirement for additional *R*-genes or modifiers for resistance (Concibido et al. 2004; Matson and Williams 1965).

15.2.1 Genomic Organization of R-genes

R-genes are often found in multi-gene clusters within the genome, arising from tandem gene duplication events (Dodds et al. 2001; Ellis et al. 2000; Lawrence et al. 2010; Martin et al. 2003; McDowell and Simon 2006; Michelmore and Meyers 1998; Radwan et al. 2008; Seah et al. 2004). This high level of duplicity is thought to be related to the aquisition of novel recognition specificities, in response to ever changing pathogen populations. Not surprisingly, four of the eight cloned nematode *R*-genes are also members of multigene clusters. The potato *Gpa2* gene lies within a cluster of four structurally related genes, of which two are described as functional *R*-genes. Interestingly, these two genes mediate recognition to phylogenetically distinct types of organisms. While *Gpa2* confers resistance to *G. pallida*, *Rx* confers resistance to potato virus X (van der Vossen et al. 2000). The *Gro-1* locus contains about 14 closely related genes but only *Gro1-4* is known to confer disease resistance (Paal et al. 2004).

Tomato *Hero A* is the only member with a described role in defense of a cluster of 14 NB-LRR genes localized within a 118 kb region (Ernst et al. 2002). The *Mi* gene family consists of seven paralogs arranged into two clusters of three and four members, respectively (Seah et al. 2004, 2007; Vos et al. 1998). This genomic organisation is thought to have occurred via a genetic duplication event (Seah et al. 2004). Only one of these genes, *Mi-1.2*, confers broad-spectrum resistance including resistance to three species of root-knot nematodes (Milligan et al. 1998), potato aphids (Rossi et al. 1998), whiteflies (Nombela et al. 2003), and psylids (Casteel et al. 2006). Although most of the remaining *Mi-1.2* paralogs are transcribed to detectable levels, it remains to be seen whether these genes confer resistance to nematodes and/or other types of pathogens or pests (Seah et al. 2004). Similar to the *Mi-1* family, the heat-stable root-knot nematode resistance gene *Mi-9* and its six paralogs seem to be clustered in two subclusters (Desmond and Kaloshian unpublished result).

15.2.2 Functional Domains and Autoinhibition of NB-LRR R-proteins

The NB and LRR domains are common structures of R-proteins. In NB-LRR proteins, the highly conserved NB domain is a part of a larger domain known as the NB-ARC that contains 3 sub-domains: the NB, ARC1 and ARC2 (Takken et al. 2006). The ARC1 domain consists of a 4-helix bundle while the ARC2 domain consists of a winged helix fold. The NB domain binds ADP or ATP depending on its activation state (Tameling et al. 2006; van der Biezen and Jones 1998). In contrast, the LRR domain is the most variable region and this characteristic is thought to be related to recognition of diverse pathogen elicitors. Its high variability likely originates from point-mutations combined with positive selection (Ellis et al. 2000; Jones and Dangl 2006; Martin et al. 2003; Nimchuk et al. 2003).

Most R-proteins have the potential to trigger hypersensitive response (HR) associated cell death, an effective defense response against biotrophic pathogens and some insect pests (Klingler et al. 2009; Martin et al. 2003). Thus, their activity must be tightly controlled. It appears that prior to pathogen recognition, R-proteins exist in an inactive state maintained by autoinhibition. In fact, domain swapping between the proteins encoded by *Mi-1.2*, the functional copy of the *Mi-1* locus, and its closely linked non-functional paralog *Mi-1.1* resulted in chimeras, a subset of which lacked this autoinhibition yielding a constitutive cell death phenotype when transiently expressed in *Nicotiana benthamiana* leaves. In addition, in *Agrobacterium rhizogenes* tomato transformation assays, expression of these chimeras inhibited the formation of hairy roots indicating lethality (Hwang et al. 2000; Hwang and Williamson 2003). Using Mi-1 and other NB-LRR R proteins, it was shown that physical interaction of the ARC2 domain and the LRR domain exerts negative regulation of R-protein activity (Rairdan and Moffett 2006), in a process directly related to ADP/ATP binding (Takken and Tameling 2009; Tameling et al. 2002). In the

inactive state, ADP is bound to the NB domain of R-proteins. This facilitates physical interaction between the LRR and ARC2 domains, stabilizing the inactive state. Recognition of an Avr effector or a host factor targeted by the effector, disrupts the association of the LRR and ARC2 domains and creates an open structural state that facilitates nucleotide exchange of ADP to ATP. By activation through binding to ATP, R-proteins recruit additional associates to form a signalosome that triggers defense responses resulting in incompatible interactions; however, the mechanism of this activation remains unclear (Takken and Tameling 2009).

15.2.3 Interactions of R-proteins with Nematode/Pathogen Effectors

In the past decade, a large number of pathogen Avr proteins from viral, bacterial, fungal, and oomycete origins have been identified that elicit resistance through NB-LRR proteins (Grant et al. 2006; Hogenhout et al. 2009; Kamoun 2006; Stergiopoulos and de Wit 2009). However, these Avr proteins are of diverse nature and share no conserved structural motifs. In the absence of R-proteins, it is believed that Avr effectors contribute to pathogen virulence by suppressing pathogen associated molecular patterns (PAMP)-triggered immunity. Another emerging theme is that interaction between R and cognate Avr effector(s) and recognition of pathogen attack can be indirect. Although in some cases direct binding of Avr factors to their cognate R-proteins has been demonstrated, R-proteins seem often to be guarding the pathogen virulence target ("guard model") or interacting with a protein that resembles the virulence target ("decoy model") (Dangl and Jones 2001; van der Hoorn and Kamoun 2008). Both models predict that R-proteins can be indirectly activated by interaction of effectors with host proteins that they target. Since R-proteins recognizing nematodes are similar to those recognizing pathogens, it is likely that R and Avr interactions in this pathosystem can also be indirect. It is hypothesized that such indirect modes of "altered-self" recognition provide the host with an evolutionary advantage over pathogens. In this case the host may retain more efficient adaptive control by reacting to effector-triggered disturbances of its regulatory circuits, rather than specific pathogen structures that could be quickly selected against in pathogen populations (Dangl and McDowell 2006). In addition, several pathogen effectors may interfere with the same host protein enabling a single R-protein to mediate resistance to multiple types of pathogens (Mackey et al. 2002). This may be a plausible mechanism for *Mi-1.2*-mediated recognition, as it allows perception of several root-knot nematode species as well as three distinct insect pests (aphid, whitefly and psyllid).

A number of root-knot and cyst nematode effector proteins have been identified that are synthesized in the oesophageal glands and secreted into the plant host (Chaps. 13 and 14). However, only modest advances have been made in identifying nematode effectors that determine or are associated with avirulent nematode populations (Bekal et al. 2003; Castagnone-Sereno et al. 2009; Lambert et al. 2005;

Neveu et al. 2003). To date, two examples exist where a direct role of such effectors has been demonstrated in regulating avirulence or triggering *R*-mediated resistance.

The *Cg1* gene, present in an *Mi-1*-avirulent RKN population, has been linked to avirulence on resistant tomato (Gleason et al. 2008). Avirulent *M. javanica* become virulent on *Mi-1*-containing tomato upon silencing of *Cg1*. Interestingly, silencing *Cg1* did not appear to alter nematode virulence on the susceptible host and therefore does not fit the expected role of an Avr protein. The lack of a definitive open reading frame in the *Cg-1* sequence suggests it may encode a non-coding RNA (Gleason et al. 2008). More investigation is needed to identify the nature and role of Cg1.

Recent work has shown a direct role for *G. pallida* RBP-1 as an Avr effector recognized by the potato *R*-gene *Gpa2* (Sacco et al. 2009). *Gp-Rbp-1* is expressed in the dorsal oesophageal gland and the encoded protein contains a secretion signal peptide and a SPRY (SP1a and RYanodine receptor) domain that is related to the GTpase-associated protein, Ran-Binding Protein in the Microtubule-organizing center (RanBPM) (Blanchard et al. 2005). Since the N-terminal CC domain of Gpa2 binds to Ran GTpase-activating protein 2 (RanGAP2), Sacco et al tested whether Gp-RBP-1 could be involved in this interaction (Rehman et al. 2009; Sacco et al. 2009). Transient expression of *Gp*-RBP-1 in leaves of transgenic *N. benthamiana* expressing Gpa2 resulted in activation of resistance responses and a HR while silencing *RanGAP2* abolished activation of these defense responses indicating that *Gp*-RBP-1 is the Avr effector recognized by the *Gpa2*-mediated resistance and confirming an essential role for RanGAP2 in Gpa2 function (Sacco et al. 2009). However, it is not clear whether RanGAP2 is the Gp-RBP-1 virulence target and attempts to show direct interactions between RanGAP2 and Gp-RBP-1 have not been successful (Sacco et al. 2009).

15.2.4 Resistance Proteins, Activation Complexes and Early Signaling

R-proteins seem to form hetero-multimeric recognition complexes that include heat-shock protein (Hsp) 90, Sgt1 and Rar1. The association with these accessory proteins is crucial for keeping R-proteins in an inactive but signaling-competent state (Azevedo et al. 2006; Boter et al. 2007; Kadota et al. 2008; Lu et al. 2003). The chaperon Hsp90 assists in proper folding of polyproteins, prevents unproductive intramolecular interactions and premature functioning of proteins. The co-chaperon Sgt1 may function as an Hsp90 client adaptor protein that recruits specific proteins to Hsp90-containing complexes. Rar-1 also interacts with Hsp90 and is involved in R-protein stability (Kadota et al. 2008).

The *Hsp70* and *Hsp90* genes were found to be transcriptionally induced during incompatible interactions with nematodes in soybean and cotton, respectively (de Deus Barbosa et al. 2009; Klink et al. 2009). *Hsp90* was also differentially regulated during incompatible tomato-root-knot nematode interaction and a role for *Hsp90* in *Mi-1.2*-mediated nematode defense was elucidated (Bhattarai et al. 2007, 2008).

The functional characterization of *Mi-1.2* defense responses is based primarily on virus induced-gene silencing (VIGS) assays. This powerful tool allows examination of the function of genes, by knocking-down their expression, and analyzing the resulting phenotype. Although VIGS-mediated gene silencing is frequently used to knock-down gene expression in above ground plant parts, silencing genes in roots remains challenging for unidentified reasons (Bhattarai et al. 2007, 2010). Using the tobacco rattle virus (TRV)-based VIGS system, the roles of several genes known to be required for the function of other *R*-genes, were explored in *Mi-1.2*-mediated resistance. Silencing *SlHsp90-1* attenuated *Mi-1.2*-mediated resistance to both aphids and nematodes, while silencing *SlSgt1-1* affected only aphid resistance. However, efficient silencing of *SlSgt1-1* resulted in lethality and *SlSgt1-1* silencing is likely to be inefficient in plants surviving attempted silencing of this gene. Thus, lack of any measurable effect on nematode resistance in *SlSgt1-1* silenced plants may simply reflect insufficient silencing. Therefore, it can not be excluded that *SlSgt1-1* is also required for *Mi-1.2*-mediated nematode resistance. *Rar-1,* which is required for the function of a subset of *R*-genes, is dispensable for *Mi-1.2* resistance to both aphids and nematodes (Bhattarai et al. 2007).

Another gene that seems to act early in *Mi-1.2*-resistance signaling is *Rme1*. *Rme1* was identified in a genetic screen of *Mi-1.2*-containing tomato with *M. javanica*. A recessive mutation in the *rme1* locus completely abolished resistance to not only *Mi-1*-avirulent *M. javanica, M. incognita,* and *M. arenaria*, but also to avirulent potato aphids and whiteflies (Martinez de Ilarduya et al. 2001, 2004). In *rme1* mutant plants, expression of an autoactive form of *Mi-1.2*, *MiDS4*, still activates defense responses (Martinez de Ilarduya et al. 2004), indicating that the defense pathway is intact in the mutant plants and suggesting that the *Rme1* gene product participates in *Mi-1.2*-mediated resistance at the same genetic step or prior to *Mi-1.2*. These observations are consistent with the possibility that *Rme1* is an effector target or bait molecule that is guarded by *Mi-1.2*. However, *Rme1* has not been cloned and nematode, aphid, or whitefly effectors have not been identified. Therefore, interactions between *Rme1*, *Mi-1.2*, and/or nematode/pest effectors remain to be determined.

15.3 Defense Responses During Incompatible Interactions

15.3.1 Transcriptional Changes and ROS During Incompatible Interactions

Nematode infections induce massive transcriptional reprogramming in both susceptible and resistant host roots. Although a wealth of information is available on transcriptional changes during the compatible interactions (Chap. 9), transcriptional changes and defense signaling pathways operating during the incompatible host-nematode interactions are poorly understood.

Large-scale gene expression analyses of host plants during incompatible interactions with nematodes have been performed with only a few plant species such as tomato (with root-knot nematodes) (Bhattarai et al. 2008; Schaff et al. 2007), soybean (with soybean cyst nematode) (Klink et al. 2007a, b, 2009), and cotton (with root-knot nematode) (de Deus Barbosa et al. 2009). None of these studies used whole genome arrays or comprehensive genome resources and are therefore incomplete. Bhattarai et al. utilized the TOM1 cDNA array representing 8,642 genes which does not include cDNAs from nematode infected tissues, while Schaff et al. used a cDNA array representing 1,547 genes preselected based on predicted roles in plant-nematode interactions. The studies by Klink et al. were performed with Affymetrix oligonucleotide arrays representing 35,611 genes while de Deus Barbosa et al. simply sequenced 1,826 cDNAs generated from resistant or susceptible cotton roots infected with *M. incognita*. Additional differences exist among these studies in sample preparation for the array probes (inoculated using seedlings in semi *in vitro* systems) or generation of the cDNA libraries (inoculated using seedlings in sand) and the time of tissue harvest after nematode inoculations (range from hours to days). In addition, the studies in tomato and cotton utilized entire roots while those in soybean utilized selected root cell types isolated by laser capture microdissection. These factors should be kept in mind when comparing gene expression data from these distinct studies.

In spite of these notes of caution, several general principles can be gleaned from these gene expression studies. Genes encoding lipoxygenase (LOX) enzymes were highly induced during incompatible soybean-*H. glycines* and tomato-*M. javanica* interactions (Bhattarai et al. 2008; Klink et al. 2007a, b, 2009). In addition, *LOX* genes were also induced in resistant pea roots and suppressed in susceptible roots at 48 h after *Heterodera goettingiana* inoculation suggesting a role for *LOX* in *R*-gene-mediated resistance (Leone et al. 2001; Veronico et al. 2006). LOX enzymes are involved in the synthesis of oxylipins that act as precursors for the hormone jasmonic acid (JA), and have been implicated in diverse functions within plants, including wound response, germination, development, senescence and resistance to pests and pathogens (Feussner and Wasternack 2002). Similarly, the *ZmLOX3* knockout mutant *lox3-4* was more susceptible to root-knot nematode compared to wild-type parent. Taken together these results suggest a positive role for *LOX* genes in nematode defense.

Several families of transcription factor (TF) genes are differentially regulated in incompatible soybean and tomato-nematode interactions. These include members of *Myeloblast* (*MYB*), *Basic helix-loop-helix* (*bHLH*), *Basic-leucine zipper* (*bZIP*) and *WRKY* families. Among these, defense-related roles have only been demonstrated for *SlWRKY72a* and *SlWRKY72b* in tomato, which act as positive regulators of both basal defense and *Mi-1*-mediated resistance to root-knot nematodes as well as to potato aphids (Bhattarai et al. 2010). In Arabidopsis *AtWRKY72* appears to control defense responses that are independent of SA-dependent immune processes (Bhattarai et al. 2010). Such processes may also be utilized by *Mi-1.2*-mediated resistance.

Genes encoding peroxidase enzymes were differentially regulated in both soybean and tomato by nematodes (Bhattarai et al. 2008; Klink et al. 2007b; Schaff et al. 2007). Members of the multigene family of plant class III peroxidases (*Prx*)

are involved in defense-related processes such as lignin and suberine deposition, cross-linking of cell walls, phytoalexin synthesis and production of reactive oxygen species (ROS) and reactive nitrogen species (Almagro et al. 2009). Several *Prx* genes, *TaPrx111*, *TaPrx112* and *TaPrx113*, are also differentially regulated in resistant and susceptible wheat cultivars by *Heterodera avenae* infection, with *TaPrx112* and *TaPrx113* showing higher induction during the incompatible interaction (Simonetti et al. 2009).

Production of ROS is well-characterized in roots during both compatible and incompatible tomato-root-knot nematode interactions, with major differences observed in timing and localization of their accumulation between both interaction types (Melillo et al. 2006). Transient ROS production has been observed during root-knot nematode penetration of both susceptible and *Mi-1*-resistant tomato roots and probably reflects a general response to wounding. However, sustained accumulation of ROS was only detectable in *Mi-1*-resistant plants, where it was specifically localized to cells and intracellular spaces surrounding the nematode head where a HR was observed (Melillo et al. 2006). Furthermore genes encoding hypersensitive-induced response (HIR) proteins were up-regulated in incompatible tomato and cotton by root-knot nematodes (Bhattarai et al. 2008; de Deus Barbosa et al. 2009). Over-expression of the pepper *CaHIR1* in *Arabidopsis* led to spontaneous necrosis and transcriptional induction of SA-responsive, but not JA-responsive, defense genes as well as elevated levels of H_2O_2 and SA (Jung and Hwang 2007), suggesting a role for SA in root-knot nematode defense. Another member of a gene family implicated in the HR process, glucosyltransferase, was also up-regulated in resistant tomato by root-knot nematode and is required for *Mi-1.2*-mediated root-knot nematode resistance (Schaff et al. 2007).

15.3.2 Defense Hormones

Three phytohormones SA, JA and ethylene (ET) are known to regulate both basal and *R*-gene mediated defense response. In general JA and ET signaling pathways seem to work synergistically whereas SA and JA/ET signaling pathways are antagonistic and communicate mainly through negative cross talk (Glazebrook 2005). To test for a role for SA in *R*-gene-mediated nematode defense, the *NahG* gene, encoding a salicylate hydroxylase enzyme that degrades SA into catechol, was used in tomato containing *Mi-1.2* root-knot resistance gene. In *NahG*-expressing hairy roots from nematode resistant tomato plants, decreased levels of SA were observed (Branch et al. 2004). These roots also exhibited partial loss of *Mi-1.2*-resistance to root-knot nematodes. In conflict with this result, *Mi-1.2*-resistant tomato plants expressing *NahG* were not measurably compromised in root-knot nematode resistance (Bhattarai et al. 2008). This discrepancy could be due to residual SA levels in the roots of *NahG Mi-1.2* tomato plants and may indicate that a low threshold level of SA is sufficient for *Mi-1.2*-mediated defense activation (Bhattarai et al. 2008).

Ethylene biosynthetic and/or ET responsive genes are differentially regulated during incompatible tomato- and soybean- nematode interactions (Bhattarai et al. 2008; Klink et al. 2007b; Schaff et al. 2007). However, there is currently no evidence for a role of ET in nematode defense. In fact, JA, ET and the phytohormone auxin seem all to promote susceptibility to nematodes (Bhattarai et al. 2008; Goverse et al. 2000; Grunewald et al. 2009; Karczmarek et al. 2004; Wubben et al. 2001, 2004). This may suggest that signaling pathways dependent on these hormones may be manipulated by virulent nematodes during the initiation of feeding structures, (Chaps. 9 and 16).

15.4 Resistance Mechanisms

The processes of penetration of host roots by root-knot nematodes and cyst nematodes and their migration towards the vascular bundle to establish a suitable feeding site are usually similar during both compatible and incompatible interactions (Cai et al. 1997; Das et al. 2008; Dhandaydham et al. 2008; Melillo et al. 2006). Recognition of the nematode is thought to occur after they pierce host cells and inject oesophageal gland secretions during initiation of the feeding site. During incompatible interactions, the subsequent defense response manifested as a HR can occur as early as 24 h after nematode inoculation as is the case with *Mi-1.2*-containing tomato (Dropkin 1969b; Paulson and Webster 1972; Riggs and Winstead 1959). This rapid resistance mechanism can be identified by a zone of cell death at the anterior ends of the invading nematode juveniles, effectively cutting them off from their nutrient supply.

In contrast, resistance can also be manifested later, after successful establishment of a feeding site. Infection of tomato plants containing *Hero A* with *G. rostochiensis* or *G. pallida*, potato plants containing *Gpa2* with *G. pallida,* or sugar beet plants containing $Hs1^{pro1}$ with *H. schachtii* results in establishment of a syncytium and allows the nematodes to become sedentary, a status that is indistinguishable from the compatible interaction (Cai et al. 1997; Holtmann et al. 2000; Koropacka 2010; Sobczak et al. 2005). However, in *Hero A*-, *Gpa2-, or* $Hs1^{pro1}$-containing plants, cells surrounding the syncytia eventually become necrotic and this is followed by the deterioration of the syncytia, preventing successful completion of the nematode lifecycle (Chap. 4). The timing of these effects suggests that, compared to *Mi-1.2*-mediated defenses, these *R*-genes act in a delayed manner possibly reflecting that their products only weakly recognize the respective nematode effectors (Sacco et al. 2009). It is also possible that the effectors recognized by these R genes are not produced or are not active until a later stage in the host-parasite interaction. During some of these delayed resistance responses the intruding juvenile nematodes predominantly develop into males, an epigenetic phenomenon observed under unfavorable conditions (Trudgill 1967).

Resistance to nematodes can also occur without an obvious HR or delayed necrosis, as observed in *Medicago truncatula* accession DZA045. In this case, infec-

tive juveniles that infect roots either simply die or develop as males (Dhandaydham et al. 2008). During interactions between resistant cowpea containing the *R*-gene *Rk* and *M. incognita*, normal nematode feeding occurs and nematodes develop to later juvenile stages but never reach maturity. The gradual deterioration of the feeding sites in these resistant cowpea plants and the reported lack of ROS production, points to a nematode resistance mechanism that does not involve a typical HR (Das et al. 2008). This diversity in mechanisms of resistance indicates the complex nature of plant interactions with nematodes.

15.5 Concluding Remarks

There is still a lack of information on many aspects of incompatible plant-nematode interactions. A major handicap is the absence of nematode resistance in the model plant species *Arabidopsis*, which is an invaluable resource for research on biological plant processes including disease resistance. As such, work on incompatible nematode-host interactions must be done with crop species, for which only limited genetic and genomic resources are available, such as near isogenic and mutant lines, completed genome and transcriptome sequences as well as commercially available full genome array platforms. In spite of these difficulties, our understanding of incompatible plant-nematode interactions is steadily growing. Improvements in crop transformation techniques, and the development of high-throughput sequencing technologies and novel approaches for functional analysis, such as the use of VIGS and tilling mutants, will enable identification of key nematode resistance signaling components. Currently, there is a shift within the plant research community toward a greater focus on crop systems and the use of knowledge gained from model systems (translational genomics) to solve problems in agriculture. This will continue to improve resources available for crop systems, an obvious advantage for future work on incompatible plant-nematode interactions.

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Chapter 16
The Role of Plant Hormones in Nematode Feeding Cell Formation

Aska Goverse and David Bird

16.1 Introduction

Phytohormones are small signalling molecules that regulate plant growth and development. In addition to the five classical plant hormones: auxin (reviews by Chapman and Estelle 2009; Friml 2010), ethylene (review by Lin et al. 2009), cytokinin (review by Werner and Schmulling 2009), gibberellic acid (reviews by Yamaguchi 2008; Hirano et al. 2008) and abscisic acid (Hirayama and Shimozaki 2007), five more hormones have recently been identified: brassinosteroids (Symons et al. 2008), jasmonic acid (Wasternack 2007), salicylic acid (Loake and Grant 2007), strigolactones (reviews by Dun et al. 2009; Westwood and Bouwmeester 2009) and nitric oxide (Grun et al. 2006). Moreover, it is becoming increasingly apparent that families of peptides regulate many plant processes, including organogenesis, meristem function and long-range signalling (Ohyama et al. 2008; Butenko et al. 2009; Wang and Fiers 2009), and rightfully should be considered to be hormones. Any comprehensive understanding of plant biology must incorporate the contributions of all these hormone classes.

Genetic screens and functional characterisation have revealed many proteins involved in hormone perception and signalling, including the receptors responsible for auxin, ethylene, cytokinin, gibberellic acid and jasmonate recognition, as well as the receptors for some classes of peptide hormones (Clark 2001). Comparison between hormones revealed that cytokinins, brassinosteroids and ethylene are perceived by the membrane-associated receptors CRE1, BRI1 and ETR1/ETR2/EIN4/ERS1/ERS2 respectively, and activate well-known signalling cascades. By contrast, auxin, gibberellic acid and jasmonate signalling involves the nuclear receptors TIR1, GID1 and COI1, respectively, and the ubiquitin-proteasome pathway (review by Santner and Estelle 2009).

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Plant hormones play pivotal roles in the integration of internal and external stimuli, including coordination of the plant's response to biotic signals emanating from mutualistic and parasitic symbionts. It seems very likely that for these organisms to establish an intimate interaction with their host must require that they manipulate the hormone balance in specific cell types, resulting, for example, in the development of root nodules in case of symbiotic rhizo-bacteria or highly specialized feeding structures in case of endoparasitic nematodes such as cyst and root-knot nematodes (CN and RKN respectively). For these nematode parasites it is widely believed that the primary signal for feeding site induction is the release of secretory proteins (described in Chap. 14) into the apoplast and/or symplast to induce a multinucleated cell complex that functions as a nutrient sink (described in Chaps. 4 and 5). The formation of such feeding cells is the result of dramatic changes in plant gene expression and the concomitant reprogramming of (un)differentiated root cells. Because plant hormones are important factors involved in transcriptional regulation of genes, manipulation of the local hormone balance (either directly or indirectly) is a likely means for nematodes to redirect gene expression patterns in plant cells. In fact, this is not a new idea. Older studies demonstrated the occurrence of auxin and its precursors in galls, egg masses and nematode juveniles, leading to suggestions that plant hormones may be involved in gall formation (Balasubramaniam and Rangaswami 1962; Viglierchio and Yu 1968; Bird 1975; Jones 1981; Bird 1996). Here we discuss recent advances in the role of plant hormones in the molecular mechanisms underlying feeding cell formation both by CN and RKN. The specific role of plant hormones in the incompatible interaction between these endoparasitic nematodes and their host plant species is discussed in Chap. 15.

16.2 Phytohormone-Associated Gene Expression Profiles in Feeding Cell Formation

To study the developmental changes in gene expression during nematode feeding cell formation, various high-throughput gene discovery techniques including micro-array based transcriptome profiling have been employed on infected root material. An excellent review describes the different techniques and their application in several endoparasitic nematode infected plant tissues (Li et al. 2009). Classification of the differentially expressed genes into different functional groups based on sequence homology to known genes as well as mapping to the Gene Ontology revealed that a significant proportion could be linked to plant hormones. This was shown both for plant tissue infected by CN (Swiecicka et al. 2009; Ithal et al. 2007a, b; Puthoff et al. 2007) and by RKN (Jammes et al. 2005; Fuller et al. 2007; Li et al. 2009), suggesting that hormones play a central role in the regulation of gene expression and the establishment of feeding cells. We note that it is important to distinguish between genes involved in hormone-regulated pathways and those genes that encode components involved in hormone synthesis and physiology. It is clear

from several studies that the expression of phytohormone-related genes is altered (induced or suppressed) at the onset of the parasitic interaction, and as noted below, genetic and biochemical data are consistent with this conclusion. This suggests that plant hormones are central to the nematode responsiveness of genes irrespective the plant-nematode combination and involved in two early steps in the compatible interaction, *viz.* the modulation of plant immunity and the initiation of a feeding cell.

Many genes differentially expressed in response to nematodes have annotation paths linking them to the hormones auxin and ethylene, and a minority is associated with other hormones, including GA, cytokinin and ABA (reviews by Li et al. 2009; Gheysen and Mitchum 2009). The role of auxin and ethylene in feeding cell development—especially CN-induced syncytia—has been investigated in quite some detail, and the emerging picture of an auxin and ethylene mediated regulatory network is comprehensively discussed below. Similarly, peptide hormones have been implicated in syncytial formation (Mitchum et al. 2008), and as discussed below, analysis of the available RKN genomes suggests that their role may be highly significant in giant cell induction. Although less is known about the role of cytokinin, this hormone is nonetheless important, and the current understanding is presented. The function of the other plant hormones in nematode-plant interactions remains poorly understood, and they will be excluded from this review; further analysis clearly is required.

16.3 Auxin

Auxin or IAA (indole-3-acetic acid) is a small secondary plant metabolite that is involved in numerous developmental processes. For example, auxin is a key factor in the formation of lateral roots and is involved in apical dominance and gravitropism. This plant hormone is primarily produced in the apical part and young leaves of the shoot and is transported to the root in a polar fashion from cell to cell (polar auxin transport or pat). Auxin influx into the cell is dependent on a proton pump and the efflux of auxin and its distribution in various tissues is mediated by so called PIN proteins. In the root apex, auxin levels reach a maximum in the stem cell niche, where it is required for stem cell specification and meristem formation.

16.3.1 Auxin-Mediated Regulatory Networks in Nematode Feeding Cells

The fact that nematode infection of roots leads to the transcriptional activation or suppression of genes points to the occurrence of local changes in endogenous hormone levels. For auxin, the first evidence for a role in feeding cell development was obtained from biochemical studies that showed an accumulation of indole com-

pounds in galls induced by the RKN species *Meloidogyne incognita*, *M. hapla* and *M. javanica* (Balasubramaniam and Rangaswami 1962). In addition, an increase in local auxin in nematode feeding cells was detected by the early activation of the auxin-responsive promoter GH3 (Hutangura et al. 1999) and the synthetic auxin-responsive promoter DR5 (Karczmarek et al. 2004). The DR5 promoter contains multiple Auxin Response Elements (*AuxRE*) from GH3 and is considered to be a marker for changes in auxin levels in roots (Ulmasov et al. 1997). This *AuxRE* consists of a 5'-TGTCTC-3' DNA sequence motif, which is required for auxin responsiveness of genes (Guilfoyle et al. 1998). Proteins encoded by the ARF (Auxin Response Factor) multigene family bind to this or similar DNA motifs, resulting in the activation or repression of gene expression. ARF-dependent transcription is controlled by Aux/IAA proteins, which define another large family of transcription factors. They do not bind to DNA directly, but form heterodimers with the ARF proteins resulting in the repression of auxin-regulated expression. Aux/IAA proteins are short lived and their degradation, which is promoted by auxin, leads to de-repression and subsequent activation of auxin-induced gene expression (reviews by Santner and Estelle 2009; Chapman and Estelle 2009).

In silico analyses of nematode-responsive genes implies that some are auxin responsive. In particular, auxin-response elements were found in the promoter sequences of genes responsible for cell wall degradation in early stages of feeding cell formation, including the beta-endoglucanases Nt-Cel7 (Wang et al. 2007), Sl-Cel7 (Karczmarek et al. 2008; Swiecicka et al. 2009) and At-Cel2 (Wieczorek et al. 2008). This suggests that a local accumulation of auxin upon infection results in the transcriptional activation of genes involved in specific developmental pathways during feeding cell formation. Hence, auxin is one of the key regulatory factors involved in coordinating the dramatic changes in gene expression observed upon nematode infection (a schematic overview is presented in Fig. 16.1a). Early observations of differential expression of the auxin-repressed genes ADR6, 11 and 12 in soybean roots infected by soybean cyst nematode (SCN: *Heterodera glycines*) support this hypothesis (Hermsmeier et al. 1998). However, in case of the auxin-inducible WRKY23 gene—which acts downstream of the Aux/IAA protein SLR/IAA14 in non-infected *Arabidopsis* plants—it was postulated that auxin-independent signals might be involved in the activation of its expression in early stages of feeding cell development (Grunewald et al. 2008).

Genetic analysis, in which a series of *Arabidopsis* mutants was screened for the effect of auxin transport, auxin homeostasis and auxin responses, points to a clear role for auxin in syncytium development. A significant reduction in beet cyst nematode (BCN: *Heterodera schachtii*) development was observed for the dominant gain-of-function mutant *axr2*, and the two mutants *pin1-1/ttg-1* and *pin2/eir1-1* (Goverse et al. 2000b). The *axr2* mutant is auxin insensitive due to a mutation in the AXR2 gene, which encodes for the protein IAA7. AXR2/IAA7 is a member of the auxin-inducible Aux/*IAA* (indole-3-acidic acid) gene family, which controls auxin transcriptional responses. Apparently, AXR2/IAA7 is an important transcription factor in controlling auxin-mediated responses in cyst nematode induced feeding cell development. The role of IAA and ARF in auxin signalling during feeding

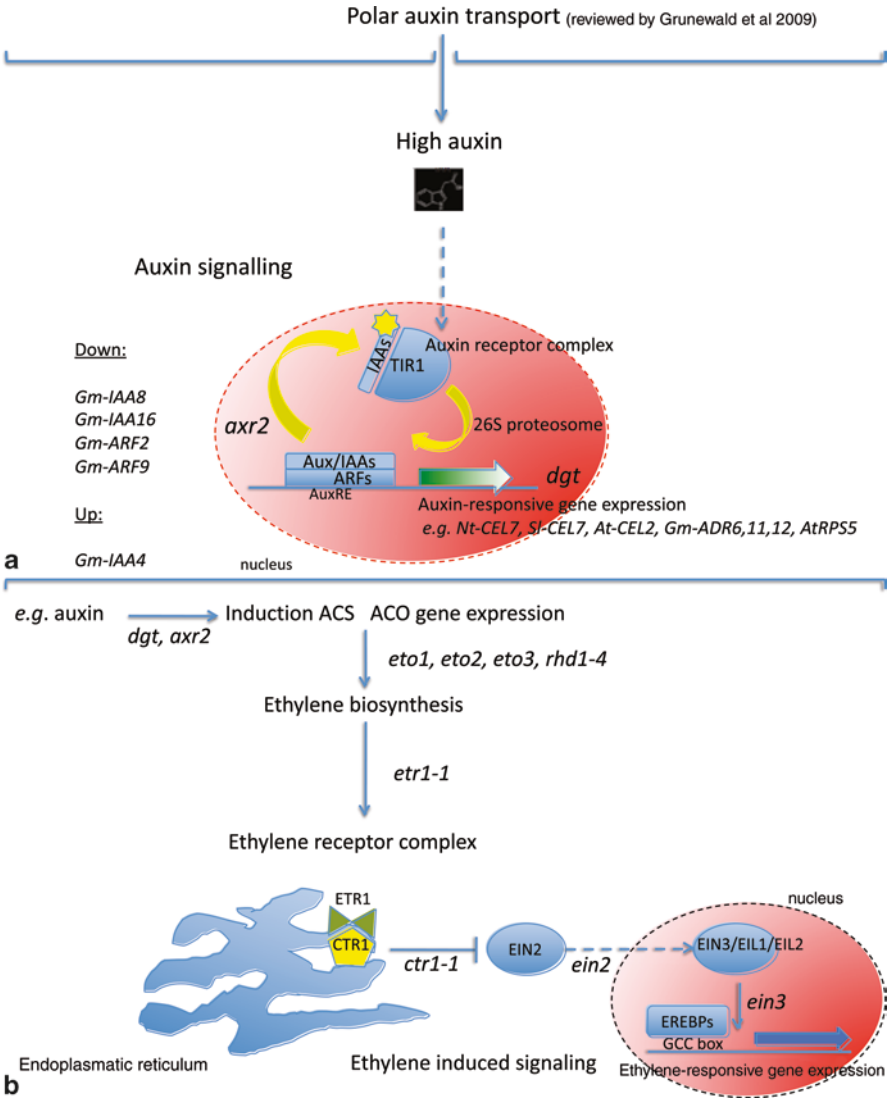

Fig. 16.1 Schematic overview of the role of auxin and ethylene in feeding site development. High-throughput transcriptome profiling has facilitated the identification of an increasing number of novel hormone related genes, which are differentially expressed during feeding cell development. Together with dedicated functional studies, a picture has emerged which points to these gene expression changes most likely being due to a local increase in auxin (Fig. 16.1a) and ethylene (Fig. 16.1b) concentrations. Such changes are sensed by the corresponding hormone receptors, thus eliciting activation of signalling pathways and redirection of gene expression. In case of auxin it is apparent that interference in polar auxin transport causes a local increase in auxin levels, whereas for ethylene *de novo* biosynthesis seems to be responsible for elevated levels. How nematodes are able to manipulate these processes remains to be resolved, but further characterisation and functional analysis of secreted nematode proteins (Chap. 14) may allow us to elucidate the underlying mechanisms

cell development is supported by a transcriptome profiling study of soybean roots challenged with *H. glycines* (Ithal et al. 2007b). The IAA4/AU gene was found to be strongly up-regulated at the onset of feeding, whereas an additional set of IAA (IAA8 and IAA16) and ARF (ARF2 and ARF9) encoding genes were shown to be rapidly down-regulated during the infection process.

To further examine the role of auxin in feeding cell development, a natural tomato auxin insensitive mutant *Diageotropica* (*dgt*) was challenged both with CN and RKN (summarized by Gheysen and Mitchum 2008). These studies demonstrated that *M. incognita* (Richardson and Price 1984) and the potato cyst nematode (PCN: *Globodera rostochiensis*) both were impaired in their development due to an arrest in early feeding cell formation (Goverse et al. 2000b). These results support the observations that auxin is involved in both syncytium and giant cell formation. The *dgt* mutant contains a unique single point mutation in the coding sequence of the cyclophilin gene *LeCyp1*, resulting in a strong reduction in LeCYP1 levels (Oh et al. 2006). It was shown that LeCYP1 is required for auxin-induced expression of the early auxin response genes LeIAA10 and LeIAA11, which is impaired in the *dgt* mutant. Apparently, the peptidyl-prolyl isomerase activity of the tomato CYP1 protein is important for auxin signalling in nematode feeding cells.

16.3.2 Polar Auxin Transport (Pat) Is Involved in Feeding Cell Establishment

The *Arabidopsis* mutants *pin1-1/ttg1* and *pin2/eir1-1* are hampered in polar auxin transport. So, the observed arrest in syncytium indicated that not only auxin-mediated signalling is important in nematode feeding cell development, but also auxin transport and its distribution. PIN proteins are asymmetrically localised plasmamembrane proteins involved in auxin efflux. Shuttling of these proteins between the plasmamembrane and the endosomal parts of the cell allows dynamic changes in their localisation and the redirection of auxin to other parts outside the cell. In this way, auxin is transported from cell-to-cell from its main production site in the shoot apex towards the roots, where it is distributed into the root meristem. Extensive analysis of this protein family in root development has elucidated the role of each PIN protein in specific developmental processes and cell types (review by Friml 2010).

Disruption of dynamic auxin transport in these mutants could account for the lack of auxin accumulation upon the induction of a feeding cell, which may lead to a reduced auxin response important for proper feeding cell development. Alternatively, an impaired polar auxin transport system may block the specific re-distribution of auxin during feeding cell development and the subsequent loss of pattern formation involved in feeding cell morphogenesis. This hypothesis is supported by the formation of disorganised syncytia by the potato cyst nematode in tomato roots upon the application of the auxin transport inhibitor naphthylphthalamic acid, whereas in untreated control roots normal syncytia were induced via a so called

cortical bridge (Goverse et al. 2000b). Hence, auxin could act as a morphogen in syncytium expansion towards and alongside the vascular tissue by progressive cell wall dissolution and the incorporation of neighbouring cells. The gradual and transient accumulation of auxin in cells adjacent to the initial syncytial cell may point at a role for auxin in the reprogramming of neighbouring root cells before incorporation into the expanding syncytium (Grunewald et al. 2009b). This hypothesis is supported by the transcriptional activation of the auxin-responsive 5-1E1 promoter in cells, which are about to be incorporated in *H. schachtii* induced syncytia upon infection of *Arabidopsis* (Goverse et al. 2000b). The 5-1E1 promoter controls the expression of the *RPS5A* gene, which encodes the Ribosomal Protein S5 involved in protein translation during the proliferation of cells (Weijers et al. 2001).

To understand the mechanisms underlying the role of polar auxin transport in nematode-induced feeding cell development, the expression of members of the PIN protein family was investigated extensively in *Arabidopsis* roots infected with BCN (Grunewald et al. 2009a). Members of the PIN protein family are auxin efflux carriers, which are asymmetrically localised at different sides of the cell resulting in a specific, partially overlapping polar distribution pattern for each PIN protein. Infection of transgenic *Arabidopsis* plants harbouring the gus reporter gene fused to either the PIN1, 2, 3, 4 and 7 promoter resulted in the detection of a strong activation of the PIN3 and PIN 4 promoter in young feeding cells, whereas PIN1 and PIN7 showed an opposite response suggesting downregulation of the encoding genes upon cyst nematode infection. Interestingly, PIN3 was also shown to be upregulated in SCN-infected *Glycine max* roots (Ithal et al. 2007b). Furthermore, the relocation of PIN3 and 4 was demonstrated in a time course experiment and challenging of a series of single and double pin mutants revealed that both acropetal auxin transport mediated by PIN1 and PIN7, as well as induced translocation of auxin by PIN3 and PIN4 is required for syncytium development. An integrated model for cyst nematode manipulation of the auxin transport machinery in plant roots is presented in Grunewald et al. (2009b).

16.3.3 Modulation of Local Auxin Concentrations by Endoparasitic Nematodes

Apparently, a local increase in auxin in feeding cells of both giant cells and syncytia is one of the earliest responses upon nematode infection. This leads to the question how such a change in auxin is achieved by the nematodes. It has been hypothesized that manipulation of polar auxin transport upon nematode infection could cause the observed accumulation of auxin in young feeding cells. However, how this is achieved remains to be resolved. It has been proposed that the production of isoflavonoids upon nematode infection could result in a local change in auxin as they function as polar auxin transport inhibitors (Hutangura et al. 1999). If so, one could expect a similar arrest in nematode development upon infection of plants, which are compromised in isoflavonoid production, as observed for the pat mutants. However,

testing a series of mutants impaired in isoflavonoid biosynthesis did not result in a significant effect on nematode development or feeding cell formation, neither syncytium development (Jones et al. 2007; Goverse, unpublished results) nor giant cell formation (Wuyts et al. 2006; Wasson et al. 2009). An alternative explanation is that an increase in flavonoid production occurs as part of a local defence response upon nematode invasion of the root as proposed by Jones et al. (2007). The recent discovery of nematode-induced rearrangements in PIN and AUX/LAX proteins led to the hypothesis that secretory proteins or peptides directly interfere with auxin transport regulators like PID and PP2A, or that auxin itself is involved in this process (Grunewald et al. 2009a).

Another explanation for a local increase in auxin is the release of indolic compounds in the root by the nematodes upon feeding cell induction. The presence of auxin in nematode saliva was first postulated by Goodey (1948) and circumstantial evidence was obtained in a series of experiments with various plant parasitic nematode species (review by Goverse et al. 2000a). In 2005, De Meutter et al. (2005) confirmed the presence of auxin in secretions from the BCN under axenic conditions. Alternatively, instead of mimicking auxin by the secretion of indolic compounds, infective nematodes may manipulate auxin homeostasis in a more indirect way *via* the release of enzymes that interfere with local auxin biosynthesis in plants. The identification and functional analysis of a cyst nematode gene encoding chorismate mutase, an enzyme involved in the conversion of the auxin precursor tryptophan, suggests that nematodes may be able to redirect the biosynthesis of auxin *in planta* (Jones et al. 2003).

Besides the manipulation of auxin homeostasis or polar transport, nematodes might also cause a change in auxin sensitivity and perception by targeting auxin-receptors or auxin signalling components. For example, the rapid auxin-independent induction of WRKY23 expression in less than 12 h post infection could be explained by the hijacking of plant gene expression by the release of signalling molecules of the parasitic nematode into the initial feeding cell. This hypothesis is supported by the detection of low molecular weight compounds in PCN secretions, which were shown to stimulate tobacco protoplast proliferation in the presence of both auxin and cytokinin (Goverse et al. 1999). Recently, transcripts were identified in the dorsal oesophageal glands of *G. rostochiensis*, encoding small peptides called SECPEPs and NEMPEPs (van Bers 2008). Preliminary data suggest that overexpression of these nematode peptides can interfere with plant developmental processes, but their exact role in feeding cell establishment needs to be resolved. We return to the topic of nematode-encoded mimics of plant peptide hormones below.

16.4 Ethylene

Ethylene or ethene is a simple gaseous compound, which can be produced by all plant cell types in response to developmental or environmental cues, either biotic or abiotic. Ethylene production is often associated with wounding and stress, but plays

also an important role in meristematic tissues and during fruit ripening (Abeles et al. 1992). Despite its simple nature, the biosynthesis of ethylene is complex. This is illustrated by the complexity of the regulation of ethylene production by the activity of two key enzymes, ACC synthase and ACC oxidase encoded by large multigene families.

16.4.1 Ethylene Biosynthesis in Feeding Cell Formation by Cyst Nematodes

It has long been known that ethylene is produced upon nematode infection of roots (Glazer et al. 1983, 1985), but this was thought to be a secondary response to biotic stress upon root invasion. However, functional analysis of *Arabidopsis* mutants compromised in ethylene production and signalling established that this hormone is essential for proper feeding cell formation by BCN (a schematic overview is presented in Fig. 16.1b). Infection of the ethylene overproducing mutants *eto1*, *eto2* and *eto3* resulted in hyper-susceptibility (Wubben et al. 2001; Goverse et al. 2000b), indicating that increased ethylene levels promote the initiation of syncytia in roots. Histological analysis showed the enlargement of the feeding structure and enhanced cell wall dissolution between neighbouring cells. An increase in the formation of syncytial cell wall ingrowths along the vascular tissue suggests that solute exchange is enhanced in these syncytia, which is supported by the development of enlarged adult females on the mutant roots. Hence, a primary role for ethylene in cell wall modification during syncytial development was postulated (Goverse et al. 2000).

The *eto2* and *eto3* mutants contain mutations in the C-termini of two ACC oxidase (ACO) isoforms ACO5 and ACO9, key enzymes involved in the conversion of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) into ethylene. The mutant ACO isozymes show an increased enzymatic activity, resulting in elevated ethylene production (Chae et al. 2003). The *eto1* mutant is a negative regulator of ethylene production, as ETO1 binds directly to ACS5 thereby inhibiting its enzymatic activity (Wang et al. 2004; Yoshida et al. 2005). If *de novo* ethylene production is essential for syncytial development, an induction of genes involved in the ethylene biosynthetic pathway is expected upon infection of the roots. In a recent semi-quantitative real time PCR study, Tucker et al. (2010) investigated the gene expression patterns of 17 members of the ACC synthase (ACS) multigene family during feeding cell formation by *H. glycines*. ACS codes for the enzyme ACC synthase, which converts S-adenosyl-methionine (SAM) into the ethylene precursor ACC, a rate-limiting step in ethylene biosynthesis (Yamagami et al. 2003). For a subset of five ACS genes, they could show a transient increase in transcript abundance in early stages of the infection process followed by prolonged elevated transcript levels during later stages of the interaction up to 20 days post infection.

An unexpected down regulation of other ACS and ACO genes was observed in soybean roots infected with *H. glycines* (Puthoff et al. 2007 and Ithal et al. 2007b).

A plausible explanation for these results is that gene expression was determined for a different set of genes and in a later stage of syncytium development compared to the previous study. The expression of ACS and ACO family members is tightly regulated during developmental processes in plants by external and internal stimuli. Hence, differential expression of specific ACS and ACO genes in nematode infected roots may reflect distinct roles for each family member in different stages of syncytium formation. To elucidate their exact role in nematode infected roots, more detailed studies are required to determine the local spatial and temporal distribution of specific ACS and ACO transcripts in feeding structures. Furthermore, functional analysis of these genes will elucidate their role in nematode parasitism. It is interesting to note that expression of several auxin-responsive ACS genes was reduced in the tomato mutant *dgt* (Abel and Theologis 1996) and the *Arabidopsis* mutant *axr2* (Abel et al. 1995), so the observed arrest in nematode feeding cell development on these mutants as described above could be the result of a defect in ethylene biosynthesis (Goverse et al. 2000b).

Interestingly, Wubben et al. (2001) identified an *Arabidopsis* mutant *rhd1-4* with phenotypic similarities to the *eto* mutants, including hyper-susceptibility to *H. schachtii.* To assess the role of ethylene in feeding cell development, the authors tested several chemical compounds on infected wild type plants to inhibit ethylene synthesis (aminoethoxyvinylglycine and silver nitrate) or to promote ethylene production by the application of the ethylene precursor, ACC. This study supported the findings that ethylene plays a role in susceptibility to CN. Further examination of the infection process on roots of the *rhd1-4* and *eto3* mutants revealed that the hyper-susceptibility phenotype may be caused by root exudates released by these mutants, which seem to enhance host attraction of *H. schachtii* juveniles prior to root penetration. The mutation in the UDH-glucose-4-epimerase gene RHD1, which resulted in hyper-susceptibilty to *H. schachtii* was shown to be dependent on EIN2 and EIN3 mediated ethylene signalling, but not on the auxin efflux factor EIR1. Furthermore, it was demonstrated that in wild type plants RHD1 gene expression was reduced upon infection of the roots with *H. schachtii*. This suggests that RHD1 is negatively regulated by ethylene, and as such it could explain the hyper-susceptibility of the loss-of-function mutant *rhd1-4* to beet cyst nematodes (Wubben et al. 2004).

16.4.2 Ethylene-Mediated Regulatory Networks in Feeding Cell Development

A positive role for ethylene mediated signalling in cyst nematode-induced feeding cell development became apparent after the screening of several *Arabidopsis* mutants compromised in ethylene sensitivity (Wubben et al. 2001). A significant reduction in *H. schachtii* development was observed upon infection of the dominant ethylene insensitive mutant *etr1-1,* which encodes a two-component histidine kinase-like receptor located in the membrane of the endoplasmatic reticulum. The

mutant form is unable to bind ethylene, which indicates that ethylene perception and downstream signalling via ETR1 is an important aspect in early syncytium formation. This is supported by the results obtained with the *ein2* mutant, as infection of the *ein2* mutant also resulted in an arrest in nematode development. EIN2 (ETHYLENE INSENSITIVE2) acts as a positive regulator of the ethylene signalling pathway downstream of the receptor-CTR1 complex (Alonso et al. 1999). Similar results were obtained for *ein3*, which encodes the transcription factor EIN3 involved in the amplification of ethylene responses in the nucleus (Solano et al. 1998). These findings are supported by the induction of the tomato gene encoding the EIN3-like protein EIL1/EIL2 during the formation of syncytia by PCN (Swiecicka et al. 2009), for this protein is also involved in the transcriptional regulation of ethylene-responsive genes (review by Yoo et al. 2009).

The occurrence of ethylene-mediated signalling in nematode induced feeding sites results in the transcriptional activation of ethylene-responsive gene expression as shown in various transcriptome analyses. This is achieved by the binding of EREBPs, a family of plant transcription factors, to the GCC box, which is an ethylene responsive promoter element. Interestingly, a differential display study of susceptible *Arabidopsis* roots challenged with *H. schachtii* revealed changes in the abundance of transcripts with sequence homology to genes encoding an AP2 domain DNA binding protein and the ethylene response element binding protein AtEBP. Both the AP2 and the EBP bind to ethylene response promoter elements and hence, belong to the EREBP family (Hermsmeier et al. 2000). Furthermore, the differential regulation of the gene *GmEREBP1* upon *H. glycines* infection of soybean roots (Mazarei et al. 2002) provides additional support for the hypothesis that ethylene responsive gene expression in feeding sites is mediated by this type of transcriptional regulators. Specifically, GmEREBP1 was shown to be down-regulated in susceptible plants, whereas it was induced upon infection of resistant plants. This suggests that GmEREBP1 acts as a positive regulator of ethylene-mediated defence responses (Mazarei et al. 2007).

The role of ethylene in RKN infection is less clear. In one experiment a transgenic *Lotus japonicus* line expressing the *Arabidopsis etr1-1* gene was challenged with *M. incognita*. Although the dominant *etr1-1* allele (Chang et al. 1993) confers both an ethylene resistant and a rhizobial hyper-nodulation phenotype to the *Lotus* plants, infectivity of the transgenic lines by the nematode was indistinguishable from wild-type *Lotus* (Lohar and Bird 2003).

16.5 Cytokinin

Cytokinins are a family of adenine-like molecules generally assumed to be essential for meristematic cell division, presumably through their influence on the cell cycle (Redig et al. 1996; Zhang et al. 1996), where they can act at the G1/S-phase transition to initiate division in non-cycling cells (Francis and Sorrell 2001). Cytokinins are synthesized primarily in root tips from nucleotide triphosphates and

subsequently transported to the shoot via the xylem (Davies 1995). Genetically identified cytokinin response genes have been cloned from *Arabidopsis* (Schmülling 2002) and examination of their expression, particularly of the *Arabidopsis* Response Regulator (*ARR*) genes, has been used to map the cytokinin response *in planta* (D'Agostino and Kieber 1999).

16.5.1 Cytokinin-Mediated Regulatory Networks in Feeding Cell Development

During the 1960s, a number of whole plant studies (e.g., Krupasagar and Barker 1969) revealed elevated cytokinin levels in RKN-infected plants. However, experiments in which cytokinin was applied to plants failed to show increased infection by RKN (Dropkin et al. 1969), although application of exogenous cytokinin to a tomato cultivar carrying the *Mi* gene, which conditions resistance to certain RKN species, resulted in loss of resistance (Dropkin et al. 1969). Collectively, these studies implied a role for cytokinin in the nematode-plant interaction, but the mechanism underlying this effect was not apparent. Nonetheless, because giant cell formation clearly involved re-entry into the cell cycle (although not cellular division), researchers continued to implicate cytokinin. In particular, the use of bioassays showed that RKN could produce biologically active cytokinin (Bird and Loveys 1980; De Meutter et al. 2003), although the role of such activity in the parasitic interaction remains questionable.

Using the *ARR5* promoter driving reporter constructs in transgenic plants, Lohar et al. (2004) undertook a comprehensive analysis of cytokinin responses during plant development and RKN infection. A response was not evident upon RKN penetration, nor during migration in the cortex, but once the juveniles reached the differentiating vascular bundle, which is the site of giant cell induction, strong *ARR5* expression was observed. Intriguingly, it appeared that the cytokinin response occurs before the juveniles reach the differentiation zone. Unfortunately, spatial mapping of *ARR5* expression was not sufficient to establish if a cytokinin response occurs in those vascular parenchyma cells destined to become giant cells (Lohar et al. 2004), but that seems likely. Experiments with cell cycle inhibitors (de Almeida Engler et al. 1999) revealed an initial transient requirement for cell cycle activation during giant cell formation, whereas later application of hydroxyurea and oryzalin failed to impact nematode development. This observation is consistent with a transient requirement for cytokinin in giant cell formation. In an elegant experiment that exploited the fact that the *Mi* gene is temperature sensitive, Dropkin (1969) demonstrated that the ability of the *Mi* locus to confer resistance to RKN also is temporally restricted to the initial period of giant cell induction. Taken together with the aforementioned observation that exogenous cytokinin application suppresses resistance (Dropkin et al. 1969), this result points to a transient need for cytokinin at the initiation of giant cells.

To more directly assess the role for cytokinin in feeding cell formation, Lohar et al. (2004) employed the transgenic expression of cytokinin oxidase genes to directly modulate cytokinin levels. Not surprisingly, these experiments revealed a correlation between the number of feeding sites induced by RKN, and the *in planta* level of cytokinin. More recently, microarray experiments have been employed to directly measure the expression of cytokinin-related genes in SCN infected roots (Ithal et al. 2007b), revealing several differentially-regulated examples. Placing these genes into the appropriate regulatory cascades will likely be quite informative as to the precise role of cytokinins in the nematode-plant interaction.

16.6 Peptide Hormones

The first plant peptide recognized as being a *bona fide* hormone was the tomato protein systemin (McGurl et al. 1992). Systemin functions as a long-distance signal to activate defenses against herbivores, and is processed to its active, 18 amino acid form from a 200 residue pre-pro-protein. It is now appreciated that peptide signaling plays a significant role in plant growth and development and plants encode large families of trans-membrane receptor kinases that perceive the hormone ligands.

16.6.1 CLAVATA Elements as Plant Developmental Regulators

Originally discovered as a regulator of apical meristem identity (Clark et al. 1996), the Clavata system (CLV) is a paradigm for a plant hormone-receptor complex. Briefly, CLV has three components: a 12 amino acid peptide hormone (CLV3), a transmembrane, leucine-rich repeat receptor-like kinase (CLV1), and a membrane-anchored receptor (CLV2). CLV1 and CLV2 form a heterodimer to bind the CLV3 ligand secreted into, and proteolytically-processed in, the apoplast, thereby effecting short-range, cell-to-cell communication. Perception of CLV3 initiates a signal transduction cascade culminating in the activation of the Wushel (WUS) transcription factor (Carles and Fletcher 2003). The ligand, CLV3, is actually a member of a gene family collectively termed Clavata-Like Elements (CLE); there are 32 CLEs in *Arabidopsis* and 47 CLEs in rice (Kinoshita et al. 2007). Given the number of CLEs and also the large number of transmembrane receptors in plants, opportunities for CLV-type regulation are large. Such receptor complexes are likely to be involved in maintaining the balance between cell proliferation and differentiation in the root apical meristem. Evidence also suggests that there may be two counteracting pathways in peptide-receptor signalling: one that promotes stem cell differentiation and one that inhibits it. For instance, CLV3 represses stem cell proliferation in the meristem and enhances tissue differentiation. In contrast, another CLE peptide, Tracheary elements Differentiation Inhibitory Factor (TDIF), functions as its name suggests to maintain the meristematic nature of vascular parenchyma cells (Oldroyd and Downie 2008). Interestingly, it has been hypothesized that giant cells are partially differentiated tracheary elements (Bird 1996).

Although the role of CLV-signalling in roots has not been exhaustively explored, mis-expression of AtCLE40 from the 35S promoter results in terminal differentiation of the root meristem, while *cle40* loss-of-function mutants show an enhancement of root waving (Hobe et al. 2003). Applying synthetic peptides corresponding to CLV3, CLE19, and CLE40 to *Arabidopsis* roots mimics the over-expression phenotypes of root meristem consumption (Fiers et al. 2005). In a more comprehensive experiment, Whitford et al. (2008) classified *Arabidopsis* CLEs as either promoting cell differentiation in root and shoot apical meristems (class A) or not (class B). Instead, class B peptides (e.g., CLE41) may inhibit root differentiation, such as of *Zinnia elegans* tracheary elements.

16.6.2 Nematode-Encoded CLE Mimics

A picture is emerging to suggest that RKN and CN also encode and secrete peptides that can mimic plant peptide-hormones. Although this model was proposed some time ago (Bird 1996), the first corroborative evidence came from a computational screen revealing that the SYV-46 peptide from *H. glycines* likely encoded a CLE-like ligand (Olsen and Skriver 2003). SYV-46 had previously been experimentally identified as a protein secreted from the SCN stylet (Gao et al. 2001), consistent with it playing a direct role in parasitism. Not only does the SYV-46 protein bind CLV2, but the *syv-46* gene also is able to complement the *Arabidopsis clv3-1* mutant (Wang et al. 2005), strongly implicating this peptide as being a genuine CLE. The *syv-46* gene appears to have undergone a recent duplication, as the SCN genome contains a second copy, differing by just 3 bases outside the CLE domain. In PCN, the CLE family has expanded and diversified further (Lu et al. 2009). Not surprisingly, cyst nematode CLEs are the subject of ongoing and active research (Davis et al. 2008; Mitchum et al. 2008; see Chap. 14).

The precise role of CLEs in the RKN-host interaction is less well established. Examination of the *M. incognita* (Abad et al. 2008) and *M. hapla* (Opperman et al. 2008) genomes reveals five and eight candidate CLE loci respectively. Mapping these genes to their presumed analogues in *Arabidopsis* implies that the RKN genomes encode both type A and type B CLEs (Fig. 16.2). Included in this tally is the *M. incognita* 16D10 gene, which, like the *H. glycines* SYV-46 protein was originally isolated as an anonymous secreted protein (Huang et al. 2003) and later computationally identified as having sequence similarity to the CLE motif (Huang et al. 2006). Transgenic over-expression of 16D10 gave a root developmental response and it was found that the nematode ligand bound to two host SCARECROW-LIKE (SCL) proteins; this result was confirmed in a yeast 2-hybrid assay (Huang et al. 2006). SCL are members of the GRAS class of transcription regulators, which play central roles in root meristem specification and also are central to rhizobial nodulation (Hirsch et al. 2009), which is a process with many molecular and developmental similarities to giant cell induction (Bird 2004; Weerasinghe et al. 2005).

The rather surprising finding of 16D10 binding to SCL raises the question "is 16D10 a CLE?" and this is discussed in some detail elsewhere (Mitchum et al. 2008).

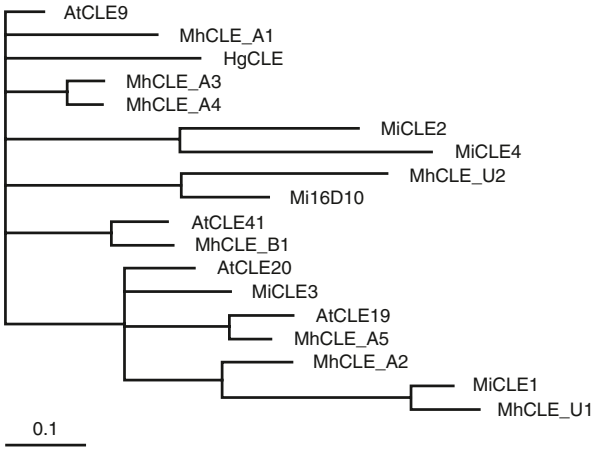

Fig. 16.2 Un-rooted tree from a Bayesian analysis showing relationships between *M. hapla* (Mh), *M. incognita* (Mi), *H. glycines* (Hg) and *Arabidopsis* (At) CLE peptide hormone domains. To avoid simply recreating an *Arabidopsis* tree, only four of the 32 At CLEs are included, numbered according to Whitford et al. (2008). The Mi CLEs are arbitrarily numbered, except the CLE domain from 16D10, which is labelled as such. Mh CLEs also are arbitrarily numbered, but with additional coding into predicted type A, type B or unclassified (U), based on similarity to *Arabidopsis* CLEs (Whitford et al. 2008)

It is important to note that the protein expressed by Huang et al. (2006) was a truncated form of 16D10 targeted to the *Arabidopsis* cytoplasm. By contrast, plant CLEs are expressed as pre-pro-proteins and are secreted into the apoplast, where they are processed into functional hormones to be recognized by receptor complexes on adjacent cells. Each of the RKN CLE candidates (including 16D10) is predicted to encode a simple pre-protein (i.e., no "pro" domain), consistent with the nematode injecting an active form of the peptide hormone directly into the apoplast. As the presence of any RKN protein in the host symplast is yet to be observed, demonstration of RKN-expressed 16D10 in the plant nucleus would be a major discovery. It is worth noting that the 16D10 sequence conforms to the form "PXXPXP," which is a motif known to mediate transcription factor protein-protein interactions (Dintilhac and Bernués 2002). It would be interesting to know that, if expressed in the cytoplasm, the other CLEs which contain the PXXPXP motif also can bind SCL. Similarly, does the *M. hapla* CLE most similar to the *M. incognita* 16D10 protein also specifically bind SCL, despite the two peptides differing by 5 of the 12 core CLE amino acids? Understanding the fate of each of the RKN CLEs within the root apoplast (or elsewhere) will likely be informative, as will understanding their evolutionary origin. Although peptide correspondence can be established between each RKN and an *Arabidopsis* CLE domain, evidence for evolutionary homology is lacking, suggesting that the nematode CLEs may have arisen convergently (Mitchum et al. 2008).

Genetic analysis in *Lotus japonicus* has implicated the CLV1-like receptor, *har-1*, as playing a role in modulating the number of root galls induced by *M. incognita* (Lohar and Bird 2003), and similar findings have been made for rhizobial

nodulation (Schnabel et al. 2005). Intriguingly, grafting experiments showed that the signal revealed by the *har-1* mutation originates in the shoot rather than the root (Buzasa and Gresshoff 2007). This implies that, in contrast to the canonical CLV system, the ligand must function over a long distance. Indeed, two Lotus CLEs (LjCLE-RS1 and LjCLE-RS2) have been directly implicated in regulating nodulation, and are strong candidates for being long-range, root-derived signalling hormones (Okamoto et al. 2009). Genetic analysis also points to a role of transmembrane receptor kinases in the RKN-host interaction, but their specific function in feeding site formation remains arcane (Weerasinghe et al. 2005).

16.6.3 RKN-Encoded CEP Mimics

Recently, a new class of 15-amino acid peptide, named CEP (C-terminally Encoded Peptide) has been discovered in *Arabidopsis* (Ohyama et al. 2008). CEPS are expressed in lateral root primordia, and based on the inhibition of lateral root development by transgenic over-expression or exogenous application of synthetic CEP peptide, are postulated to be hormone ligands. In the original report (Ohyama et al. 2008), five CEPs were revealed, but an additional protein with five CEP motifs and another with two motifs can be found in the *Arabidopsis* genome. Consistent with a role in regulating lateral root development, CEPs are widely distributed amongst monocots and dicots but appear absent from moss (*Physcomitrella*) or unicellular green algae (*Chlamydomonas*).

Interrogation of the *M. incognita* and *M. hapla* genomes revealed 8 and 9 CEP genes respectively. CEPs were not found in any other animal genera, including CN. An alignment of *M. hapla* and *Arabidopsis* CEP domains is shown in Fig. 16.3. Like their plant analogues, each RKN gene encodes a signal sequence at the amino

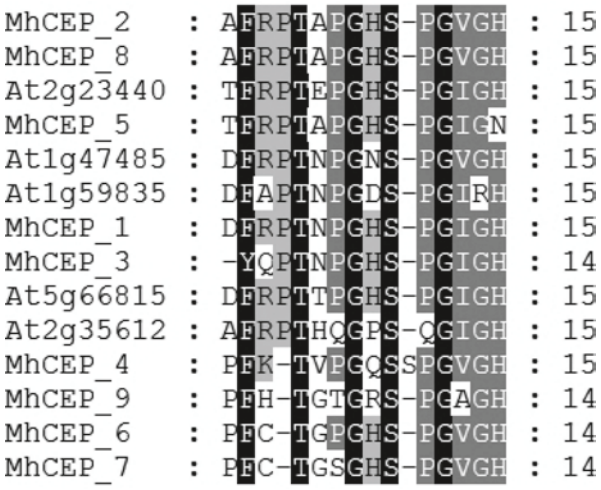

Fig. 16.3 Multiple sequence alignment of *M. hapla* and *Arabidopsis* CEP domains, ranked according to degree of sequence similarity. The full TAIR gene name is given for each of the *Arabidopsis* CEP domains. *M. hapla* CEPs are arbitrarily numbered. Shading indicates conservation of residues across the alignment

terminus and single CEP motif at the carboxyl terminus. As is the case for the CLEs, plant CEPs include a domain between the signal sequence and the hormone domain, which most likely represents a pro-protein domain that is proteolytically removed in the apoplast. Like RKN CLEs, RKN CEPs lack this domain, consistent with direct injection into the apoplast. As with the CLEs, extensive experimentation will be required to fully understand their role (if any) in the nematode-host interaction.

16.7 Perspectives

We are now beginning to understand the regulatory role of auxin and ethylene in the reprogramming of gene expression during feeding cell formation, and have identified a need for cytokinin, although the precise role(s) of other classical plant hormones like GA and ABA remains elusive. Transcriptome wide gene expression analysis of SCN-induced syncytia in soybean revealed differential expression of genes regulated by a range of hormones, including auxin, ethylene, gibberellin, jasmonic acid and cytokinin (Ithal et al. 2007), consistent with a complex interplay of plant hormones. Similarly, the analysis of nematode-responsive promoters (Wieczorek et al. 2008) also points to a role of plant hormones being involved in the formation of a feeding site. The presence of ABA and GA response elements in the promoter of a plant beta-endoglucanase, which is upregulated in syncytial development (Wieczorek et al. 2008), suggests that both hormones could be involved in modulating cell wall modifications during this process. Future studies on the functional role of GA and ABA responsive genes will lead to resolve their importance in the compatible interaction between endoparasitic nematodes and their hosts.

In addition to the classical plant hormones, it will be interesting to determine the role of novel hormones like strigolactones (review by Westwood and Bouwmeester 2009) and peptide hormones like the CLEs (review by Wang and Fiers 2009) and CEPs (Ohyama et al. 2008) in the formation of nematode feeding cells. Recently, it was shown that strigolactones act as a long distance messenger of auxin (Dun et al. 2009) and a role in nodule formation by *Rhizobium* has been demonstrated (Soto et al. 2010). It has been postulated by several authors that nodulation and nematode feeding cell development respond to similar developmental cues and that overlapping signalling pathways may occur (Bird 1996; Koltai et al. 2001; Grunewald et al. 2009; Mathesius 2003). Perhaps strigolactones play a similar role in endoparasitic nematode-induced feeding cell development.

Furthermore, it will be a challenge to understand how the different hormones act in concert during feeding cell formation. Plant hormones operate often as agonists or antagonists, resulting in enhanced activity or opposite effects. For example, an increase in auxin may lead to the production of ethylene, whereas cytokinin is known to repress both auxin signalling and transport. So, cross-talk between signalling pathways creates complex regulatory networks, which are difficult to untangle. A first glimpse of the cross-talk between various hormone mediated signalling

pathways in feeding cell development is shown in recent high-throughput studies showing differential expression of genes related to various types of hormones.

The observed key regulatory role of plant hormones in the establishment of a proper feeding site and the reprogramming of root cells into a specialised feeding structure suggests that they act via an ancient and conserved mechanism. The fact that similar roles are observed for hormones like auxin and ethylene in the formation of syncytia by various cyst nematodes, including *H. schachtii*, *G. rostochiensis* and *H. glycines* in different taxonomically unrelated plant species like soybean, tomato and *Arabidopsis* support this hypothesis. Hence, it is anticipated that plant hormones have a mutual regulatory role in giant cell development by various RKN species in a wide range of plant species. Whether plant hormones play a similar role in syncytium and giant cell formation remains elusive, as comparison between these two feeding cell structures is seriously hampered by the lack of dedicated studies in which both cyst and RKN species were included. A comparative high-throughput transcriptome analysis followed by functional studies would allow us to unravel common steps in the phytohormone regulatory networks involved in giant cell and syncytium development in the future.

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Chapter 17
Unravelling the Plant Cell Cycle in Nematode Induced Feeding Sites

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17.1 Introduction

17.1.1 The Plant Cell Cycle

In the past years, detailed molecular, cellular, biochemical, genetic, genomic and developmental approaches have been employed to investigate the cell division cycle, leading to better understanding of the different processes taking place. Although analogous to other eukaryotes, plants occasionally use different processes to regulate the cell cycle. A number of key events and a defined set of cell cycle genes are unique to plant cells and participate in plant-specific processes (Vandepoele et al. 2002). Conservation of core cell cycle genes in eukaryotes has been confirmed with genome-wide studies in *Arabidopsis* (Vandepoele et al. 2002). A typical cell cycle is characterized by a succession of four phases: G1, a preparative gap phase linking mitosis and DNA replication in which conditions are monitored to determine whether they are favourable for a new round of DNA replication; S-phase, during which genome duplication occurs (also named "DNA synthesis cycle"); G2, the gap between DNA replication and mitosis during which the cellular state is monitored in order to determine whether the cell can go through mitosis; mitosis, which includes the "chromosome segregation cycle" followed by the separation of genetic material including cytoplasmic division; and finally the "cytokinesis cycle" (De Veylder et al. 2003; Gutierrez 2009). Prior to the initiation of the S-phase, G1 cyclins build up and stimulate cyclin dependent kinase (CDK) activity. This inhibits repressors of cell cycle progression, leading to the up-regulation of G1/S specific genes which are controlled by activator transcription factors and thus initiates the cell cycle. Infrequently cells may subsequently exit the cell cycle and initiate either a cell differentiation program or enter an alternative cell cycle, the endocycle (see Sect. 17.1.2).

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Distinct pathways are involved in regulating the availability of cell cycle components. These include precise activation or repression of gene expression, variation in sub-cellular localization, posttranslational modifications and selective proteolytic degradation via the proteasome. Cyclin-dependent kinases and their regulatory cyclin subunits play a key role in cell cycle control. In *Arabidopsis thaliana*, 12 CDKs (named A to F) and other CDK-like genes are present in the genome (Gutierrez 2009). A single CDK regulates the G1/S and the G2/M transitions and CDKBs and CDKA cooperate to regulate the G2/M transition. To date, 52 cyclin-related proteins (in seven different subclasses, A–D, H, P and T) have been identified and around 30 appear to have a putative function in the control of the cell cycle (Vandepoele et al. 2002; Wang et al. 2004). At the transcriptional level, the majority of cyclins show a robust oscillatory behavior, especially the A and B types. Cyclins are regulated both transcriptionally and posttranslationally, mainly by controlled protein degradation. By regulating the abundance of specific cyclins, CDK activity is precisely tuned and targeted to substrates in a spatial and temporal manner (Verkest et al. 2005). Compared to cyclins, CDKs do not display much cell cycle phase-dependent transcriptional regulation (Inzé and De Veylder 2006), with the exception of the B-type CDK genes which peak during G2 and M phases (Fobert et al. 1996; Mironov et al. 1999; Menges and Murray 2002; Andersen et al. 2008). The CDK/cyclin complexes phosphorylate a number of substrates thereby triggering the transition from one cell cycle phase into the next. The CDK subunit (CKS) proteins are scaffold proteins of CDKs that function as adaptors for targeting CDKs to mitotic substrates (Hayles et al. 1986; Tang and Reed 1993). Although the *Arabidopsis* CKS proteins have been implicated in cell division control and meristem maintenance (De Veylder et al. 2001a) the precise function and localization of plant CKS proteins still await further investigation.

Due to its roles in growth and development CDK-cyclin activity is strictly controlled. In yeast, mammals and plants one of the major regulators of CDK activity are CDK inhibitory molecules (CKIs), which bind and inhibit or sequester CDKs. These proteins are homologous to animal Kips and therefore the seven CKIs found in the *Arabidopsis* genome were named Kip-related proteins (KRPs; De Veylder et al. 2001b). KRPs have been demonstrated to participate in control of CDKs by decreasing their activity. The capacity of KRPs to trigger the onset of the endocycle (see below) in dividing tissues has been confirmed by the specific overexpression of Arath;KRP2 in proliferating tissues, causing an inhibition of mitotic CDK activity and a premature onset of endoreduplication.

17.1.2 The Endocycle

As a reaction to a diverse range of physiological or developmental signals, proliferating cells are able to impede their transition to mitosis after they have terminated chromosome replication. This block is a prerequisite for the switch from the cell cycle to an alternative cycle, the endoreduplication cycle. Here, successive rounds

of complete genome replication take place in the absence of an overruling mitosis (Gutierrez 2009). Therefore, through bypassing nuclear division, an exponential increase in genome ploidy level is achieved. Although endoreduplication is described for all eukaryotes (Edgar and Orr-Weaver 2001), it seems especially important for plant development. The endoreduplication cycle, or endocycle, consists of two key phases: the exit from the cell division cycle and the continuation of a recurring endoreduplication cycle (Fig. 17.1). Repeated endocycles are often correlated with an increase in cell size (Melaragno et al. 1993) and may also be important in the augmentation of metabolic activity of particular cells. This appears to be the case during maize endosperm development (Traas et al. 1998; Joubès and Chevalier 2000; Kondorosi et al. 2000) and, eventually, in feeding cells induced by sedentary nematodes. The endocycle is also needed to initiate specific developmental pathways, as has been demonstrated for *Arabidopsis* hypocotyl cells and trichomes (Hülskamp et al. 1994; Larkins et al. 2001; Boudolf et al. 2004; Castellano et al. 2004). However, this may not be a generalized situation (Beemster et al. 2002) and is still a matter of debate (Kondorosi et al. 2000; Sugimoto-Shirasu and Roberts 2003). It appears that some regulators controlling cell cycle progression also play crucial roles in the regulation of endoreduplication (Gutierrez 2005; Caro et al. 2008). Work to date suggests that CDK activity, via CDKA-cyclin complexes, is a major regulator of endoreduplication (Gutierrez 2005; De Veylder et al. 2007). The role of CDKA in the endocycle has been recognized by the observed reduction in the mean C value obtained by inactivation of CDKA activity in developing maize endosperm (Leiva-Neto et al. 2004). CDK activity at the G1 to S transition is probably crucial to activate the mechanisms that trigger DNA replication via the retinoblastoma (Rb)-E2F pathway and genes in this pathway regulate the G1 to S

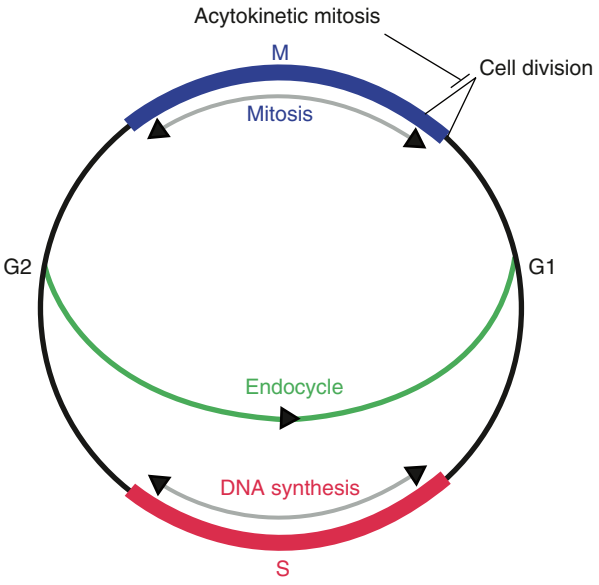

Fig. 17.1 Schematic view of the mitotic cycle and the endocycle. During the mitotic cycle, plant cells pass through *G1, S* and *G2* phases, subsequently dividing and generating two daughter cells. Throughout each endocycle, the *M* phase is absent, cells double their nuclear DNA content and this is often associated with increased cell size

transition (Vlieghe et al. 2007). E2Fa and E2Fb promote cell division by inducing the expression of genes required for DNA synthesis (De Veylder et al. 2002; Rossignol et al. 2002; Kosugi and Ohashi 2003; Sozzani et al. 2006). Dimerization of the E2Fs with DP proteins is a prerequisite for tight sequence-specific binding to the promoter regions of the E2F-responsive genes. Another class of E2F genes, which bind DNA as monomers, has been named E2FdDEL2, E2FeDEL1 and E2FfDEL3. (de Jager et al. 2001; Kosugi and Ohashi 2002; Mariconti et al. 2002; Vandepoele et al. 2002). Several genes are targeted by E2F-DP including those encoding the DNA replication-licensing factors CDC6 and CDTI (Castellano et al. 2001, 2004). The transition from mitotic division to endoreduplication has been correlated with a decrease in expression of mitotic cyclins during *Arabidopsis* leaf and tomato fruit development (Joubès et al. 1999; Beemster et al. 2005). The onset of endoreduplication has been linked with the inhibition of M-phase-associated CDKB1;1 activity (Grafi and Larkins 1995) and the A-type cyclins seem to be good candidates to function in collaboration with CDKB 1;1 (Yu et al. 2003). CDK-inhibitory proteins (CKIs) have also been shown to be involved in the regulation of the endocycle. Low levels of both ICK1/KRP1 and ICK2/KRP2 positively control the endocycle onset, whereas high levels result in a decrease in DNA ploidy levels (Verkest et al. 2005; Weinl et al. 2005). CDKA;1 is the main target of ICK/KRPs and their phosphorylation by CDKB1;1 controls the inhibition of CDKA activity. ICK/KRP2 phosphorylation by CDKs triggers its destruction by the proteasome. In addition, when CDKB1;1 activity decreases, ICKs/KRPs will become stabilized and will inhibit CDKA activity. Therefore, a harmonized decrease in CDK activity may be required to allow the onset of the endocycle (Verkest et al. 2005). Mammalian CDK activity is negatively regulated via the activity of the WEE1 kinase, and homologs of *WEE1* that probably play a role in the onset of the endocycle have been identified in plants (Sorrell et al. 2002; Gonzalez et al. 2004). Another way to control CDK activity is via the activation of the ubiquitin-proteasome pathway. Complexes formed during this pathway will control proteolysis of mitotic cyclins and the overproduction of APC-activating protein CCS52A triggers their destruction, resulting in endoreduplication. Conversely, downregulation of CCS52A expression significantly reduces the DNA ploidy level (Cebolla et al. 1999; Vinardell et al. 2003; Tarayre et al. 2004). Yet another factor that might inactivate mitotic activity and downregulate CDKA activity is the E2Fe/DEL1, a member of the E2F family of transcription factors which inhibits the endocycle in *Arabidopsis*. Ectopic expression of E2Fe/DEL1 diminishes endoreduplication whereas loss of function results in increased ploidy levels.

Downregulation of S-phase inhibitors is needed for endoreduplicating cells. An example is the retinoblastoma protein (RB) whose activity is neutralized by cyclin/CDK complexes (Hunter and Pines 1994; Sherr 1994). Phosphorylation of Rb-related (RBR) protein by an S-kinase results in the transcription of S-phase genes (Grafi et al. 1996). All data together stress the relevance of promoting the G1/S transition and inhibiting mitotic activity in order to activate the endocycle. Mutant analysis will help us to dissect the endocycle under various environmental conditions including during attacks by plant parasitic nematodes.

17.1.3 The Cell Cycle in Nematode Roots Infected by Parasitic Nematodes

Sedentary nematodes infect plant roots and trigger the formation of specialized feeding sites by substantial reprogramming of the development of root cells. Symptoms of plant infestation by root-knot (RKN) and cyst nematodes (CN) include knot- or gall-like (Fig. 17.2a) formations and syncytium (Fig. 17.2b) development in the roots, respectively. Although feeding sites such as galls and syncytia have the same function, nourishing the nematodes, they display a complex and distinct ontogeny. Root knot nematodes pierce and inject secretions into six to eight cells of the root vascular cylinder, inducing the formation of giant multinucleate cells (Fig. 17.2c) (Bird 1961; Jones and Northcote 1972; see also Chap. 5). These cells undergo repeated rounds of mitosis (Starr 1993; de Almeida Engler et al. 1999) with interrupted and blocked cytokinesis (de Almeida Engler et al. 2004; Caillaud et al. 2008). These feeding cells are surrounded by parenchymatic vascular cells and xylem ele-

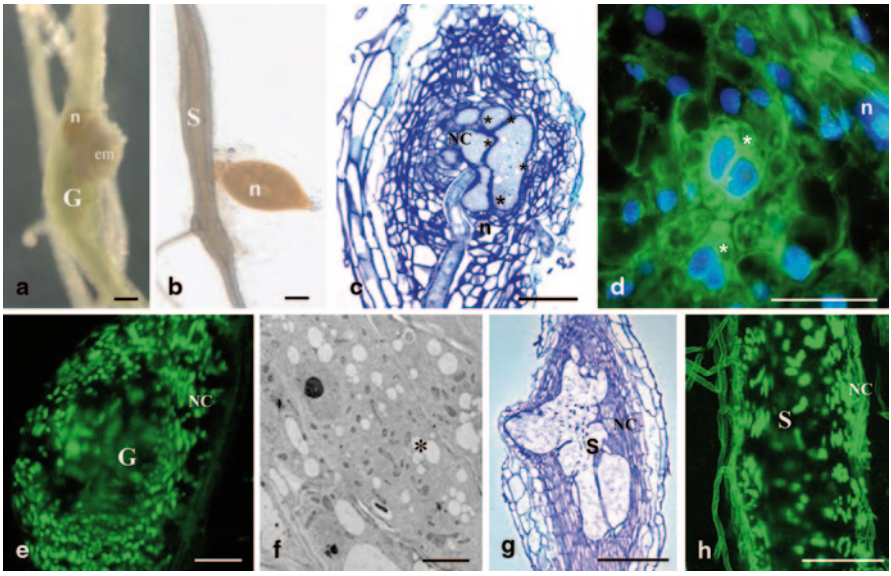

Fig. 17.2 Nematode feeding sites induced by the root-knot nematode *Meloidogyne incognita* or the cyst nematode *Heterodera schachtii* in (**a**–**c**, **e**–**h**) *Arabidopsis thaliana* and (**d**) *Pisum sativum*. **a** An infected seedling containing a gall with a female and an egg mass. **b** An infected seedling containing a syncytium with a cyst filled with eggs. **c** Section of a gall stained with toluidine blue. **d** A newly induced gall showing two binucleate giant cells in pea with enlarged DAPI stained nuclei (*blue*) and microtubules labelled with anti-α-tubulin (*green*). **e** A whole gall harbouring the Histone 2B-YFP fusion with green fluorescing nuclei. **f** Electron micrograph of a giant cell showing large nuclei and a cytoplasm rich in organelles. **g** A section of a syncytium stained with toluidine blue. **h** A whole syncytium harbouring the Histone 2B-YFP fusion with green fluorescing nuclei. *G* gall, *n* nematode, *em* egg mass, *S* syncytium. *Asterisks* giant cell, *NC* neighbouring cells. Bars = 100 μm (**a**–**e**, **g**, **h**), 50 μm (**f**)

ments that divide assymetrically in a disordered manner, causing a root swelling or gall (Fig. 17.1c). One of the first visual effects of giant cell induction is the first nuclear division and the enlargement of daughter nuclei (Fig. 17.2d). Although solid evidence is lacking, it is believed that the enlarged nuclei may be the result of endoreduplication events, but incomplete mitoses or nuclear fusion might also play a role in controlling the size of nuclei in giant cells. Throughout giant cell development and expansion multiple and often synchronous mitotic events take place ending up in an enlarged multinucleate cell with outsized nuclei (Fig. 17.2e, f; Starr 1993). Typical cellular features of these highly metabolically active giant-feeding cells are a dense cytoplasm filled with numerous organelles and small vacuoles (Fig. 17.2f).

Cyst nematodes pierce one single root cell and secretions of the nematode trigger the formation of a large feeding cell called "syncytium" (Fig. 17.2g; see also Chap. 4). Syncytium formation involves a massive reprogramming of root cell development as neighbouring cells (NC) first divide and then are incorporated into the syncytium through cell wall dissolution along the plasmodesmata and later by *de novo* development of cell wall openings (Grundler et al. 1998) (Fig. 17.2g, h). The syncytium is highly metabolically active and has a dense cytoplasm in which organelles proliferate (Fig. 17.2g). Syncytia most likely become multinucleate solely due to the incorporation of nuclei from adjacent cells after cell wall degradation and cell fusion rather than via mitotic activity. Expanding syncytia show enlargement of nuclei and nucleoli (Endo 1970; Bleve-Zacheo and Zacheo 1987; Golinowski et al. 1997) possibly as a result of endocycles (de Almeida Engler et al. 1999).

It has been shown that the regulation of the cell cycle in NFS involves changes in the expression and activation of a significant number of core cell cycle genes (Niebel et al. 1996; de Almeida Engler et al. 1999, unpublished data). In this chapter we will summarize which cell cycle genes are expressed during nematode feeding site development with the aim of clarifying how the cell cycle progresses in galls and syncytia induced by RKN and CN respectively. Results were obtained from DNA synthesis analysis, expression studies by mRNA *in situ* hybridisation and monitoring promoter activity of four cell cycle genes. Cell cycle phase-specific chemical blockers have been used to dissect the cell cycle in galls and syncytia. Finally, ongoing work to extend the examination of the cell cycle in NFS is also discussed.

17.2 Transcriptional Activity and Transcript Levels of Cell Cycle Genes in Nematode Feeding Sites

To monitor steady state transcript levels in NFS, whole-mount *in situ* hybridisation (WISH) and *in situ* hybridizations on sections have been performed at different developmental stages of galls and syncytia with probes for key cell cycle genes. The promoter activity of the same genes was analyzed using the *GUS* reporter system (de Almeida Engler et al. 1999). The cyclin-dependent kinase *CDKA;1* gene of *Arabidopsis* is transcribed throughout the cell cycle at a constant level (Hemerly

et al. 1993, 1995), whereas *CDKB1;1* is preferentially expressed from the S phase to the G2 phase (Segers et al. 1996). The steady state mRNA level of *CYCA2;1* increases during S phase and peaks at the end of the G2 while the transcript level of the *CYCB1;1* increases during the G2 phase, peaking at the G-to-M transition. *CYCA2;1* expression is linked with cell division and competence to divide (Burssens et al. 2000) while *CYCB1;1* is predominantly expressed in dividing cells (Ferreira et al. 1994). In the 1990's the *A. thaliana* marker lines containing promoter-GUS fusions of *CDKA;1, CDKB1;1, CYCB1;1* and *CYCA2;1* were used to examine the activity of the promoters of these genes in nematode-feeding cells (Niebel et al. 1996; de Almeida Engler et al. 1999). In root meristems of non-infected *Arabidopsis* seedlings, strong (*CDKA;1, CDKB1;1*) or patchy expression (CYCB1;1 in Fig. 17.3a and *CYCA2;1*) is observed. A patchy pattern reveals cell cycle phase specific gene expression. During penetration and migration of RKN or CN within the roots, decreased promoter activity of *CDKB1;1* and *CYCA2;1* is often observed at the root tip. *GUS* expression in feeding sites induced by RKN or CN is detected predominantly at initial stages of feeding site development (Fig. 17.3b, c), while in

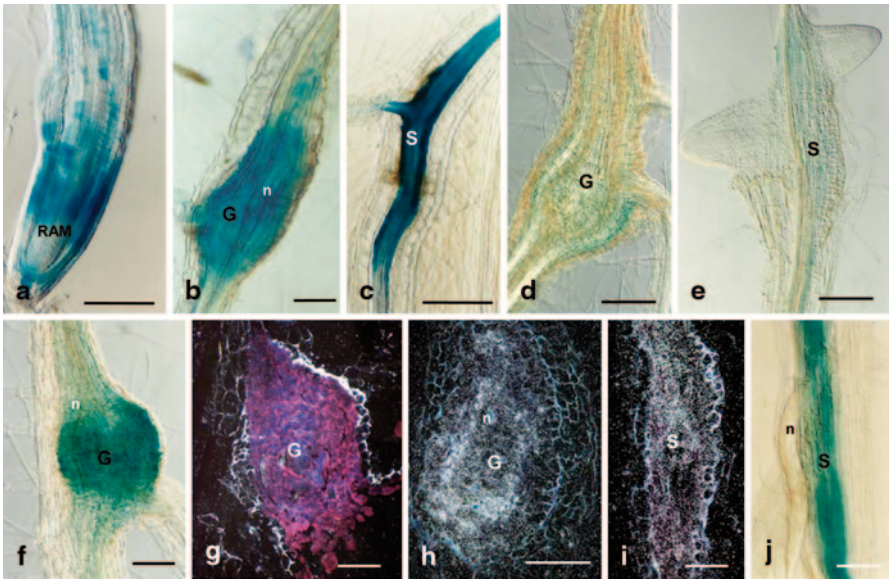

Fig. 17.3 Expression analysis of cell cycle genes in galls induced by *Meloidogyne incognita* or syncytia induced by *Heterodera schachtii* in *Arabidopsis*. **a** Whole root apical meristem showing patchy promoter activity of *CYCB1;1*. **b** A gall 7 dpi showing promoter activity of *CYCB1;1*. **c** A syncytium 7 dpi showing promoter activity of *CYCB1;1*. **d** A gall 21 dpi showing low promoter activity of *CYCB1;1*. **e** A syncytium 21 dpi showing low promoter activity of *CYCB1;1*. **f** A gall 10 dpi showing promoter activity of *CKS2*. **g** A gall section 10 dpi showing promoter activity of *CKS2* (*red*). **h** *CKS2* expression in a gall 10 dpi revealed by mRNA *in situ* hybridization (*white dots*). **i** *CKS2* expression in a syncytium 7 dpi as shown by mRNA *in situ* hybridization. **j** A syncytium 7 dpi showing promoter activity of *CKS2*. *RAM* root apical meristem, *G* gall, *n* nematode, *S* syncytium, *nu* nuclei. Bars = 100 µm

fully developed feeding cells, GUS staining is significantly decreased (Fig. 17.3d, e). Promoter activity of *CDKA;1, CDKB1;1, CYCB1;1* and *CYCA2;1* is also detected in cells surrounding the giant cells or syncytia indicating their competence for mitotic stimulation. In transgenic potato plants harbouring a *CYCB1;1* promoter-*luc* construct (Verhees et al. 1998) inoculated with the potato cyst nematode *Globodera rostochiensis*, LUC activity was observed around 16 h after inoculation and a maximum was reached at 6 days after inoculation. Surprisingly, the strongest activity was observed in the central region of the syncytium where the initial feeding cell was located and the expression was lower at the periphery of the growing syncytium. Although this observation has confirmed activity of G2/M phase specific genes, a higher transcriptional activity was expected to occur at the expanding edges. These findings could reflect the blockage of syncytium into the G2 phase of the cell cycle.

Analysis of mRNA levels by *in situ* hybridization on whole mounts, thick and semi-thin tissue sections (Fig. 17.3h, i) complemented information obtained by GUS analysis in galls and syncytia (Fig. 17.3f, g, j). During initial gall and syncytium development, high mRNA levels of the *CYCB1;1* gene suggest the prevalence of the G2 phase (Fig. 17.3b, c). Transcripts of *CDKA;1, CYCB1;1*, and *CYCA2;1* in vascular root tissue cells surrounding the initiating giant cells and syncytia also demonstrate the competence for division of these vascular parenchyma cells which are required for gall and syncytium development (de Almeida Engler et al. 1999). Different stain intensities for the *CYCB1;1* and the cyclin genes within individual giant cells in the same gall confirm that they can be at different stages of the cell cycle, as has been previously suggested (Bird 1961; Rubinstein and Owens 1964). As expected, expression levels of *CDKB1;1, CYCB1;1* and *CYCA2;1* in galls and syncytia are more transient than *CDKA;1* which shows a broader expression profile. At later stages of gall development (~9 days post inoculation-dpi), little or no mRNA of *CDKB1;1* or the two cyclin genes, and low levels of *CDKA;1* mRNA are detected. The absence of mitotic figures at this stage suggests that nuclear division is no longer required once giant cells are sufficiently developed. The comparable stage of syncytium development shows that cells neighbouring the feeding cell still express both CDKs and cyclins although inside the syncytium there is no further promoter activity of the genes analysed (de Almeida Engler et al. 1999). Therefore, cells neighbouring the feeding site are still cycling until late stages, allowing syncytia to reach a critical size required for the cyst nematodes to complete their life cycle and produce eggs.

Since cell cycle activation, and thus mitosis and DNA replication, seem to be essential for feeding site initiation, we performed a more complete analysis of the temporal and spatial expression patterns of different classes of core cell cycle genes in non-infected roots and nematode infected *Arabidopsis* plants. mRNA *in situ* hybridizations were performed using 61 core cell cycle genes and the results are being analysed and compared between normally dividing root cells and NFS (see below, de Almeida Engler et al. unpublished data).

To establish the relevance of the upregulation of a set of genes identified by *in situ* analyses, further functional analysis is needed. Suggested changes in gene

expression are currently being validated and, in addition, overexpressing lines of a set of genes that are highly expressed in NFS are being characterized. Van de Cappelle et al. (2008) generated transgenic *Arabidopsis* lines containing inverted repeats of *CDKA;1* cDNA under the control of the *AtWRKY23* promoter (Barthels et al. 1997). This promoter is highly expressed in feeding sites at the early stages of development (Grunewald et al. 2008). When knockdown lines are infected with *Meloidogyne incognita*, significantly fewer galls and egg masses develop on the roots of the transgenic compared to wild type plants. Infection of the *AtCDKA;1* silenced lines with *Heterodera schachtii* results in a significantly lower number of cysts compared to the control roots and seedlings show no developmental phenotype. This suggests that knockdown of cell cycle genes such as *AtCDKA;1* can be exploited as a strategy to produce transgenic plants less sensitive to plant-parasitic nematodes.

Our recent analysis reveals strong promoter activity of other *Arabidopsis* cell cycle genes, such as *CKS2* (Fig. 17.3f, g) and the cell cycle inhibitors *KRP2* and *KRP6* mainly during early feeding site formation (unpublished data). High CKS2 expression may be associated with high mitotic activity observed in developing giant cells. Preliminary expression data suggest that cell cycle inhibitors (KRPs) are expressed differently in feeding cells. Mutant analysis will reveal if this differential expression plays a role in the establishment of the compatible interaction between plants and nematodes. Similarly, it will be interesting to conduct validations for other candidate genes principally involved in the S phase which is essential for DNA synthesis during the mitotic and endocycles. Genes most likely to be involved in DNA synthesis in giant cells and syncytia include *DEL1, CYCAs, CYCDs, E2F*s and *MCM*s. It will also be interesting to perform comparative studies between giant cells and syncytia to determine the specific role of the cell cycle in mitotic versus amitotic feeding cells.

17.3 *In situ* Profiling of Cell Cycle Genes in Uninfected *Arabidopsis*: A Useful Source of Information for Nematode Feeding Sites

To better understand the role of the cell cycle genes during plant development, a thorough analysis of the temporal and spatial expression pattern of different classes of core cell cycle genes in *A. thaliana* seedlings has been conducted. The spatial localization of 61 core cell cycle genes analyzed by mRNA *in situ* hybridization (ISH) can be queried via an on-line database which contains ~1,800 images which are now publicly available (de Almeida Engler et al. 2009). Practically all genes analysed are expressed in the vascular root tissue where nematodes normally establish their feeding sites. In addition, the increased expression of 27 cell cycle genes in the root elongation zone suggests their involvement in the switch from cell division to differentiation. Interestingly, the root elongation zone is the preferred site for nematode penetration and feeding site initiation.

Endoreduplication has been demonstrated to occur in the cortical cells of the hypocotyl in *Arabidopsis* and to be differentially regulated in light and dark (Gendreau et al. 1997, 1998). Cells reach a maximum of 8C in the light, whereas in the dark the hypocotyl cells undergo an additional round of DNA replication resulting in 16C cells. *E2Fb, ORC1a, CDC6a, PIN1At* and the *KRP6* genes had the highest expression in the cortical cells at the apical hook of the hypocotyl, suggesting their involvement in endoreduplication and cell elongation. For that reason they are important candidates to be analyzed in nematode feeding cells.

Based on our *in situ* analysis in *Arabidopsis* seedlings we can hypothesise that genes predominantly expressed in meristematic tissues may operate only during the mitotic cycle, whereas those expressed in dividing and differentiating tissues might play a role in both the mitotic and the endoreduplication cycles. This resource of information will be valuable to find out how the cell cycle might progress in NFS and how this is reflected in cell cycle gene expression patterns. Indeed mechanisms which can be found in nematode induced feeding cells such as the uncoupling of mitosis from cell division, as well as the potential induction of multiple endoreduplication cycles, should have a profound effect on how the cell cycle genes are expressed. Therefore the data generated on the spatial localization of core cell cycle genes *in planta* can be used as reference for a similar analysis of nematode feeding cells.

17.4 DNA Synthesis and the Endocycle in the Multinucleate Giant Cells and Syncytia

As mentioned in Sect. 2.2, cell expansion and differentiation partly rely on the endoreduplication cycle. This process is likely to underlie the development of nematode feeding sites containing enlarged nuclei. Analysis of DNA synthesis using ^{3}H-thymidine provided evidence that cells and tissues in feeding sites at different developmental stages go through the S phase of the cell cycle. ^{3}H-thymidine incorporation in nuclei of vascular parenchyma cells is low in uninfected root regions where the galls or syncytia generally develop. DNA synthesis is activated in young giant cells (2 dpi), as shown by a faster accumulation of signal as well as an overall increase in signal density over the nuclei of NC (Fig. 17.4a). These observations in *A. thaliana* are in agreement with observations in giant cells of tomato (Rubinstein and Owens 1964) and cotton (Rohde and McClure 1975). When a short pulse (3 h) of ^{3}H-thymidine is applied to nematode infected seedlings, a preferential labelling occurs in giant cells. Short pulse ^{3}H-thymidine incorporation experiments have shown that DNA synthesis in giant cells occurs in intense peak(s) while DNA synthesis seems more spread over time in the neighbouring cells. This indicates that the mitotic cycle in young giant cells may be faster than in uninfected root cells and that alternating endoreduplication and mitotic cycles could occur. Similarly, young syncytial cells (5 dpi) show higher ^{3}H-thymidine incorporation as compared to the actively dividing NC, suggesting additional cycles of DNA synthesis in the feeding

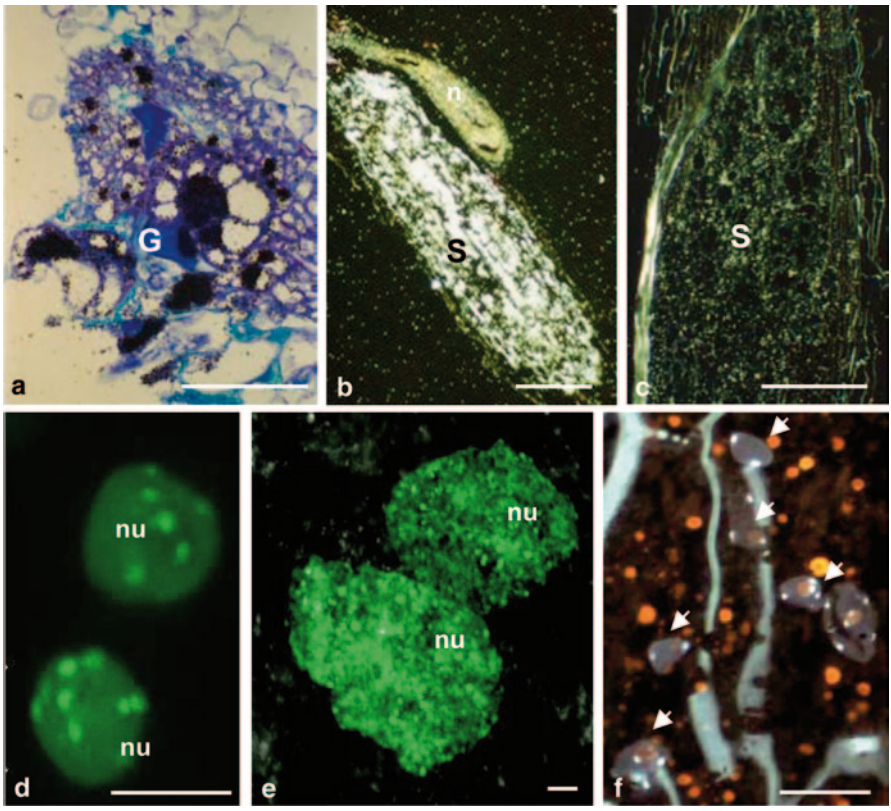

Fig. 17.4 Nuclear activity and morphology in giant cells and syncytia in *Arabidopsis*. **a** DNA synthesis (*black dots*) in a gall stained with toluidine blue under bright field optics. **b** DNA synthesis (*white dots*) in a syncytium 7 dpi under dark field optics. **c** DNA synthesis in a syncytium 21 dpi. **d** nuclei (*green*) of wild type root cells harbouring the Histone 2B-YFP fusion showing chromocenters (*greener dots*). **e** nuclei (*green*) of a giant cell showing numerous chromocenters of various sizes (*greener dots*). **f** nuclei (*white arrows*) of a syncytium stained with DAPI showing chromocenters (*white spots*). *RAM* root apical meristem, *G* gall, *n* nematode, *S* syncytium, *nu* nucleus. Bars = 100 µm (**a–c**), 5 µm (**d–e**), 20 µm (**f**)

cell (Fig. 17.4b). At later stages of syncytium development more label was observed at the expanding edges (Fig. 17.4c)

All interphase nuclei of giant cells harbour more than the typical ten chromocenters that can be observed in diploid *Arabidopsis* cells (compare Fig. 17.4d in normal cells with 17.4e in giant cells). A chromocentric nuclear organization is often observed in plants with small chromosomes and low DNA content (Lafontaine 1974; Nagl and Fusenig 1979). GC chromocenters are larger than those present in the nuclei of NC and, as previously suggested, large nuclei might result from nuclear fusion, incomplete mitosis or polyteny (de Almeida Engler et al. 1999). The number of chromocentres increases in polyploid nuclei whilst the size of the chromocentres enlarges in polytene nuclei. On the other hand, chromocentres can fuse resulting in

fewer and larger chromocentres (Kabir and Singh 1989). Once a gall is fully developed (~14 days after infection) DNA synthesis is slowed down or stopped and no mitotic activity is observed, implying that giant cells already attained a sufficient level of DNA required to sustain a high metabolic activity until completion of the nematode's life cycle. Consistently, acytokinetic mitosis was rarely observed in mature giant cells of pea, tomato, lettuce, and broad bean (Starr 1993).

In contrast to galls, syncytia and their neighboring cells still showed ^{3}H-labeled nuclei at later stages (15 dpi) of development, suggesting that syncytia are subject to a more continuous development over a longer period than galls. For syncytia, the number and size of chromocenters is highly variable (Fig. 17.4f). Mitosis (or mitotic chromosomes) has never been observed in syncytia suggesting that DNA replication (endoreduplication) might be the main mechanism for DNA amplification in syncytia leading to nuclear hypertrophy. Cytological analysis has shown that nuclei of NC were also larger than those in cortical root cells, indicating that endoreduplication cycles might occur in these cells before syncytium incorporation (de Almeida Engler et al. 1999). Cell division and nuclear hypertrophy seem to precede incorporation of NC into the syncytium until late stages of development (Golinowski et al. 1996).

We are beginning to decipher the role of the endocycle in the development of NFS. Our comprehensive analysis of the temporal and spatial expression patterns of all classes of core cell cycle genes in NFS induced in *Arabidopsis* allowed us to select and focus on a set of genes potentially involved in the endocycle of feeding cells. Of these, candidate genes such as *DEL1;1*, the three *CCS52* genes and *HYP7* (Cebolla et al. 1999; Vlieghe et al. 2005; Sugimoto-Shirasu et al. 2005) were chosen for further analysis. Promoter activity of *CCS52a* was observed in galls induced in *Medicago truncatula* plants (Favery et al. 2002). In addition, transgenic *A. thaliana* lines harbouring hairpin constructs of the *AtCCS52* gene have been generated recently. These lines have a reduced *AtCCS52* expression, a lower ploidy level and were shown to be less susceptible to nematode infection as compared to controls (Van De Capelle et al. unpublished data). Another candidate gene with possible involvement in the endocycle is the cell cycle inhibitor, *KRP2*. Overexpression of Arath; KRP2 in proliferating tissues triggers an inhibition of mitotic CDK activity and a premature onset of endoreduplication (Verkest et al. 2005). Preliminary mutant analyses suggests that *KRP2* is involved in giant cell development.

17.5 Cell Cycle Inhibitors Influence DNA Synthesis and Mitosis in Feeding Cells

To further analyse the specific involvement of particular phases of the cell cycle in feeding site development, two cell cycle inhibitors have been used: hydroxyurea (HU), a cytostatic drug acting as a specific inhibitor of DNA synthesis (Young and Hodas 1964), and the herbicide oryzalin, an inhibitor of plant microtubule polymerization which arrests cells at mitosis. The effect of applying these chemicals at dif-

ferent time points after nematode inoculation on feeding site development has been evaluated (de Almeida Engler et al. 1999). Control experiments demonstrated that these chemical inhibitors were harmless to nematodes (Orum et al. 1979; Glazer and Orion 1984; de Almeida Engler et al. 1999).

Blocking DNA synthesis in nematode infected roots strongly affects the establishment and development of both galls and syncytia and of the feeding nematodes. After HU treatment, feeding cells have smaller nuclei and a much less dense cytoplasm. HU treatment of seedlings early after infection (3 dpi) with root-knot nematodes arrests mitosis, as well as nuclei enlargement in giant cells and gall expansion. Application of HU at later stages results in normal development of NFS and nematodes. Blocking DNA synthesis at early stages of syncytium development blocks cell incorporation into the syncytium. Cytological analysis showed that nuclear hypertrophy and cell division of NC precede their incorporation into the syncytium from early (3 dpi) until late stages of development (15 dpi) (Magnusson and Golinowski 1991; Golinowski et al. 1996). These findings strongly suggest that endoreduplication cycles may occur in these cells before incorporation into the syncytium. All records clearly point to DNA replication as being crucial for syncytium and giant cell development. Chemical treatments with HU when the NFS are developed allow nematodes to complete their life cycles.

It is generally accepted that giant cells become multinucleate due to sequential mitosis, while the surrounding NC are formed by successive divisions forming a root gall. As expected, inhibition of mitosis by oryzalin arrests giant cell development confirming mitosis as a key step for giant cell development. Treatment of infected seedlings at later stages (9 dpi) showed that NFS were sufficiently developed at that time to allow nematode maturation (de Almeida Engler et al. 1999). Although nuclear divisions seem not to occur in syncytia, oryzalin treatment affected syncytium induction. Blocking mitosis in syncytia by oryzalin treatment at early stages (5 dpi) lead to decreased syncytium expansion and only a fraction of the cyst nematodes completed their life cycle. The effect of a mitotic block seems to affect NC division and consequently a lower number of cells are available for syncytium expansion. When applying oryzalin at later stages of infection, the maturation rates of cysts are still lower compared to untreated infected roots demonstrating that at this stage syncytium expansion is still disturbed by the treatment. Even in the absence of mitosis within the syncytium itself, cell division of surrounding cells appears essential for the radial enlargement of the feeding site (de Almeida Engler et al. 1999).

The heterogeneous distribution of nuclei in different size and shape dispersed along the dense cytoplasm is characteristic of a mature syncytium. Several cytological reports support the idea that mitotic activity is absent in syncytia (Endo 1964, 1987; Endo and Veech 1970; de Almeida Engler et al. 1999) although an initial mitotic stimulation cannot be excluded during feeding site initiation as proposed by Piegat and Wilski (1963). Differently, giant cells have their nuclei clustered and at times organized in a semicircle, representative of successive rounds of mitosis. Cell wall stubs are observed more frequently between the nuclei in HU-treated giant cells as compared to untreated samples. Mitotic figures are rarely seen in giant cells

as compared to NC of *Arabidopsis* indicating that mitotic events are less frequent in feeding cells or that cell cycle phases are shorter than in a normal cell. Therefore, by slowing down cell cycle progression with chemical treatments some mitotic events might attempt to proceed in GCs as for uninfected cells, resulting in the higher frequency of cell wall stubs indicative of attempted giant cell division (Navarrete et al. 1979; Clain and Brulfert 1980; de Almeida Engler et al. 1999). Jones and Payne (1978) reported that alignment of cell plate vesicles in giant cells of *Impatiens balsamina* progress until vesicles become dispersed and cytokinesis is arrested. Based on the results of cell cycle blockers, DNA synthesis and mitosis are essential for the primary establishment of galls and syncytia. Previous studies have applied several agrochemicals with cell cycle-inhibiting properties to manage nematode infection in crop species. HU, oryzalin, colchicine and other inhibitors such as maleic hydrazide, morphactin, BAS 083, chlorpropham, *S*-ethyl dipropylthiocarbamate and dimethyl tetrachloroterephthalate were tested without detailed cytological analysis (Davide and Triantaphyllou 1968; Gershon 1970; Orion and Minz 1971; Romney et al. 1974; Griffin and Anderson 1979; Orum et al. 1979; Glazer and Orion 1984, 1985; Stender et al. 1986). Therefore, no solid statement can be made about their effect on feeding cell ontogeny.

17.6 Concluding Remarks

We have started to understand and dissect cell cycle components in the plant cell during a successful nematode interaction. Part of this machinery has been deciphered but more elements are under investigation. When a mitotic blocker is applied but DNA replication is allowed, gall development is arrested, demonstrating that endoreduplication cycles or other ways of DNA amplification are not sufficient to drive giant cell expansion. In syncytia, a mitotic block only affects the radial expansion by preventing NC from dividing. However their longitudinal expansion via cell wall dissolution is not disturbed since they expand via the already existing root vascular parenchymatic cells. Consequently, less developed NFS result in incomplete maturation of the infecting nematodes that depend on abundant nutrient supply from completely developed syncytia. So, one could say that a minimal syncytium “per se” can develop without mitotic cycles. This is in contrast with giant cells that need not only DNA replication but also mitotic activity to make a minimal giant cell. Genome multiplication is essential to the formation of both style of feeding cells.

Further characterization of cell cycle components involved in NFS development will be essential. This could involve silencing those cell cycle genes that are strongly expressed in galls or syncytia. A promising strategy might be to repress the endocycle and therefore feeding site development at its initial stage. Since the cellular features of feeding sites in different hosts are very similar, biotechnological approaches that address the cell cycle could be promising in engineering resistance to a broad range of nematode species.

Finally, it will be fascinating to elucidate how cell cycle events in feeding cells are influenced directly or indirectly by nematode secreted proteins. Plant parasitic nematodes have been shown to secrete hundreds of different proteins, the majority of still unknown function (Bellafiore et al. 2008). Some of these proteins might specifically block (syncytia) or activate (giant cells) the activity of M phase–promoting factors and/or induce S phase–related genes. Alternatively, nematode secreted proteins might influence the cell cycle by changing ubiquitination pathways in NFS (Gao et al. 2003; Tytgat et al. 2004).

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Chapter 18
The Plant Cytoskeleton Remodelling in Nematode Induced Feeding Sites

Janice de Almeida Engler and Bruno Favery

18.1 Introduction

18.1.1 The Plant Cytoskeleton

The plant cytoskeleton is a highly dynamic and versatile intracellular scaffold composed mainly of microtubules (MTs) and actin filaments (F-actin or microfilaments, MFs) as well as intermediate filaments. Microtubules and MFs play a central role in many aspects of plant cell growth and differentiation, including cell division, cell differentiation, cell expansion and intracellular organization and motility (Staiger 2000; Wasteneys and Galway 2003). Both MTs and MFs are made of globular protein subunits that can assemble and disassemble rapidly within the cell. Globular actin (G-actin) molecules are associated with one tightly bound Ca^{2+}, which stabilizes its globular conformation, and one noncovalently bound ATP. The ATP terminal phosphate is hydrolyzed when the G-actin polymerizes to form a MF. MFs consist of two strands of globular molecules twisted into a helix. MTs consist of molecules of tubulin, which is a dimer composed of α-tubulin and ß-tubulin. When tubulins assemble into MTs, they form protofilaments aligned in rows, with the ß-tubulin of one dimer joined to the α-tubulin of the next one. Usually 13 protofilaments are arranged side by side around a central core forming a hollow tube of 25 nm external diameter. As with actin, the nucleotide-triphospate hydrolysis (here GTP) has a crucial influence on the kinetics of polymerization. MFs and MTs are polar structures. Their two distinct ends grow and depolymerize at very different rates and are defined as the fast-growing (+) end and the slow-growing (−) end.

The cytoskeleton also contains many different proteins that interact with subunits or filaments, link the filaments to one another or to other cell components, or influence the rate and extent of the filament polymerization. The main actin-

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and microtubule-binding proteins are described below. Understanding cytoskeleton dynamics requires knowledge of how plant cells control localized filament polymerization, the organization of filaments into networks, filament depolymerization and the maintenance of the pool of monomers required for rapid response to stimuli (Vantard and Blanchoin 2002). MTs and MFs form motile structures that appear at specific stages or in response to external signals and then disappear. In addition, regions of the plant cell cytoskeleton can be reorganized in response to local stimuli. Even though we discuss MTs and MFs independently, it is clear that these components must be linked together and their functions coordinated. The small GTP-binding proteins of the Rho family (known as Rop in plants) are likely to be among the critical switches regulating actin and MT dynamics (Yalovsky et al. 2008). In higher plants, various model systems such as tip-growing cells (e.g. pollen tubes and root hairs) (Hepler et al. 2001), trichomes (Mathur et al. 1999) and morphogenetic mutants, mainly in *Arabidopsis*, have been used to investigate the function of the cytoskeleton during plant development. Host-pathogen interactions may also provide additional interesting model systems.

18.1.2 MTs and Microtubule-Associated Proteins

Microtubules play an important role in ensuring that wall precursors are inserted in appropriate sites at appropriate times. Plants have four main MT assemblies (Lloyd and Hussey 2001; Wasteneys and Galway 2003). During interphase, the majority of cytoplasmic MTs are dispersed over the cell cortex lying close to the inner face of the plasma membrane. These cortical MTs are oriented mainly perpendicular to the axis of cell elongation and form a MT array which encircles the cell (Fig. 18.1). By directing precursors to the appropriate sites, this cortical MT array helps regulate the direction in which wall materials are deposited and, indirectly, the direction in which the cell expands. There is substantial evidence to indicate that cortical MTs provide tracks for cellulose microfibril deposition (Gutierrez et al. 2009). At the onset of mitosis, a transient preprophase band (PPB) of cortical MTs, which looks like a bunched-up ring-like version of the interphase array, determines the plane of cell division (Van Damme et al. 2007) (Fig. 18.1). Upon entry into mitosis, all cortical MTs disappear to be replaced successively by two kinds of bipolar microtubular structures: the spindle MTs and the phragmoplast (Fig. 18.1). The anastral mitotic (or meiotic) spindle assembles in prometaphase and ensures the accurate segregation of chromosomes. Spindle formation is complete during metaphase when the chromosomes have been aligned in the metaphase plate of the spindle. Once all the chromosomes are aligned, the cell enters anaphase and the sister chromatids are separated moving toward their respective poles. In late anaphase, bipolar spindle MTs reorganize into double rings of MTs, constituting the phragmoplast that guide secretory vesicles to the new expanding cell plate formed between daughter nuclei. During telophase, the cytokinetic phragmoplast grows out centrifugally, leading the

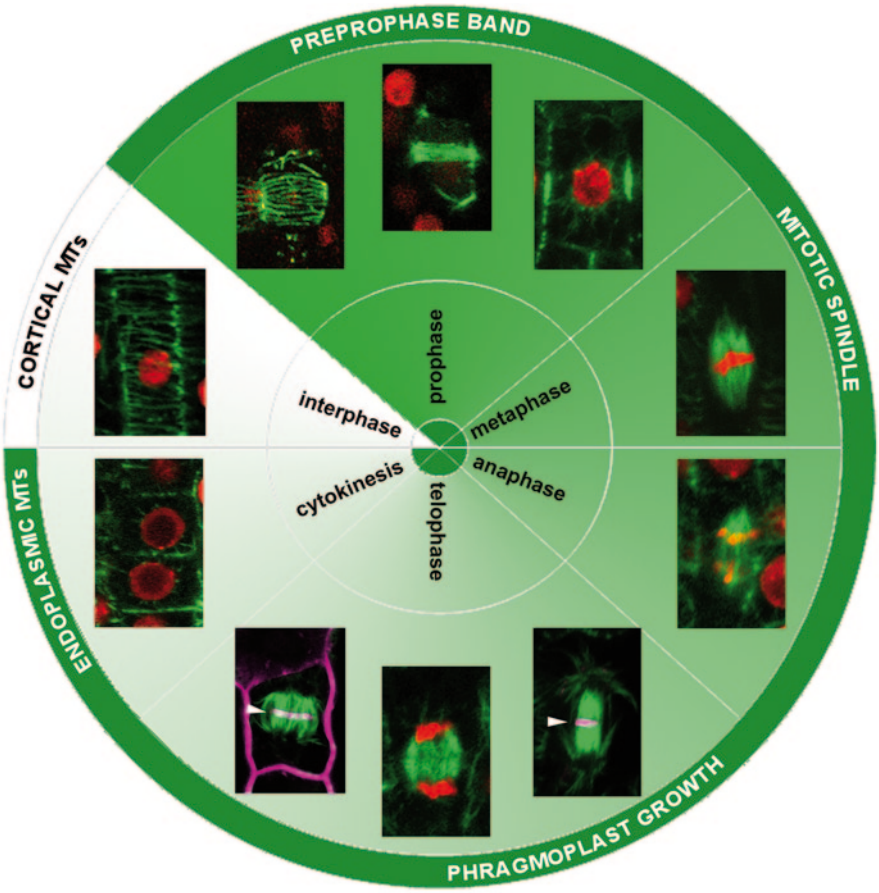

Fig. 18.1 Organization of the plant MT arrays during mitosis. Images were obtained by *in vivo* confocal microscopy of *Arabidopsis* root cells co-expressing two marker proteins: microtubule-binding domain (MBD):GFP (in *green*) and nuclear Histone 2B-YFP (in *red*). Pink coloration corresponds to the fluorescent membrane dye FM4-64. Arrowheads show cell plate. Modified from (Caillaud 2009)

cell plate towards sites foretold by the transient placement of the PPB. Once cytokinesis is complete, MTs extend from the nucleus toward the cell cortex (Fig. 18.1).

The organization and dynamics of MT arrays are controlled by microtubule-associated proteins (MAPs). Different classes of plant MAPs have been discovered and functional studies have started to reveal how they might work (Lloyd and Hussey 2001; Wasteneys 2002). MAPs have been categorized into (i) structural MAPs that form filaments which cross-bridge MTs. These include MAP65s (Jiang and Sonobe 1993; Chan et al. 1999; Smertenko et al. 2000) like MAP215/MOR1 (Whittington et al. 2001); (ii) motor proteins that move MTs or move cargo along them such as

kinesins (Reddy and Day 2001a) and dyneins (King 2002); (iii) proteins involved in MT nucleation and release such as γ-tubulin (Binarova et al. 2000) and katanin (Stoppin-Mellet et al. 2002); and (iv) a loose-grouping of other MT-interacting proteins: TAN1, HSP90 and MAP190 (Igarashi et al. 2000; Hussey et al. 2002).

18.1.3 Actin and Actin-Binding Proteins

The plant actin cytoskeleton is involved in different cellular processes essential for plant development and responds to internal and external signals. These processes include cell morphogenesis, cell growth and differentiation, cell division, responses to wounding and pathogen attack and hormone distribution (Staiger 2000; Wasteneys and Galway 2003; Hussey et al. 2006; Staiger and Blanchoin 2006). Actin MFs are involved in these processes acting as molecular rail tracks for myosin-based cytoplasmic streaming, allowing the translocation of organelles, such as Golgi bodies and peroxisomes, and anchoring chloroplasts in response to light (Staiger and Hussey 2004). In most interphase plant cells, three contiguous MF arrays, which support particle movement throughout the cell, can be discerned: subcortical unipolar MF-bundles, which support long-range translocations, short cortical (probably) non-bundled MFs, and a perinuclear MF network. A fine network of MFs is also found at the plasma membrane. In diffusely expanding cells this network is consistent with the actin cytoskeleton presence near sites of rapid exocytosis during tip growth and wound wall formation (Wasteneys and Galway 2003). During mitosis, MFs are present in the PPB and phragmoplast structures and could in fact contribute to the formation and organization of the MTs that are the major components of both structures. Thus, MFs cooperate with MTs to mark the nascent cell division site and provide guidewires to direct the expanding cell plate to the final site of fusion with the mother cell wall during cytokinesis. When the PPB MTs degrade, cortical actin disappears at the position of the PPB, leaving behind an actin depleted zone that lasts throughout mitosis (Van Damme et al. 2007).

The spatial distribution and activity of actin-binding proteins (ABPs) are essential in controlling actin dynamics. Analysis of plant genome sequences reveals highly conserved sequences with some of the known ABPs in other eukaryotes (Deeks et al. 2002; Vantard and Blanchoin 2002; Staiger and Blanchoin 2006). However, other well known ABPs are absent or poorly conserved in plants. Notable absences include thymosin ß4, WASp, α-actinin, spectrin, filamin and tropomyosin (Staiger and Hussey 2004). Identified plant ABPs can be grouped into (i) monomer-binding proteins that regulate polymerization and depolymerisation including actin-depolymerizing factors (ADF)/cofilins and profilins which act synergistically (Didry et al. 1998); (ii) cross-linking and bundling proteins that form orthogonal networks or bundles such as fimbrins and villins; (iii) capping proteins that control the availability of filament ends for subunit addition; (iv) nucleation factors that seed polymerization of new filaments such as actin-related protein 2/3 (ARP2/3) complex (Vantard and Blanchoin 2002) and the unique conserved class of poly-L-

proline profilin-binding proteins in plants, formins or formin-homology (FH) proteins (Deeks et al. 2002); (v) other MF-binding proteins: AIP1, annexin and the molecular motor myosins (Reddy and Day 2001b).

18.1.4 The Cytoskeleton and Plant-Microbe Interactions

Specific changes in plant cytoskeleton organization have been demonstrated in various plant-pathogenic or symbiotic microbe interactions, although the significance of these rearrangements is generally unknown. These changes are complex and varied and many have not been fully elucidated (Takemoto and Hardham 2004; Lipka and Panstruga 2005; Hardham et al. 2007; Schmidt and Panstruga 2007). However, it has become increasingly clear that the cytoskeleton plays a crucial role in many plant–microbe interactions.

Given the central role that the cytoskeleton plays in innate immunity in mammals and the specific targeting of MTs (Radtke et al. 2006; Yoshida et al. 2006) or actin machinery by pathogens (Guiney and Lesnick 2005; Trosky et al. 2008), it was hypothesized that the plant cytoskeleton is likely to play a central role in host defense responses and may represent a virulence target. The reorganization of the actin cytoskeleton has been assumed to contribute to the first line of plant defense against pathogenic fungi and oomycetes (Hardham et al. 2007). In response to infection of *Arabidopsis* by oomycete pathogens, actin MFs are actively re-arranged and form large bundles in cytoplasmic strands focused on the penetration site (Takemoto et al. 2003). The importance of the cytoskeleton in early responses to pathogens is also indicated by the fact that treatments with actin cytoskeleton-disrupting agents, such as cytochalasins, permitted various fungi, which normally fail to infect, or have a very low penetration efficiency, to penetrate non-host plants (Kobayashi et al. 1997; Yun et al. 2003; Shimada et al. 2006). The actin cytoskeleton functions not only in non-host resistance but also in basal defense and in race-specific resistance (Opalski et al. 2005; Miklis et al. 2007). Resistance gene-dependent plant cell death (the hypersensitive response, HR) to rust fungi was specifically inhibited by cytochalasin in cowpea, whereas additional MT arrays that surrounded the invasion hypha were observed in infected plant cells (Skalamera and Heath 1998). The barley *rpg4* gene was recently shown to encode an ADF functioning, together with *Rpg5*, to confer resistance to several stem rust races (Brueggeman et al. 2008). Moreover, actin dynamics were also linked with the activation of gene-for-gene resistance to the bacterial pathogen *Pseudomonas syringae* in *Arabidopsis* (Tian et al. 2009).

In contrast to the behaviour of the actin MFs, the MT cytoskeleton response is more variable in different plant–pathogen systems and appears to be less important in the plant defense response (Takemoto and Hardham 2004). Thus, in nonhost, incompatible, and compatible interactions of *Arabidopsis* with oomycetes (*Phytophthora sojae* or different races of *Hyaloperonospora parasitica*), there was no evidence of cortical MT bundles being specifically focused on the penetration site (Takemoto et al. 2003). The most obvious change was the appearance of diffuse fluorescence

around the penetration site most likely due to tubulin subunits. This accumulation could be due to increased tubulin synthesis, or to localized MT de-polymerization, as reported in the parsley–*Phytophthora* interaction (Gross et al. 1993). MTs play important roles in viral infection. Many viruses hijack the MT cytoskeleton to transport viral proteins, nucleic acids and virus particles. The best characterized example is the transport of animal viruses (Radtke et al. 2006). However in plants, rare MT–virus interactions have been reported, and their specific role in the viral cycle remains disputed in most cases (Takemoto and Hardham 2004). The best-known example is the interaction between the Tobacco mosaic virus movement protein and MTs (Boyko et al. 2000). Interestingly, a recent study showed that plant MTs are involved in the formation of transmission-specific inclusion bodies of Cauliflower mosaic virus (Martiniere et al. 2009). This unexpected function in a virus life cycle suggests that MTs act not only on immediate intracellular or intra-host phenomena, but also on processes ultimately controlling inter-host transmission. In addition, TMV movement does not require an intact actin cytoskeleton, but ER-associated MFs and ABPs could play a role in controlling its efficiency (Hofmann et al. 2009).

Microfibre and MT reorganization also accompany mutualistic plant-microbe associations. The study of the cellular responses elicited in the host plant root associated with the preparation of the epidermal cell layer for penetration by arbuscular mycorrhizal fungi revealed the formation of a pre-penetration apparatus, comprising host MTs, MFs and endoplasmic reticulum. This transient cytoskeletal structure directs hyphae through the epidermis (Genre et al. 2005). Significant changes in the dynamic behaviour of MT arrays were found to be associated with all early steps of symbiotic interactions, including infection thread formation and growth, as well as the activation of root pericycle and cortical cells that initiates nodule primordia organogenesis (Timmers et al. 1999). Rearrangement of the actin cytoskeleton has also been shown to mediate invasion of legume roots by symbiotic bacteria. Treatment of legume root hairs with Nod factor leads to rapid changes in the polymerization pattern of actin filaments (Weerasinghe et al. 2005). The long actin bundles extending into the root hair apical tips were observed to undergo fragmentation, and fine bundles of actin filaments accumulated. A recent survey of *Lotus japonicus* mutant lines with a defective infection process identified two genes, NAP1 and PIR1, involved in actin rearrangements and essential for infection thread formation and colonization of roots by *Mesorhizobium loti* (Yokota et al. 2009). These results support the hypothesis that the Nod factor-dependent reorganization of MTs and MFs is a prerequisite for successful symbiotic interactions.

18.1.5 Plant-Nematode Interactions and Feeding Cells

Plant-parasitic nematodes are obligate biotrophs that can only feed on living cells. Two of the most economically damaging groups are the sedentary endoparasites

root-knot nematodes (RKNs, *Meloidogyne* spp.) and cyst nematodes (CNs, *Globodera* spp. and *Heterodera* spp.). Both types of nematodes establish an intimate relationship with their host plants inducing the redifferentiation of root cells into specialized feeding sites. RKN induces feeding sites containing five to seven giant cells, whereas CN induces a syncytium. Details of these processes are provided in Chaps. 4 and 5. The first sign of giant cell induction by a RKN is the formation of one or several binucleate cells. The multinucleate status is reached via successive nuclear divisions (karyokinesis) followed by failure to undergo complete cytokinesis (Jones and Payne 1978; Wiggers et al. 1990; Caillaud et al. 2008a). Normal divisions of the surrounding cells result in the formation of the typical gall (Fig. 18.2a). Conversely, in a syncytium the initial feeding cell expands within the vascular tissue by progressive cell wall openings and incorporation of adjacent cells (Golinowski et al. 1997) (Fig. 18.2b). Comparisons of nematode-infected host transcription patterns using a variety of techniques (Gheysen and Fenoll 2002; Caillaud et al. 2008b), in particular recent genome-wide expression profiling of isolated giant cells or syncytia (Barcala et al. 2010; Szakasits et al. 2009), identified many genes

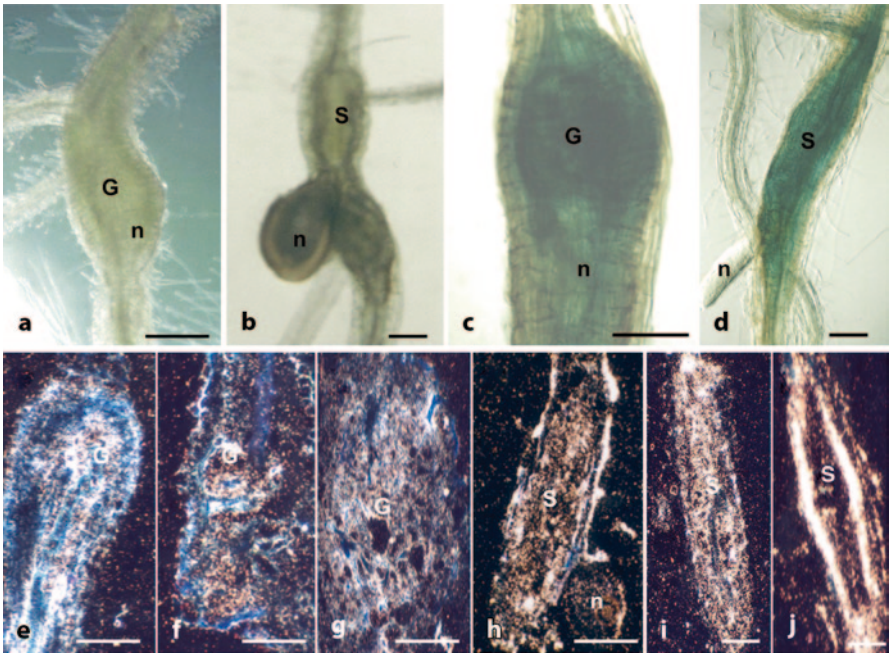

Fig. 18.2 Expression analysis of actins and tubulins in nematode infected Arabidopsis roots 10 dpi. **a** A gall induced by *Meloidogyne incognita*. **b** A syncytium induced by *Heterodera schachtii*. **c** Promoter activity of *ACT7* in a gall. **d** Promoter activity of *ACT7* in a syncytium. **e** mRNA *in situ* localization of α-tubulin in a gall (*white dots* represent the hybridization signal). **f** mRNA localization of β-tubulin in a gall. **g** mRNA localization of γ-tubulin in a gall. **h** mRNA *in situ* localization of γ-tubulin in a syncytium. **i** mRNA localization of β-tubulin in a syncytium. **j** mRNA localization of γ-tubulin in a syncytium. G, gall; n, nematode; S, syncytium. Bars = 100 μm

differentially expressed during feeding cell formation. These genes are involved in diverse processes that include cytoskeleton regulation.

18.2 Actin and Tubulin Genes are Highly Expressed in Nematode Feeding Sites

Transcriptional activity of actin genes has been reported in nematode feeding sites (NFS) induced by the RKN *M. incognita* and the CN *H. schachtii* (de Almeida Engler et al. 2004). Two genes, *ACT2* and *ACT7*, are transcriptionally active during gall and syncytium development suggesting that a large pool of G-actins is required for cytoskeleton remodelling in the feeding cells. *ACT2* and *ACT7* transcription shows significant up-regulation several hours after nematode infection, as revealed by high GUS expression driven by the *ACT2* and *ACT7* promoters (Fig. 18.2c, d). A constant increase in expression was observed until 4–5 days post inoculation (dpi). At later developmental stages (15–20 dpi) the promoters of both actin genes remained active and particularly high transcription was reported for *ACT7*. Vascular tissue near galls and syncytia often showed stronger GUS staining than more distal tissues, showing that nematode infection affects not only the feeding sites but also the nearby vascular cylinder. Both *ACT* promoters respond to wounding, hormones (McDowell et al. 1996a) and cell proliferation (McDowell et al. 1996b; Kandasamy et al. 2001). Nematodes wound root cells in order to feed, induce changes in hormone levels (Gheysen and Fenoll 2002) and provoke ectopic mitotic events in giant cells during NFS formation. Actin gene induction has also been observed during a compatible plant-fungus interaction (Jin et al. 1999) showing that plant cellular responses promoted by both fungi and nematodes resulted in up-regulation of actin expression.

Components of the microtubular cytoskeleton undergo drastic changes upon nematode infection as well. Elevated mRNA levels of α-, β- and γ-tubulin genes have been detected *in situ* (de Almeida Engler et al. 2004). High levels of tubulin are consequently found in giant cells (Fig. 18.2e–g). These feeding cells undergo numerous cycles of mitoses and therefore high expression levels of α-, β- and γ-tubulins might provide components for the assembly of mitotic MT arrays. Tubulin mRNA levels in syncytia are lower, which is in agreement with the lack of mitotic activity in this type of feeding cell (Fig. 18.2h–j; see Chap. 17). High transcript levels were noted in cells neighbouring syncytia that undergo division before their incorporation into the feeding site (Fig. 18.2j). In other biotic interactions such as ecto- and endomycorrhizas, transcriptional activation of the α-tubulins has also been observed (Bonfante et al. 1996; Carnero Diaz et al. 1996). During gall development, tubulin expression diminished gradually towards 25 days after infection. Transcriptional activation of actin and tubulin genes implies that additional G-actins and tubulins may be required for the cytoskeleton assemblage of giant cells, syncytia and their neighbouring cells.

18.3 Cytoskeleton Rearrangements in Nematode Feeding Sites

The organization of MTs and MFs in nematode feeding cells has recently attracted considerable interest. Detailed analyses of the cytoskeleton in feeding cells demonstrate that both actin and MT cytoskeleton are concomitantly affected but the effect of CN and RKN was remarkably different. While MTs and MFs were disrupted in syncytia, in giant cells a functional mitotic apparatus and disorganized cortical MTs and MFs were still present. This section describes up to date findings on the rearrangements of the MT and actin plant cytoskeleton in both types of feeding sites.

18.3.1 The Microtubular Cytoskeleton in Nematode Feeding Cells

Rearrangements of the plant cytoskeleton in giant cells and syncytia have been described by de Almeida Engler et al. (2004). The MAP4 MT-binding domain (MBD)-GFP marker protein was used to visualize dynamic changes in the organization of the MTs in living cells (de Almeida Engler et al. 2004; Caillaud et al. 2008a). It was shown that the MT cytoskeleton in nematode-infected roots is reorganized. In uninfected plants, root cells of the vascular cylinder are typically rectangular with transverse-to-oblique oriented MTs in the cell cortex. Mitotic MT arrays are rarely detected in these cells (Fig. 18.3a). During induction of giant cells and syncytia, GFP fluorescence resulting from tubulin proteins is detectable, even at low magnification (Fig. 18.3b). Penetration of RKN in the root elongation zone does not visibly affect the MTs in epidermal and cortical cells. In the course of giant cell and syncytium development, the three mitotic arrays (PPBs, spindles, and phragmoplasts) were frequently observed in cells surrounding the giant cells and syncytia. Neighbouring cells surrounding the giant cells showed curved and asymmetrically positioned phragmoplasts leading to the formation of differently shaped cells (Fig. 18.3c). Galls are characterized by giant cell expansion and ectopic cell division of neighbouring cells that result in root swelling (Fig. 18.2a, b). Using *in vivo* confocal microscopy of gall sections a complex cortical array with bundled MTs in giant cells (Fig. 18.3d) and an unstructured endoplasmic GFP signal (Fig. 18.3e) were revealed. Immunocytochemical analysis of α- and β-tubulins in fixed sections of *Arabidopsis* roots infected with both types of nematodes reinforced the *in vivo* observations carried out in whole mounts and unfixed tissue sections.

Both methods demonstrated an amorphous organization of the microtubular cytoskeleton in the cytoplasm of giant cells (Fig. 18.3e–f). Tubulin was strongly labelled around mitotic nuclei and near the cell cortex of giant cells, suggesting that the MTs link the host cell nucleus with the cell cortex (Fig. 18.3f). The endoplasmic tubulin staining and GFP signal observed in giant cells may result from the *de novo* synthesis of tubulin dimers, or depolymerization of the existing MTs. Alternatively, the

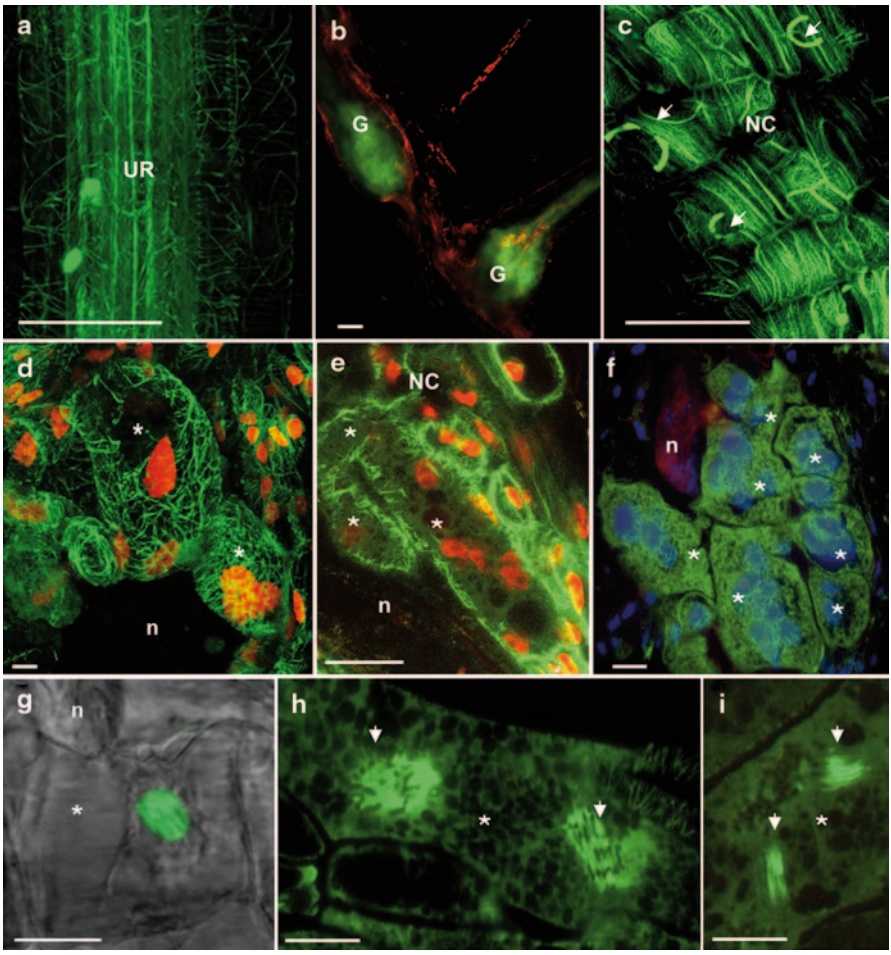

Fig. 18.3 The microtubule cytoskeleton in galls. **a** *In vivo* confocal microscopy of MTs (*green*) in an uninfected *Arabidopsis* root expressing MBD:GFP. **b** Root-knots or galls induced by *M. incognita* in *Arabidopsis* showing GFP fluorescence of MTs (*green* and autofluorescence in *red*). **c** *In vivo* confocal microscopy of MTs of gall cells neighbouring the giant cells showing curved phragmoplasts (*arrows*). **d** *In vivo* confocal microscopy of a young gall co-expressing DNA (H2B:YFP in *red*) and MT markers (MBD-GFP in *green*). Interphasic young (7 dpi) giant cells showing bundles of cortical MTs. **e** *In vivo* confocal microscopy of cortical MTs and a diffuse cytoplasmic fluorescence (*green*) in maturing (10 dpi) giant cells containing several nuclei (*red*). **f** Immunocytochemical analysis of MTs in a section of a gall in *Pisum sativum* (pea) showing a diffuse fluorescence in the cytoplasm of giant cells (MTs in *green*, autofluorescence in *red* and nuclei in *blue*). **g** A mitotic spindle in a cell neighbouring a giant cell expressing MDB:GFP. **h** Large malformed spindles (*arrowheads*) of mitotic nuclei of a giant cell. **i** Phragmoplasts (*arrowheads*) with misaligned microtubules (*green*) in a mitotic giant cell. UR, uninfected root; G, gall; NC, neighbouring cells; n, nematode; Asterisks, giant cells. Bars = 50 μm (**a, c–e**), 100 μm (**b, f**), 10 μm (**g**) and 20 μm (**h, i**)

presence of nematode secreted compounds might prevent assembly of new MTs and thereby affect the pool of free tubulin and of MT binding proteins in the giant cells. In contrast, a cortical MT array can still be observed in young giant cells (Fig. 18.3d–e). A functional mitotic apparatus containing multiple spindles and phragmoplasts is present throughout the abundant mitotic events observed in giant cells (Fig. 18.3h–i). However, PPBs were never detected. Changes in the dynamics of MTs induced by nematodes may affect or prevent the assembly of a PPB. Since giant cells do not form a cell wall following karyokinesis, the PPB array becomes superfluous and may not be produced or is prevented from forming. Mitotic figures are also often observed in cells neighbouring giant cells during mitosis (Fig. 18.3g). In giant feeding cells spindles are enlarged and often altered in shape (Fig. 18.3h; de Almeida Engler et al. 2004). Phragmoplasts are short and misaligned (Fig. 18.3i; de Almeida Engler et al. 2004). This unusual organization of the phragmoplasts might result in misalignment of the cell plate and disoriented plane of cell division. Giant cells are unable to complete cytokinesis and their phragmoplasts appear not to be fully developed. Completion of cytokinesis depends on coordinated interaction and fusion of secretory vesicles, dynamic cytoskeleton and a set of accessory proteins (Sylvester 2000). The alignment of secretory vesicles in the phragmoplast midzone is perturbed in giant cells (Jones and Payne 1978). On the other hand, failure of the phragmoplast guidance during the expansion stage may also contribute to the incomplete cytokinesis and the deposition of a mini cell plate (Caillaud et al. 2008a).

No mitotic MT arrays have been observed during syncytial cell development. Mitosis only occurs in cells of the vascular cylinder prior to their incorporation into syncytium (de Almeida Engler et al. 1999). During the early stage of syncytium expansion, the spatial organization of cortical MT changes progressively and a distinct MT orientation was seen within each syncytium zone from predominantly oblique to randomly oriented MT arrays (Fig. 18.4a; de Almeida Engler et al. 2004). The bright

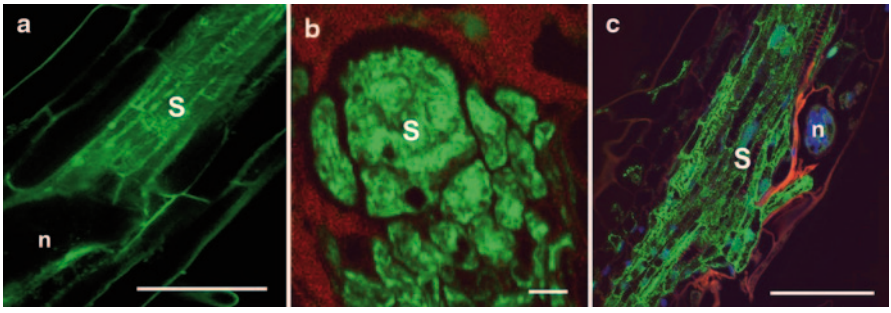

Fig. 18.4 The microtubule cytoskeleton in syncytia. **a** *In vivo* confocal microscopy of MTs (*green*) in a syncytium showing a diffuse fluorescence close to the nematode head and random distribution in neighbouring cells. **b** MTs (*green*; autofluorescence in *red*) *in vivo* in a fresh section of a syncytium. **c** Immunocytochemical analysis of tubulins in a section of a syncytium showing a diffuse fluorescence in the centre of the feeding structure and random distribution of MTs in neighbouring cells (MTs in *green*, autofluorescence in *red* and nuclei in *blue*). S, syncytium; n, nematode. Bars = 50 μm

dispersed fluorescence in the cytoplasm (or endoplasm) indicates an abundant pool of free tubulin that might originate from *de novo* synthesis and/or from depolymerization of the existing MTs (Fig. 18.4b–c). The normal microtubular organization in vascular cells adjacent to the syncytia may result from a syncytium being an isolated symplastic zone with no functional plasmodesmata (Golinowski et al. 1997). Therefore, at least in early developmental stages of syncytia, signals that trigger MT disorganization do not reach neighbouring cells until their incorporation into the syncytia.

18.3.2 The Actin Cytoskeleton in Nematode Feeding Cells

Observations of MFs labelled with GFP-tagged actin-binding proteins (GFP:mTalin or GFP:FABD) in living plant cells (Kost et al. 1998; Ketelaar et al. 2004) and immunocytochemical studies have revealed a disturbed organization of the actin cytoskeleton in nematode feeding cells (de Almeida Engler et al. 2004, 2010). Vascular root cells are characterized by a dense network of actin filaments (Fig. 18.5a).

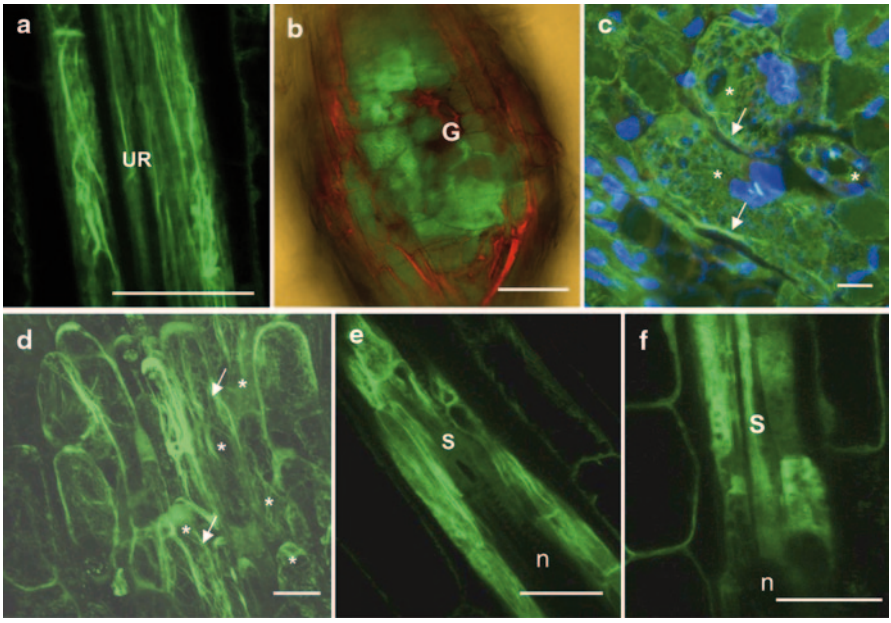

Fig. 18.5 The actin cytoskeleton in an uninfected root, galls and syncytia. **a** *In vivo* confocal microscopy of actin filaments in an uninfected root. **b** Actin fluorescence (in *green*; autofluorescence in *red*) in a fresh gall 7 dpi. **c** Immunocytochemical analysis of actin in a section of a gall 7 dpi showing a *green* fluorescence in the cytoplasm (nuclei in *blue*) and actin bundles in the cell cortex (*arrows*). **d** MFs *in vivo* in a gall 7 dpi with thick bundles in the giant cell cortex (*arrows*). **e** MFs *in vivo* in a syncytium 3 dpi showing a diffuse fluorescence close to the nematode and filaments in neighbouring cells. **f** MFs *in vivo* in a syncytium showing a diffuse fluorescence close to the nematode head 5 dpi. UR, uninfected root; G, gall; Asterisks, giant cells; S, syncytium. Bars = 50 µm

Giant- and neighbouring cells show intense GFP-ABD fluorescence, indicating an abundance of actin protein, but this actin appears to be less well organised than in other cells (Fig. 18.5b). Cells neighbouring the giant cells show randomly distributed actin filaments which often appear denser in the cells that are directly in contact with the giant cells. Thin actin filaments in the cytoplasm of giant cells appear fragmented, possibly due to depolymerization or severing (Fig. 18.5c; de Almeida Engler et al. 2004). The absence of endoplasmic MFs was also apparent during cell division (de Almeida Engler et al. 2010). MFs are also involved in phragmoplast organization and cell plate formation (Van Damme et al. 2007). Electron micrographs of phragmoplasts in giant cells show that actin filaments are disorganized and misaligned (de Almeida Engler et al. 2010). This atypical organization of the MFs in the phragmoplasts of giant cells might contribute to the disruption of cell plate synthesis via vesicle positioning and fusion, and consequently contribute to preventing future cytokinesis. Abnormally thick actin filaments have been observed in the giant cell cortex providing further evidence that the MF network responds to the presence of nematodes (Fig. 18.5d).

The actin cytoskeleton in syncytia appears more disrupted than in giant cells. *In vivo* (Fig. 18.5e–f) and immunocytochemical analyses of the feeding structures revealed a gradual decrease of cytoplasmic F-actins at early developmental stages and a mainly diffuse cytoplasmic fluorescence later on (Fig. 18.5f; de Almeida Engler et al. 2004). During syncytium induction, a radial array of actin filaments focused along the nematode head was observed, suggesting the involvement of the actin cytoskeleton in the early steps of syncytium induction (de Almeida Engler et al. 2004, 2010). In cowpea infected by *Uromyces*, MFs are consistently observed to focus on the infection site in susceptible plants (Skalamera and Heath 1998). Actin accumulation close to the nematode head might be part of a basal resistance response to nematode infection that could be later suppressed by nematode effectors during a susceptible interaction.

Up to now, all reports suggest that nematode infection causes a reshuffling of the actin cytoskeleton in both types of feeding cells throughout nematode development. The study of actin-associated proteins might help to explain how these cytoskeletal adjustments in giant cells and syncytia occur and what the consequences are for feeding cell differentiation. It is tempting to speculate that the structural changes in the actin cytoskeleton might be the consequence of specific nematode secretions injected into vascular cells, able to trigger the cytoskeletal changes needed to establish a functional feeding cell.

18.4 The Effects of Cytoskeleton-Disrupting Drugs on Nematode Feeding Sites

Studies employing cytoskeleton-disrupting drugs combined with the study of cytoskeletal mutants, demonstrate that both MTs and actin filaments are critical for different cellular processes including cell expansion (Kobayashi and Kobayashi 2007;

Takemoto et al. 2003). Therefore, expansion of feeding cells induced by parasitic nematodes should depend on the cytoskeleton. Nematode infected roots have been exposed to the cytoskeleton inhibitors taxol (which stabilizes MT), oryzalin (which destabilizes tubulins) and cytochalasin D and latrunculin A (which both destabilise actin) to investigate whether cytoskeleton changes are a prerequisite for nematode infection, feeding site initiation and development, and consequently nematode maturation (de Almeida Engler et al. 2004 and unpublished data). Control experiments have shown that treatments using low concentrations of these drugs do not interfere with nematode viability and reproduction. Breakdown of the actin cytoskeleton by cytochalasin D treatment during feeding site initiation strongly affected giant cell development whereas syncytium growth was less affected. The dissimilarity between the two types of feeding sites is possibly due to the presence of actin filaments in giant cells compared to syncytia, where no filaments were detected in the cytoplasm. Similar results have been obtained in tests in soil using taxol and cytochalasin in pepper (*Capsicum annuum*) as a host plant infected with *M. incognita*. Treatment of feeding sites (3–7 dpi) with colchicine, which inhibits MT polymerization by binding to tubulin, have confirmed data obtained with oryzalin treatment (Wiggers et al. 2002). Infected seedlings (3 dpi) treated with colchicine arrested giant cell development and consequently nematode maturation. However, more mature feeding sites (7 dpi) showed a normal rate of nematode maturation even though numbers of nuclei per giant cell were reduced compared to controls (Wiggers et al. 2002). These results suggest that the number of nuclei per giant cell present at 3 dpi was not sufficient to sustain normal nematode development. Conversely, stabilization of the MTs with taxol at 3 dpi reduced the rate of feeding site initiation and inhibited nematodes maturation and reproduction. In taxol treated giant cells or syncytia, feeding cells were devoid of their typical dense cytoplasm. This may reduce nutrient supply and inhibit juvenile nematode development. Therefore, preventing plant cytoskeleton breakdown limits the ability of newly established feeding sites to expand. Once a feeding site has been established (~14 dpi), modification of actin or MTs dynamics with chemical compounds that breakdown the cytoskeleton has no effect on feeding site development, and both RKN and CN can successfully complete their life cycle. However, application of taxol after the expansion of feeding sites delayed nematode maturation and reduced the proportion of juveniles capable of completing their life cycle. It has been shown that non-pathogenic fungi are able to penetrate barley coleoptile cells and form secondary hyphae under cytochalasin treatment (Kobayashi et al. 1997). Cytochalasin also suppresses or delays resistance reactions such as hypersensitive cell death allowing *Blumeria graminis* (syn. *Erysiphe graminis*) to go through its lifecycle in nonhost plant (Yun et al. 2003).

Nematode infection triggers alteration of MTs and actin organization in the two types of feeding cells and this is maintained throughout the nematode's life cycle. MFs and MTs interact with each other structurally and functionally and regulate universal mechanisms in plant cells. In addition, MTs contribute to cytoplasm viscosity (Gross et al. 1993) and a level of cytoskeleton fragmentation might facilitate the ingestion of the large cytoplasm volumes taken up during the nematode feeding process. Reorientation of the plant cytoskeleton in response to pathogen attack and

reuse of plant materials via cytoskeletal reorganization can also be coordinated by an invading pathogen as part of its strategy of gaining access to plant nutrients, as observed during the development of haustoria of biotrophic pathogens (Takemoto and Hardham 2004; Schmidt and Panstruga 2007). Stabilization of the MTs results in normally developed feeding sites but still leads to improper maturation of the infecting juveniles most likely by disturbing nematode feeding. Breakdown of the cytoskeleton seems to be a prerequisite to allow completion of the nematode's life cycle, as shown by drug-induced depolymerization of the actin or MTs leading to almost normal maturation of the infecting nematode (de Almeida Engler et al. 2004). Future work will determine whether nematode secretions contain proteins that could interact with MFs or MTs and account for the reorganization of the host cytoskeleton.

18.5 Cytoskeleton Interacting Proteins and Their Putative Role in Feeding Site Development

Microtubule Associated Proteins and ABPs play essential roles in controlling MTs and actin dynamics and organization. The first plant candidate proteins implicated in giant cell actin and MT cytoskeleton reorganization have been identified. Formin AtFH6 and MAP65-3 were identified by a promoter trap strategy of genes expressed in giant cells in *Arabidopsis* (Favery et al. 2004; Caillaud et al. 2008a). A candidate gene approach revealed the key role of ADF2 (Clement et al. 2009). Both MAP65-3 and ADF2 functions have been shown to be essential for giant cell ontogenesis in *Arabidopsis*. Genome-wide expression profiling of the host response to RKN infection identified also *Arabidopsis* regulated genes encoding several classes of cytoskeletal proteins including some formins, ADFs, annexins, villins, MAP65s and kinesins (Jammes et al. 2005; Fuller et al. 2007; Barcala et al. 2010).

18.5.1 Microtubule Associated Proteins in NFS

Plant MAP65s were first purified from tobacco and carrot MT preparations (Jiang and Sonobe 1993; Chan et al. 1999; Smertenko et al. 2000). Biochemical studies have shown that MAP65s bind and bundle MTs *in vitro* and nine MAP65 isoforms can be identified in the *Arabidopsis* genome (Hussey et al. 2002). Plant MAP65s differ in their activities, functions, and subcellular localization studies showed that MAP65s may be targeted to all or to specific parts of the MT arrays (Van Damme et al. 2004; Gaillard et al. 2008; Smertenko et al. 2008). EMS-induced *map65-3* mutants display cell wall stubs and multiple nuclei in the root meristem, a characteristic feature of cytokinesis-defective mutants (Muller et al. 2002, 2004). *MAP65-3* expression is induced in the initial phases of giant cell formation and rapidly declines before the development of fully mature giant cells (Caillaud et al. 2008a).

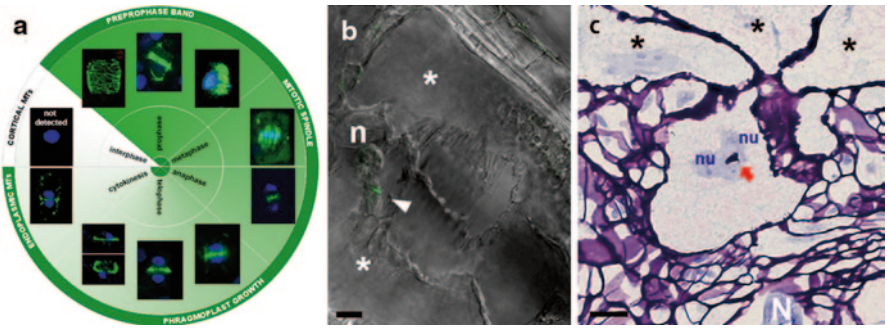

Fig. 18.6 MAP65-3 *in planta* subcellular localization. **a** MAP65-3 localization throughout the cell cycle. MAP65-3 labelled PPB in preprophase, metaphase and anaphase spindle, and phragmoplast. Images were obtained by *in vivo* confocal microscopy of *Arabidopsis* root cells co-expressing two marker proteins, MAP65-3:GFP under the control of the *MAP65-3* promoter (in *green*) and nuclear Histone 2B-YFP (in *blue*). **b** MAP65-3:GFP localization to a mini cell plate (*arrowhead*) in a giant cell 10 dpi. **c** Giant cell mini cell plate (*red arrow*) that separates daughter nuclei but did not extend across the cell. Sections through a gall at 10 dpi stained with toluidine blue. Asterisks, giant cells; n, nematode; nu, nuclei. Bars = 20 μm

When mature galls contain fully expanded and differentiated giant cells, *MAP65-3* expression was detected only in the dividing surrounding cells. Detailed functional analysis during plant development has highlighted the role of MAP65-3 in plant cell division. MAP65-3 colocalizes with all mitotic MT arrays and cell plate deposition in somatic cells. It plays a key role in their organization during both mitosis (spindle morphogenesis) and cytokinesis (phragmoplast expansion) (Fig. 18.6a). No MAP65-3 signal was detected in interphase cells. *In vivo* confocal microscopy of 10 dpi gall sections revealed that MAP65-3 was associated with mitotic MT arrays in surrounding cells, as observed for root meristematic cells. After these cells had completed cytokinesis, MAP65-3 colocalized with the newly formed cell plate separating the two daughter cells. In developing giant cells, MAP65-3 decorates the nascent cell plate, created from the phragmoplast MT array, initially lined up between the two daughter nuclei during cytokinesis initiation. However, the nascent cell plate does not expand further to complete cytokinesis in giant cells (Fig. 18.6b) due to phragmoplast restricted out-growth. Cytokinesis initiation during giant cell formation first shows a normal alignment of cell plate vesicles followed by the dispersion of these vesicles and cytokinesis arrest (Jones and Payne 1978). The giant cell-specific "mini cell plates" may form a physical barrier separating the two daughter nuclei and may be required for the multiple rounds of mitosis that occur in developing giant cells, resulting in the formation of a functional feeding site. Optical microscopy confirmed that mini cell plates, and subsequently mini cell walls, were frequently observed between two nuclei in giant cells (Fig. 18.6c). In the absence of functional MAP65-3, giant cells started to develop but failed to fully differentiate and were eventually destroyed. These giant cell defects impaired the maturation of the infecting nematodes. Giant cell mini cell plates were never observed in the *map65-3* knock-out mutant, where they were replaced by aberrant

cell wall stubs. We therefore hypothesize that the accumulation of mitosis defects during repeated mitoses prevents the development of functional feeding cells, resulting in the death of the nematode. Thus, MAP65-3 plays a critical role in giant cell mitotic MT array organization required for successful pathogen growth and development (Caillaud et al. 2008a).

18.5.2 Actin Binding Proteins in NFS

Formins, also known as formin homology (FH) proteins, are actin-nucleating proteins that stimulate MF assembly for fundamental cellular processes including cell division, adhesion, establishing polarity, and motility in eukaryotes (Faix and Grosse 2006; Kovar 2006; Staiger and Blanchoin 2006). Although FH proteins are required for organization of the actin cytoskeleton, some formins have also been found to be implicated in MT cytoskeleton regulation (Lee et al. 1999; Palazzo et al. 2001). Animal and fungal formins have been studied extensively, but little is known about the function of formins in plants. *Arabidopsis* contains 21 AtFH genes that can be grouped into two major families (Cvrckova 2000; Deeks et al. 2002). Three type-I formin genes, *AtFH1, AtFH6* and *AtFH10* are induced in developing giant cells induced by RKNs and expression persists until their final differentiated state (Favery et al. 2004; Jammes et al. 2005; Barcala et al. 2010). *AtFH6* expression was not detected thereafter in fully differentiated giant cells. In contrast *AtFH4* and *AtFH21* were shown to be repressed in dissected giant cells 3 days post infection (Barcala et al. 2010). In contrast to AtFH proteins of animals and fungi, type I AtFH proteins are unique in containing an N-terminal domain with a putative signal peptide or membrane anchor and a transmembrane domain. Subcellular localization and cell fractionation analyses showed that AtFH6 was anchored to the plant plasma membrane (Fig. 18.7a). Immunolocalization demonstrated that AtFH6 was uniformly distributed throughout the giant cell plasma membrane (Fig. 18.7b). Single *atfh6* (Favery et al. 2004), *atfh1* and *atfh10* mutants (unpublished results) were not distinguishable from wild type plants and supported similar RKN development. This lack of a visible phenotype may be explained by genetic redundancy. The role of AtFH6 in cytoskeleton organization was investigated by determining that AtFH6 functionally suppressed the budding defect of a yeast formin mutant. Thus, these three formin genes may regulate isotropic growth of hypertrophied feeding cells by controlling the assembly of actin cables at the plasma membrane (Fig. 18.7c). During giant cell formation, the observed thick actin cables would guide the vesicle trafficking needed for extensive plasma membrane and cell wall biogenesis. Interestingly AtFH1 or AtFH3 overexpression in pollen tubes induced the formation of supernumerary actin cables leading to tube broadening, growth depolarization and growth arrest (Cheung and Wu 2004; Ye et al. 2009). Overexpression of AtFH8 perturbs root hair tip growth (Deeks et al. 2005). Biochemical analysis demonstrated that AtFH1 and AtFH5 nucleate actin *in vitro* (Ingouff et al. 2005). AtFH1 FH2 domain allows barbed-end elongation and nucleation of MFs from actin monomers bound to profilin. The activity of

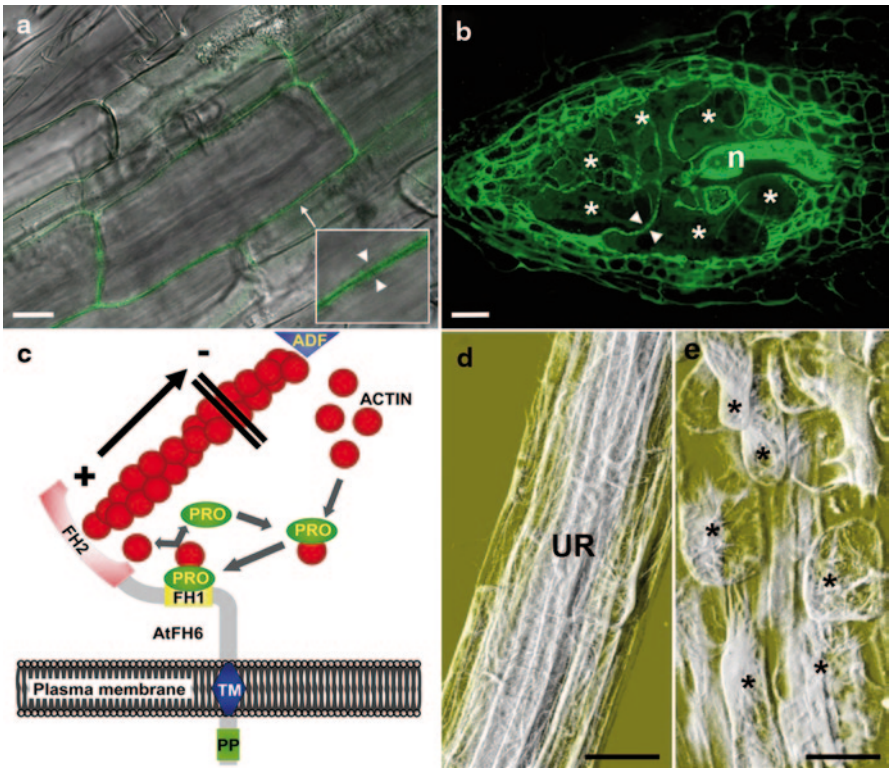

Fig. 18.7 Actin binding proteins AtFH6 and AtADF2 in galls induced by root-knot nematodes. **a** Fluorescent signal (*green*) showing plasma membrane targeting of AtFH6:GFP (*arrows*). **b** Immunolocalization of the AtFH6 protein (*green*) in *Arabidopsis* gall section 10 dpi. Fluorescent signal is detected at the plasma membrane (*arrowheads*) of giant cells and neighbouring cells. **c** Model of actin cable assembly at the plasma membrane by the formin AtFH6. The FH1 and FH2 conserved domains of membrane-anchored AtFH6 cooperate in rapidly assembling profilin (PRO)-actin at the barbed end (+) into long filaments. ADF/cofilin proteins will bind G- and F-actin and increase actin turnover by severing actin filaments and by increasing the rate of dissociation of actin monomer from the pointed ends. **d** Actin cytoskeleton in *AtADF2*-RNAi roots showing thicker vascular tissues and actin bundling particularly in vascular cells. **e** Actin cytoskeleton organization in a gall of an *AtADF2*-RNAi seedling. Thick cables are present throughout the cytoplasm of giant cells arrested in their development. TM, transmembrane domain; PP, extracellular polyproline region; UR, uninfected root; Asterisks, giant cells; n, nematode. Bars = 5 μm (**a**), 50 μm (**b, d, e**)

the FH2 domain, containing the actin binding activity, is modulated by the FH1 domain that is necessary for efficient nucleation (Fig. 18.7c). Another exciting feature of AtFH1 is its ability to organize actin filaments directly into parallel or anti-parallel MFs for the formation of actin bundles or cables. Until now, only profilins and a putative membrane protein (FIP2) have been shown to interact with FH1 domain of plant formins (Deeks et al. 2002). The identification of formin-interacting proteins in giant cells would allow the study of regulatory mechanisms and signalling molecules responsible for actin cytoskeleton reorganization.

Recently, we have shown that actin-depolymerizing factor 2 (ADF2) is required for normal *Arabidopsis* development and its role in successful RKN infection was revealed (Clement et al. 2009). ADF or cofilin is a crucial regulator of the turnover of filamentous actin (Carlier et al. 1997; Staiger 2000; Maciver and Hussey 2002). The ADF/cofilin proteins associate with monomeric and filamentous actin and increase actin turnover by severing MFs and by increasing the rate at which monomers leave the filament's pointed end (Carlier et al. 1997; Chen et al. 2000; Maciver and Hussey 2002; Andrianantoandro and Pollard 2006; Pavlov et al. 2007). The *Arabidopsis* ADF gene family comprises 11 expressed members grouped phylogenetically into four ancient subclasses (Feng et al. 2006) with distinct, but intersecting expression patterns (Dong et al. 2001; Ruzicka et al. 2007). Transcript levels and promoter activity of seven *Arabidopsis ADF* genes showed that five of them are upregulated in galls, with a specific expression of *ADF2* in giant cells and neighbouring cells (NC) up to 14 dpi (Clement et al. 2009). Microarray survey of dissected feeding sites has shown *ADF3* expression, and promoter activity has been detected in the root vascular tissue and in whole feeding sites induced by RKN and CN (Fuller et al. 2007). Histological analysis of galls revealed that *ADF3, ADF4*, and *ADF6* are feebly expressed or not present in giant cells, and stronger ADF promoter-driven GUS expression was detected in NC, which suggests that the function of these isotypes may be only partially overlapping with ADF2. Inducible downregulation of *ADF2* in RNAi lines revealed the stabilisation of the F-actin network in uninfected root vascular tissue (Fig. 18.7d), still allowing giant cell initiation though blocking further feeding cell and nematode development (Fig. 18.7e; Clement et al. 2009). The effect on giant cells caused by the knockdown of *ADF2* on the actin network was clearly caused by the decrease in F-actin turnover responsible for the stabilization and bundling of the actin cytoskeleton. This actin cytoskeleton stabilization prevented nematodes to mature into females and to generate eggs. Therefore, the high expression of *ADF2* in nematode feeding cells induced by RKN seems to be an essential factor for a successful nematode infection.

The importance of the ADF2 in actin organization and giant cell development validates former experimental data attained on feeding cell cytoskeleton under drug treatments (de Almeida Engler et al. 2004). Thus, a high concentration of ADF2 in giant cells may cause the destabilization of the MFs, decreasing actin network integrity and consequently facilitating nematode feeding and its development and maturation (Clement et al. 2009). These observations reveal that MF network reorganization regulated by actin-associated proteins is critical for a successful nematode infection of *Arabidopsis* plants. Moreover, it appears that giant cell growth and expansion depends on a dynamic actin cytoskeleton. Recent work shows the importance of *Arabidopsis* ADF4 in the control of the actin cytoskeleton rearrangement necessary for AvrPphB mediated resistance against the phytopathogenic bacterium *P. syringae* (Tian et al. 2009). Miklis et al. (2007) observed that interference with the actin cytoskeleton function is not controlled by a particular ADF isoform, but it appears to be a common feature of ectopic ADFs expression. They also reported that ectopic ADF3 expression in barley (*Hordeum vulgare*) affects actin cytoskeleton integrity and function in epidermal cells. All together, these observations demonstrate that phylogenetically divergent plant pathogens have developed a common

mechanism to control the dynamic state of the host actin filament network by up-regulation of ADF expression. Future work will be focused on the identification of other actin binding proteins implicated in the reorganization of the feeding cell actin cytoskeleton and the pathways regulating their activity.

18.6 Closing Remarks

The data presented here support the view that nematodes induce long-term cytoskeleton rearrangements in plant cells, and that these are essential for the development of a functional feeding site to allow proper nematode maturation. The observed cytoskeleton restructuring seems to be essential during nematode feeding cell initiation, as well as for the expansion of the feeding cells, since a disturbed organization of MTs and MFs can be observed both in giant cells and syncytia. Analysis of the molecular mechanism of the cytoskeletal reorganization in giant cells has been initiated by identifying MT and actin interacting proteins such as formins, ADF2 or MAP65-3. These studies lay a foundation for further in-depth studies on the regulatory mechanisms and signalling molecules involved in plant cell cytoskeleton reorganization during nematode infection. A better knowledge of the plant cytoskeleton and its responses during a compatible interaction will facilitate the discovery of potential targets to engineer resistance to parasitic nematodes in crops.

The idea that nematodes may deliver specific effector molecules into the plant cell targeting (either directly or indirectly) the cytoskeleton machinery is very tempting, and a search for these effectors is currently in progress. Knowledge of the full genome sequences of root-knot nematodes (Abad et al. 2008; Opperman et al. 2008) and, in the near future, of cyst nematodes, will be a very useful resource in the identification of the effectors and will help to gain a better understanding of the role and participation of the cytoskeleton during different types of interactions between phytopathogenic nematodes and their host plants.

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Chapter 19
Cell Wall Modifications Induced by Nematodes

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Abbreviations

DAI	day after root invasion
EGase	endoglucanase
ISC	initial syncytial cell
J2	second stage juvenile
J3	third stage juvenile
J4	fourth stage juvenile
NFS	nematode feeding site
TEM	transmission electron microscopy

19.1 Introduction

The plant primary cell wall is a complex and dynamic association of different high molecular weight polysaccharides and structural, enzymatic and catalytic proteins (Somerville et al. 2004; Lerouxel et al. 2006). Cellulose microfibrils are its main component forming an intercalated network that surrounds and wraps around the protoplast. Microfibrils are cross-linked and interconnected by chains of different hemicelluloses, pectins and proteins. The cell wall creates an outer cover for each plant cell and provides mechanical strength. It counteracts the osmotic pressure caused by vacuolar sap, thus preventing bursting of the plant cell. Each plant cell forms its own cell wall and neighbouring cell walls are connected by a middle lamella composed mostly of pectins. Although mechanical properties are the most important function of a cell wall, it cannot be a fixed structure. It has to respond and flexibly react to different biotic and abiotic stresses and developmental stimuli to allow proper development and differentiation of plant tissues and organs. Tak-

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ing these facts into consideration, it is easier to understand why the *Arabidopsis thaliana* genome contains more than 700 genes encoding glycosyl transferases and hydrolases, about 200 genes with similarity to pectin degrading enzymes and several hundred genes encoding other types of proteins involved in cell wall synthesis, degradation and modification (Somerville et al. 2004). This high number of cell wall-related genes indicates how flexible and sophisticated this organelle is and how well developed and complex the processes involved in cell wall biology are. In addition, many plant cells also deposit a secondary cell wall on the inner surface of their primary wall. Its chemical composition and structure is different from the primary wall as it may contain different secondary-wall specific components such as lignin or suberin.

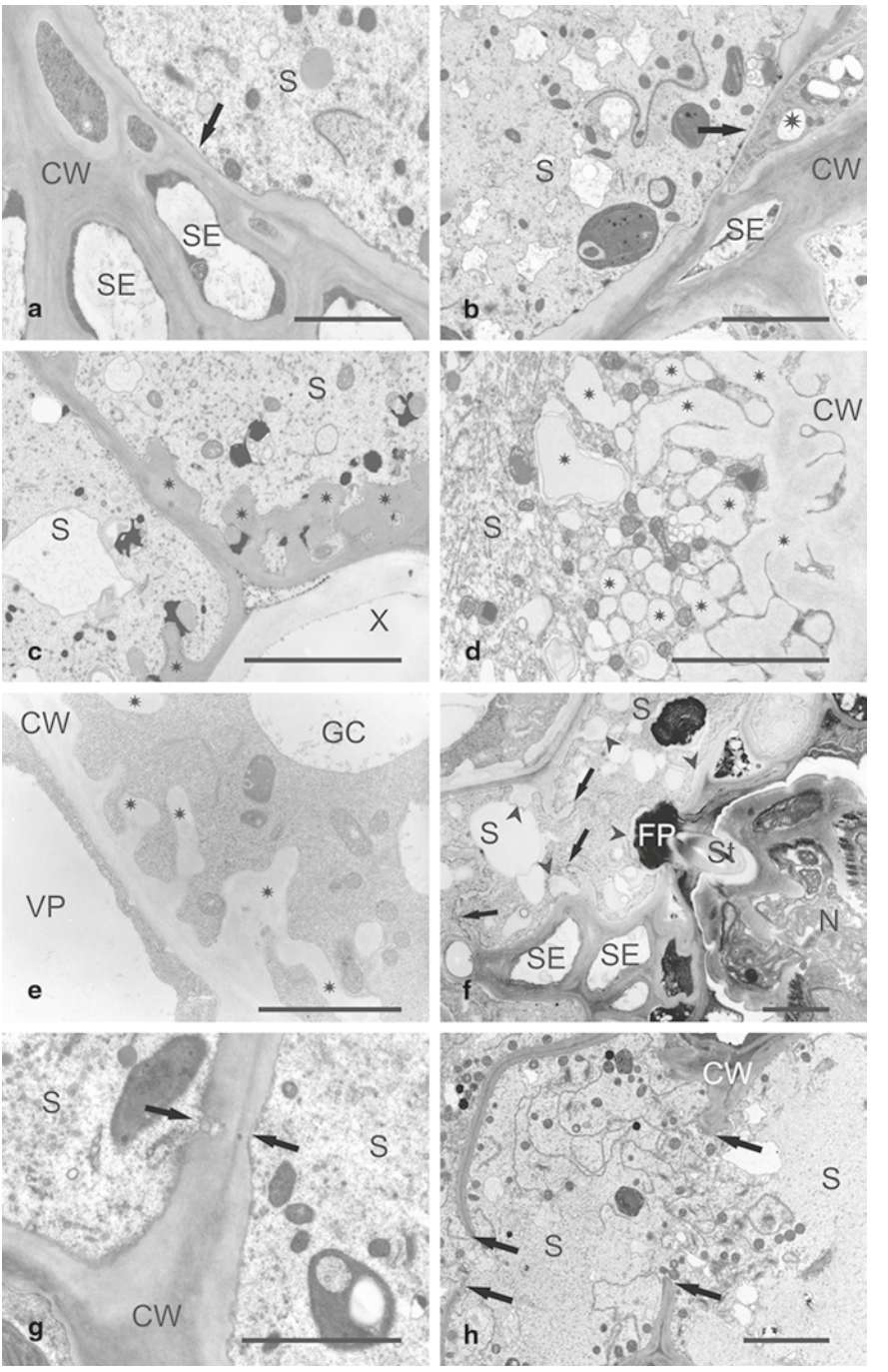
The structural and chemical complexity of the cell wall poses serious problems for endoparasitic nematodes which have to overcome the defence mechanisms and physical barriers provided by cell walls of root cells and successfully penetrate tissues and migrate inside the host. These processes are described in detail in Chap. 12. In this chapter we focus on the cell wall modifications occurring soon after the nematode reaches the initial feeding cells and starts to transform them into the feeding site. We describe the cell wall rearrangements that take place during development of giant-cells and syncytia based on microscopic observations and analyses of changes in the expression pattern of plant genes. When the nematode becomes sedentary and starts feeding, secretion of its own endogenous cell wall degrading enzymes is markedly reduced (Goellner et al. 2001). The idea that plant genes are induced in order to allow the extensive and complex cell wall remodelling that takes place in nematode feeding sites (NFS) has been discussed for many years. This hypothesis is now well supported by numerous studies on changes in plant gene expression in nematode infected roots (Ithal et al. 2007a, b; Jammes et al. 2005; Puthoff et al. 2003, 2007; Swiecicka et al. 2009; Tucker et al. 2007; Vercauteren et al. 2002; Wieczorek et al. 2006, 2008). Here, we present data concerning expression of plant genes encoding proteins involved in cell wall modifications and biosynthesis in the NFS, possible mechanisms of their activation in parasitized roots as well as the effects of silencing of particular cell wall modifying genes of plant origin on nematode and NFS development.

19.2 Ultrastructure of Feeding Site Wall in Susceptible Interactions

After migration inside the plant root, infective juveniles of sedentary root-parasitic nematodes induce the formation of a feeding site. Juveniles of *Meloidogyne* sp. induce groups of giant-cells preferentially located among procambial cells just above the root-tip meristem (Wyss et al. 1992). Juveniles of cyst forming nematodes induce syncytia composed of fused cells. Juveniles of *Heterodera* sp. usually select the initial syncytial cells (ISC) among procambial cells in the elongation and root-hair zone while juveniles of *Globodera* sp. prefer cortical parenchyma or endodermal cells for ISC induction (Golinowski et al. 1996; Sobczak et al. 2005). Six

hours after the invasive juvenile becomes motionless the ISC wall becomes locally thickened. New layers of electron translucent cell wall material, apparently formed from callose (Hussey et al. 1992), are deposited around the inserted stylet-tip and on adjacent parts of the cell wall (Fig. 19.1f). Deposition of callose also takes place locally in neighbouring cells. In spite of their different ontogeny and development (for details see Chaps. 4 and 5), giant-cells and syncytia have several structural cell wall modifications in common. After induction, the initial cells enlarge and expand while surrounding parenchymatous cells divide. Some cells differentiate into elements of regular tissues, such as functional xylem vessels or sieve tubes (Berg et al. 2008; Golinowski et al. 1996). The most remarkable feature of the feeding site cell wall is its general thickening. Histological staining and immunogold labelling indicated that cell walls of giant-cells and syncytia contain cellulose, hemicelluloses and pectins characteristic for primary cell walls. No trace of lignin, a typical component strengthening secondary walls, could be detected (Berg et al. 2008). In giant-cells thickening appears very early, but it is localised to numerous small patches on giant-cell walls. These patches expand and merge leading to the formation of large and extensive cell wall thickenings sometimes resembling bordered pits with torus. However, even in mature giant-cells the walls are never uniformly thickened, suggesting that the mechanism for cell wall deposition is asymmetrically distributed during their development (Berg et al. 2008). A different situation is observed in syncytia, where the outer wall thickens evenly throughout syncytium development. In contrast to this, the parts of the syncytial wall that face sieve tubes often remain thin for a long time, but the sieve tube walls become thickened (Grundler et al. 1998; Fig. 19.1a). A second exception is the outer syncytial wall and especially its parts that face neighbouring cells. These undergo processes leading to their incorporation into the syncytium (Fig. 19.1b). The thickened syncytial cell wall does not reveal any special structural or staining pattern when observed at high magnifications in the transmission electron microscope (TEM) (Figs. 19.1a, b). It stains in a similar manner to the primary walls of other vascular cylinder cells. Thickening of the cell wall is apparently a response to high internal osmotic pressure which, in syncytia induced by *H. schachtii* in *A. thaliana* roots, can reach 10,000 hPa (Böckenhoff and Grundler 1994).

Formation of cell wall ingrowths is another feature common between giant-cells and syncytia (Jones and Gunning 1976; Jones and Northcote 1972a, b; Golinowski et al. 1996). Cell wall ingrowths are characteristic feature of transfer cells that are specialised in short distance transport of water and nutrients in plants. They significantly increase the surface of the plasmalemma and thus the symplast-apoplast interface (Gunning 1977; Offler et al. 2002; Pate and Gunning 1972). NFS has to provide sufficient amounts of water and nutrients to ensure proper development of the associated juvenile and to keep the feeding site functional. Cell wall ingrowths are thought to facilitate this, as they initially appear as small finger-like protrusions on parts of the syncytial wall facing vessels in syncytia at 7–10 DAI (DAI–**d**ays **a**fter root **i**nvasion; Fig. 19.1c). Later, in syncytia associated with J4 or adult females, they elongate, branch and form elaborate reticulate labyrinths usually only on walls facing xylem vessels. They grow up apically but the bottom parts of ingrowths fuse together forming extensive cell wall thickenings (Fig. 19.1d). Syncytia associated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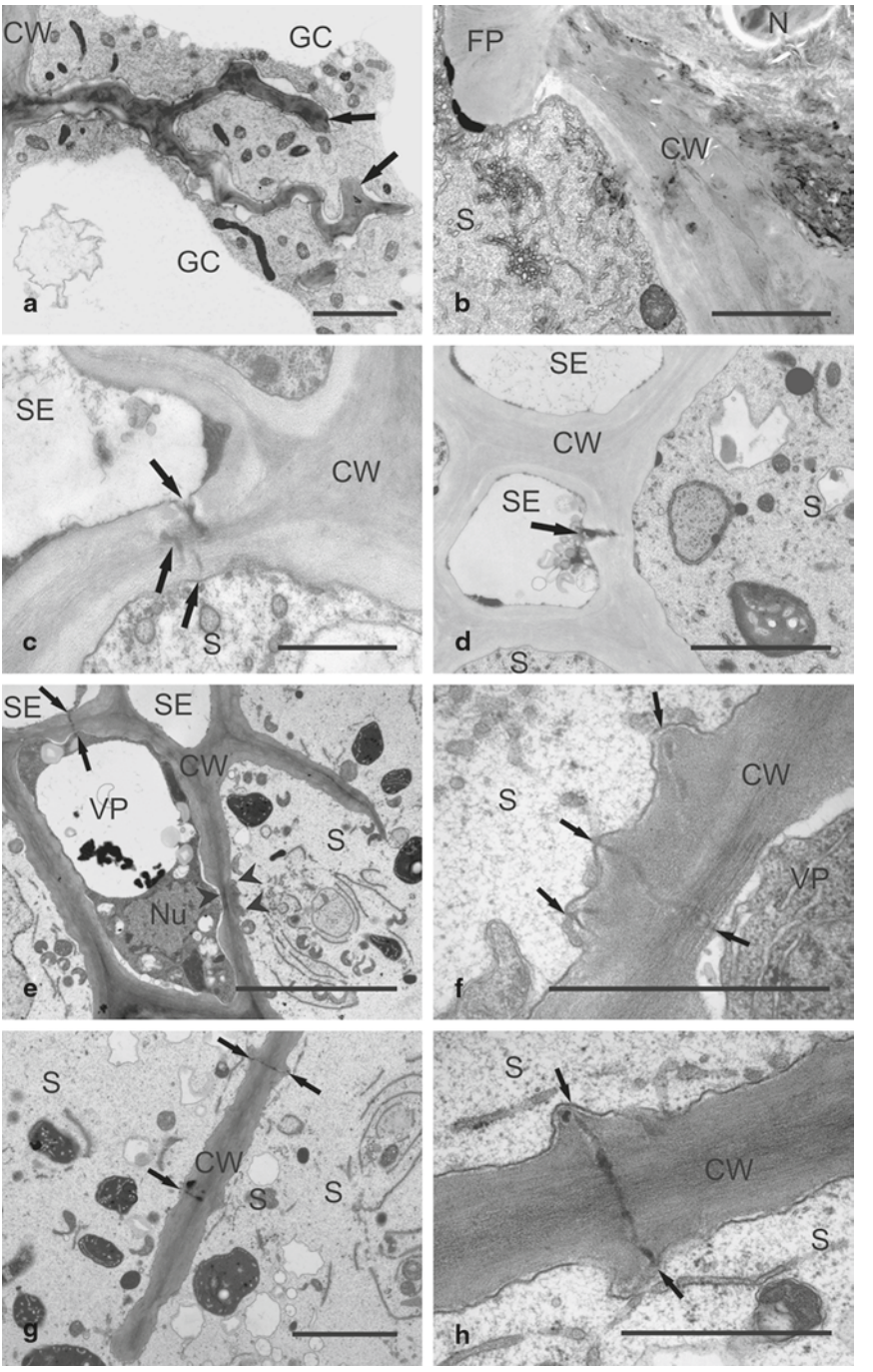
with male juveniles are induced under conditions of nutrient shortage and their cell wall ingrowths are often formed on walls between syncytial elements and on walls facing the cortex (Sobczak et al. 1997). It was calculated that an adult female of *H. schachtii* withdraws four times the volume of the syncytium each day (Müller et al. 1982). As cell wall ingrowths are typically formed at parts of wall facing xylem it seems reasonable to assume that their role is to ensure water supply. The shape of cell wall ingrowths formed in giant-cells is similar to those observed in syncytia, but in giant-cells wall ingrowths are dispersed in patches on walls facing a range of different tissues including phloem, vascular cylinder parenchyma and neighbouring giant-cells (Berg et al. 2008; Jones and Gunning 1976; Jones and Northcote 1972b; Fig. 19.1e). Cell wall ingrowths were visualised from classically aldehyde-fixed samples as slightly osmiophilic protrusions surrounded by an electron translucent layer covered by plasmalemma (Golinowski et al. 1996; Jones and Gunning 1976; Jones and Northcote 1972b). Studies implementing high-pressure freezing and freeze substitution techniques that allow avoidance of artefacts caused by chemical fixation and dehydration, show that the plasmalemma is tightly appressed to the osmiophilic part of the cell wall protrusions and an electron translucent layer does not exist. This procedure also preserved electron dense grains in wall protrusions and microtubules in cytoplasm arranged along wall ingrowths (Berg et al. 2008). Histochemical staining and immunogold labelling indicate that cell wall ingrowths are composed of hemicelluloses and cellulose distributed uniformly in wall ingrowths and of electron dense grains in wall protrusions, which stain positively for pectins (Berg et al. 2008).

The occurrence of cell wall openings is typical for syncytia only. Syncytia are formed via protoplast fusion followed by hypertrophy of cells that are pre-conditioned to be incorporated into the NFS. The first cell wall openings between the ISC and neighbouring cells are formed by widening of pre-existing plasmodesmata that

Fig. 19.1 **a** Thin portion of the cell wall (*arrow*) between sieve tubes (SE) and syncytium (S) induced by *H. schachtii* in *A. thaliana* roots (10 DAI). (CW-cell wall). Bar 2 µm. (*Courtesy of S Siddique*). **b** Thin portion of the cell wall (*arrow*) between cell (*asterisk*) being incorporated into syncytium (S) induced by *H. schachtii* in *A. thaliana* roots (10 DAI). (CW-cell wall, SE-sieve element). Bar 5 µm. (*Courtesy of S Siddique*). **c** Cell wall ingrowths (*asterisks*) formed on the cell wall facing xylem vessel (X) in syncytium (S) induced by *G. artemisiae* in *Artemisia vulgaris* root (14 DAI). Bar 2 µm. (*Courtesy of R Dobosz*). **d** Cell wall ingrowths (*asterisks*) formed on the cell wall (CW) facing xylem vessel in syncytium (S) induced by *G. artemisiae* in *A. vulgaris* root (21 DAI). Bar 2 µm. (*Courtesy of R Dobosz*). **e** Cell wall ingrowths (*asterisks*) formed on the cell wall (CW) between vascular parenchyma cell (VP) and giant-cell (GC) induced by *M. chitwoodii* in *Solanum bulbocastanum* roots (7 DAI). Bar 2 µm. (*Courtesy of J Jupowicz*). **f** Cell wall openings (*arrows*) formed by widening of plasmodesmata in young syncytium (S) induced by *H. schachtii* juvenile (N) in *A. thaliana* roots (24 h after selection of ISC). Electron translucent cell wall material (*arrowheads*) is deposited in patches on syncytial wall and over feeding plug (FP) through which nematode stylet (St) is inserted into syncytium. (SE-sieve element). Bar 2 µm. (*Courtesy of W Golinowski*). **g** Early stage of cell wall opening (*arrows*) formation by progressive dissolution of syncytial cell wall (CW) without plasmodesmata in syncytium (S) induced by *H. schachtii* in *A. thaliana* root (10 DAI). Bar 2 µm. (*Courtesy of S Siddique*). **h** Different stages of cell wall opening widening (*arrows*) by progressive dissolution of the cell wall (CW) in syncytium (S) induced by *G. rostochiensis* in *S. tuberosum* root (7 DAI). Bar 2 µm. (*Courtesy of A Karczmarek*)

have not been occluded by the thickened cell wall (Grundler et al. 1998). Young syncytia induced in the pericycle of *A. thaliana* roots are C-shaped and composed of pericyclic cells interconnected by numerous narrow cell wall openings (Fig. 19.1f), which are apparently formed by widening of the plasmodesmata that are numerous between pericyclic cells (Sobczak et al. 1997; Sobczak and Golinowski 2008). When a syncytium is established and surrounded by thickened walls, new elements are incorporated by progressive and local dissolution of the outer syncytial wall and the walls of neighbouring cells. The new cell wall openings are formed between existing syncytial elements without involvement of plasmodesmata (Fig. 19.1b, g). After the cell wall and middle lamella are digested, the plasmalemma fuses and the protoplast of the neighbouring cell is incorporated into the syncytium. The newly formed cell wall opening is usually surrounded by thin and pointed cell wall remnants (Fig. 19.1h). The number and size of cell wall openings changes during syncytium development. They are relatively few and narrow in syncytia associated with young J2, but they become gradually widened and more numerous in syncytia associated with J3, J4 and adult females. There are differences in cell wall opening sizes along the syncytium. In the region next to the juvenile head, which is the oldest part of the syncytium, the syncytial elements are most hypertrophied and interconnected by the widest cell wall openings. In contrast, distal parts of the syncytium are composed of the least hypertrophied elements with the smallest cell wall openings. Because cell wall fragments are frequently observed inside giant-cells, there was a debate as to whether giant-cells are formed via cell wall breakdown and protoplast fusion or via single cell hypertrophy. These wall fragments are rounded in giant-cells, while in syncytia they are usually sharply pointed. However, Jones and Payne (1978) showed that giant-cells arise from single cells and that cell wall stubs are the result of the abnormal cell wall formation (Fig. 19.2a). Developing giant-cells undergo repeated endomitosis without cytokinesis (de Almeida Engler

Fig. 19.2 **a** Cell wall stub (*arrows*) formed on the outer cell wall (CW) of giant-cell (GC) induced by *M. chitwoodii* in *Raphanus sativus* root (14 DAI). Bar 1 μm. (*Courtesy of G Grymaszewska*). **b** Portion of feeding plug (FP) in the outer cell wall (CW) of syncytium (S) induced by *H. schachtii* juvenile (N) in *A. thaliana* root (10 DAI). Bar 2 μm. (*Courtesy of S Siddique*). **c** Fragments of plasmodesmata and plasmodesmata-like structure (*arrows*) present in the cell wall (CW) between sieve tube (SE) and syncytium (S) induced by *H. schachtii* in *A. thaliana* root (10 DAI). Bar 2 μm. (*Courtesy of S Siddique*). **d** Fragment of widened plasmodesmata-like structure (*arrow*) present in the cell wall (CW) between sieve tube (SE) and syncytium (S) induced by *H. schachtii* in *A. thaliana* root (14 DAI). Bar 2 μm. (*Courtesy of S Siddique*). **e** Plasmodesmata (*arrowheads*) present in the cell wall (CW) between parenchymatous vascular cylinder cell (VP) and syncytium (S) induced by *G. rostochiensis* in transgenic *Solanum tuberosum* root (21 DAI). Plasmodesmata-like structures (*arrows*) are present between the parenchymatous vascular cylinder cell (VP) and sieve tube (SE). (Nu-nucleus) Bar 5 μm. (*Courtesy of K Koropacka*). **f** Magnification of Fig. 19.2e showing plasmodesmata (*arrows*) present in the cell wall (CW) between parenchymatous vascular cylinder cell (VP) and syncytium (S) induced by *G. rostochiensis* in transgenic *S. tuberosum* root (21 DAI). Bar 1 μm. (*Courtesy of K Koropacka*). **g** Plasmodesmata (*arrows*) present in the cell wall (CW) between two syncytial elements (S) induced by *G. rostochiensis* in transgenic *S. tuberosum* root (21 DAI). Bar 2 μm. (*Courtesy of K Koropacka*). **h** Enlargement of plasmodesma (*arrows*) present in the cell wall (CW) between two syncytial elements (S) induced by *G. rostochiensis* in transgenic *S. tuberosum* root (21 DAI). Bar 1 μm. (*Courtesy of K Koropacka*)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et al. 1999; Goverse et al. 2000) but in some cases the cell plate starts to develop and this can lead to the formation of thick cell wall stubs. They may undergo various modifications including branching or uneven cell wall material deposition resulting in differences in shape and thickness (Berg et al. 2008; Mordechai and Oka 2006).

The feeding plug is a structure formed locally in the host cell wall around the site of nematode stylet insertion into the syncytial wall. It has been shown surrounding the stylet of *Rotylenchulus reniformis* (Razak and Evans 1976; Rebois 1980) and cyst forming nematodes *Heterodera* sp. and *Globodera* sp. (Endo 1978; Sobczak et al. 1999; Sobczak and Golinowski 2008). There is no evidence for a feeding plug in *Meloidogyne* sp. (Hussey and Mimms 1991). The presumed function of the feeding plug is to seal the stylet as it is inserted into the syncytium in order to avoid leakage of cytoplasm (Fig. 19.1f). Ultrastructural examinations suggest that the feeding plug is structurally similar to the syncytial wall, but it is easily recognisable, especially in young syncytia where it is strongly osmiophilic (Fig. 19.1f). In syncytia associated with older juveniles it becomes larger, often multipartite and more electron translucent (Fig. 19.2b). Feeding of cyst forming nematodes occurs in cycles lasting for a few hours (Wyss 1992). After each food uptake phase, the nematode stylet is withdrawn and reinserted. When the stylet contacts the plasmalemma, it is covered with a layer of callose. Subsequently, during stylet withdrawal this material is pulled into the syncytial wall (Sobczak et al. 1999). During reinsertion materials secreted by the nematode amphids may be pushed into the feeding plug thus explaining the observed continuity between amphidial secretions and the feeding plug (Endo 1978; Sobczak et al. 1999). Histochemical analysis indicates that the feeding plug of *R. reniformis* is composed of polysaccharides but, surprisingly, it also contains lignin (Razak and Evans 1976).

The first cell wall openings between the ISC and neighbouring cells are formed by widening of plasmodesmata. Meanwhile the outer syncytial wall becomes thickened and the newly deposited cell wall material occludes all plasmodesmata. This leads to the symplastic isolation of young syncytia and nutrients are transported from phloem into syncytia apoplastically via transmembrane carriers (Böckenhoff et al. 1996; Hofmann and Grundler 2006; Juergensen et al. 2003). Recently, a set of experiments showed that the syncytium is supplied with nutrients apoplastically until about eight DAI. Later, plasmodesmata are re-opened or formed *de novo* and syncytia associated with female juveniles are supplied symplastically (Hofmann and Grundler 2006; Hofmann et al. 2007; Hoth et al. 2005). For details concerning water and nutrient transport into NFS see Chap. 20. Unfortunately, all data available at present suggesting *de novo* formation of plasmodesmata are only indirect and there is no equivocal evidence (*e.g.* TEM) to confirm this. The available images show only parts of plasmodesmata between syncytial elements and the sieve tubes (Fig. 19.2c) or greatly widened plasmodesmata-like structures or misdeveloped sieve pores (Fig. 19.2d). These are opened towards the sieve tube but this has never been shown on the side of the syncytium. However, in a single syncytium induced by *G. rostochiensis* in transgenic potato, a few clear sections of non-occluded regular plasmodesmata were found (Figs. 19.2e–h). They are present in the outer syncytial wall between the syncytium and parenchymatous vascular cylinder cells (Figs. 19.2e, f) as well as in the walls between syncytial elements next to the

cell wall opening (Figs. 19.2g, h). Plasmodesmata and plasmodesmata-like structures are also observed at early developmental stages of NFS formation when there is agreement about apoplastic syncytium uploading. While they are very rare in syncytial walls during all developmental stages of syncytia, plasmodesmata occur abundantly between parenchymatous cells surrounding the syncytium (Fig. 19.3a). Syncytia induced by *Nacobbus aberrans* are so far the only well documented example of syncytia that contain plasmodesmata (Jones and Payne 1977). Plasmodesmata are known to function as pressure sensitive valves that close when the pressure difference between neighbouring cells is higher than 2,000 hPa (Oparka and Prior 1992). Turgor pressure in syncytium was shown to reach about 10,000 hPa, a higher value that the more usual 4,000 hPa in vascular cylinder cells (Böckenhoff and Grundler 1994). There is no evidence showing different types of plasmodesmata or plasmodesmata with different size exclusion limits in syncytia associated with male and female juveniles as postulated by Hofmann and Grundler (2006). Male NFSs

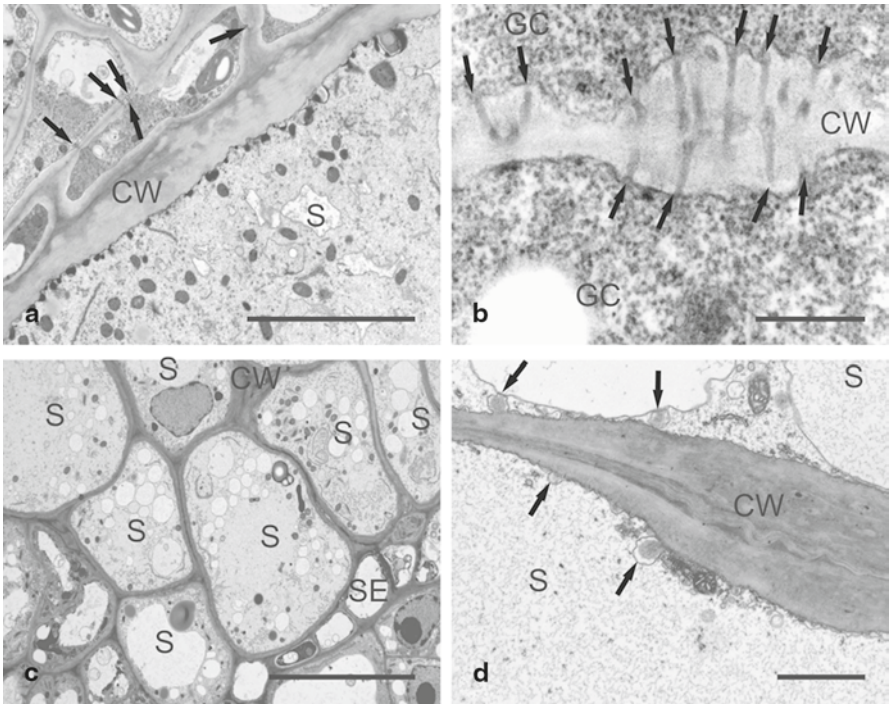

Fig. 19.3 **a** Plasmodesmata (*arrows*) present in the cell walls (CW) of parenchymatous cells abutting syncytium (S) induced by *H. schachtii* in *A. thaliana* root (10 DAI). Bar 10 µm. (*Courtesy of S Siddique*). **b** Plasmodesmata (*arrows*) present in the cell wall (CW) between two giant-cells (GC) induced by *M. chitwoodii* in *S. bulbocastanum* roots (7 DAI). Bar 1 µm. (*Courtesy of J Jupowicz*). **c** Ultrastructure of syncytium (S) induced by *G. rostochiensis* in root of transgenic *S. lycopersicum* line with silenced expression of expansin *EXPA5* (10 DAI). (CW-cell wall, SE-sieve element). Bar 5 µm. **d** Paramural bodies (*arrows*) on strongly thickened cell wall (CW) between two syncytial elements (S) induced by *G. rostochiensis* in root of transgenic *S. lycopersicum* line with silenced expression of expansin *EXPA5* (10 DAI). Bar 1 µm

rarely develop a direct interface to sieve tubes and they are usually separated from sieve tubes and xylem vessels by necrotised cells (Sobczak et al. 1997; Sobczak and Golinowski 2008; Soliman et al. 2005).

There is a general agreement that plasmodesmata are present in giant-cells at least in walls between two neighbouring giant-cells (Jones and Gunning 1976; Jones and Northcote 1972b). Recently Berg et al. (2008) showed a lack of plasmodesmata in the outer walls of giant-cells induced by *M. incognita* in *A. thaliana* roots and suggested that food is uploaded apoplastically into giant-cells via a well developed system of cell wall ingrowths. This supports observations by Hoth et al. (2005) indicating that giant-cells are symplastically isolated as there was no GFP movement from sieve tubes to giant-cells via plasmodesmata. Additionally, in different *Solanum* sp. infected with *M. chitwoodii* or *M. incognita*, plasmodesmata were not found in the outer walls of giant-cell groups, but they were present in abundance at regions of thin walls between two giant-cells (Jupowicz, Golinowski, Fudali, Sobczak-unpubl.; Fig. 19.3b).

To summarise, cell wall modifications occurring during NFS formation are very complex and include spatially and temporally synchronised processes of cell wall extension, synthesis and, in the case of syncytia, also degradation. These alterations in plant cell walls are likely to be mediated by carefully coordinated changes in host gene expression. Such changes have been investigated using a range of techniques including global analysis using microarray technology as well as detailed studies employing methods such as qRT-PCR, *in situ* hybridization and promoter:GUS fusions. These studies have revealed an extended list of plant genes encoding cell wall-modifying enzymes and proteins whose expression is modified in nematode infected roots (Ithal et al. 2007a, b; Jammes et al. 2005; Puthoff et al. 2003, 2007; Swiecicka et al. 2009; Tucker et al. 2007; Vercauteren et al. 2002; Wieczorek et al. 2006). These changes are discussed in the following section.

19.3 Expression of Genes Involved in Cell Wall Extension and Remodelling

One of the most striking hallmarks of nematode feeding sites is hypertrophy of the modified cells. In both giant-cells and syncytia the cell wall needs to be loosened to allow the turgor driven enlargement. Important players in controlling wall strength and extensibility allowing its rapid loosening are enzymes that metabolise the xyloglucan fraction of the cell wall. They catalyse the cleavage of xyloglucan backbones resulting in the addition of the half-chains to the nonreducing end of a second xyloglucan chain (Cosgrove 1999). Xyloglucan metabolising enzymes can be divided into two main groups: xyloglucan transglycosylases (XET; Smith and Fry 1991) and xyloglucan endotransglycosylases (EXGT; Nishitani and Tominaga 1992). They play an important role in a number of different processes including cell

growth, fruit softening, organ abscission, vascular differentiation and responses to pathogens (Carpita and McCann 2000).

Our knowledge about the involvement of xyloglucan transferases in the induction and maintenance of NFS is still fragmentary and mainly based on Gene Chip analyses (Barcala et al. 2009; Ithal et al. 2007a, b; Jammes et al. 2005; Puthoff et al. 2003, 2007; Tucker et al. 2007). Puthoff et al. (2003) found *XTR7* to be downregulated in *A. thaliana* 3 days after infection with *H. schachtii*. Changes in XET expression were also monitored in microaspirated syncytia induced in *A. thaliana* by *H. schachtii* (Szakasits et al. 2009). *XTR6, XTH9* and *TCH4* were strongly upregulated in 5 and 15 DAI syncytia, whereas *XTR7, XTR8, XTR9* and *At5g57530* were downregulated. Two XET genes showed modified expression in *A. thaliana* after infection with *M. incognita* (Jammes et al. 2005). *At4g30290* was downregulated in 7–21 DAI galls, whereas *At3g48580* was upregulated in 7 DAI galls but downregulated in 21 DAI galls. More recently Barcala et al. (2009) presented global Gene Chip analysis of laser microdissected 3 DAI giant-cells induced in *A. thaliana* by *M. javanica*. These studies showed the upregulation of *EXGTA1* and *XTR6* but the expression of *XTR6* was higher in galls than in giant-cells. Among 'gall distinctive' genes with the highest fold change value was *At3g48580*, which was only slightly upregulated in syncytia induced by *H. schachtii* (Szakasits et al. 2009). Interestingly, a homologue of *A. thaliana XTR6* (upregulated in syncytia and galls) was downregulated in soybean upon infection with *H. glycines* (Puthoff et al. 2007). Another Gene Chip analysis of *Glycine max* infected with *H. glycines* showed changed expression of six different XET genes (Ithal et al. 2007b). These genes were shown to be upregulated in 2 DAI syncytia. For BM568229 (*TCH4*) this result was confirmed for 5 DAI syncytia using *in situ* hybridization. Tucker et al. (2007) found *XET1* to be significantly downregulated in soybean roots infected with *H. glycines* using RT-PCR and the Affymetrix Gene Chip analysis. These numerous, but still incomplete, data demonstrate the complexity of the processes occurring in the cell wall of NFS.

Expansins are extracellular plant proteins contributing to cell wall modifications. They were discovered in studies on elongation of cucumber hypocotyls and considered one of the main factors promoting cell wall enlargement and extension (McQueen-Mason et al. 1992). Interestingly, they do not possess any enzymatic activity and it is suggested that they act by disrupting hydrogen bonds between cell wall polymers (Sampedro and Cosgrove 2005; Yennawar et al. 2006). The expansin superfamily consists of four families: α-expansins (EXPA), β-expansins (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB) (Kende et al. 2004). Based on their unique properties and their expression patterns, expansins are believed to mediate not only cell wall expansion during cell growth but also many other processes where cell wall remodelling is required including differentiation of tracheary elements, pollen tube growth and fruit ripening (Im et al. 2000; Rose et al. 1997; Sampedro and Cosgrove 2005). Thus, it is not surprising that during development of the NFS elaborate changes in the plant cell wall architecture are accompanied by upregulation of expansin genes. The elevated levels of expansin expression in roots containing syncytia or giant-cells are well documented by robust gene profil-

ing analysis and detailed studies that focus on specific members of this gene family (Fudali et al. 2008; Gal et al. 2006; Jammes et al. 2005; Tucker et al. 2007; Wieczorek et al. 2006).

Expression patterns of expansins have been best characterised in *A. thaliana* infected with sedentary endoparasites (Jammes et al. 2005; Wieczorek et al. 2006). Seven *EXPA* and two *EXPB* genes are upregulated in galls induced by *M. incognita* in *A. thaliana* roots and their expression is temporally regulated: expression of six of them was higher at 14 DAI than at 7 DAI, while one of them showed the opposite expression pattern (Jammes et al. 2005).

Very detailed and comprehensive analysis of expression of all *A. thaliana* members of the *EXPA* and *EXPB* families revealed that at least ten expansin isoforms (*AtEXPA1, -3, -4, -6, -8, -10, -15, -16, -20* and *AtEXPB3*) are represented in the transcriptome of 5–7 DAI syncytia induced by *H. schachtii* (Wieczorek et al. 2006). *AtEXPA3, -6, -8, -10* and *-16* are specifically activated in roots during syncytium induction, while *AtEXPA1, -4, -15, -20* and *AtEXPB3* are also expressed in corresponding uninfected root segments. In addition, expansins activated in syncytia were also expressed in other parts of the same root. For example, activity of *AtEXPA1, -4* and *-15* promoters was detected in the vascular cylinder of primary and lateral roots and in the root tips. In the case of *AtEXPA6*, GUS staining was observed in the root tips. Only two of the studied expansins, *AtEXPA3* and *AtEXPA16*, turned out to be syncytium specific and were not expressed in the uninfected root.

Comparing the expression of expansin genes in *A. thaliana* it becomes evident that different sets of genes are expressed in syncytia and giant-cells and that isoforms expressed in both feeding sites are expressed at different time points during development. For example, *AtEXPA7* is downregulated in syncytia but it is slightly upregulated in giant-cells and while expression of *AtEXPB1* remains unchanged in syncytia, it is increased in galls. *AtEXPA15* is expressed at the same level in 5–7 and 15 DAI syncytia but in galls it is expressed at higher level at later time points (14 DAI when compared to 7 DAI). *AtEXPA16* is expressed in syncytia at both 5 and 15 DAI while in giant-cells it is only slightly upregulated at 14 DAI (Jammes et al. 2005; Wieczorek et al. 2006).

Microarray analysis of tomato roots infected with *M. javanica* revealed only one expansin isoform, *LeEXPA5*, to be upregulated (Bar-Or et al. 2005; Gal et al. 2006). Its transcripts were localised in tissue surrounding developing giant-cells, but not in the giant-cells at 4 and 10 DAI. In contrast, in syncytia induced in tomato roots by *G. rostochiensis* five additional expansin isoforms were found to be upregulated: *LeEXPA1, -2, -4, -11* and *-18* (Fudali et al. 2008). In roots containing 3 and 5 DAI syncytia *LeEXPA4* mRNA was localised to peripheral parts of syncytia and in adjacent parenchymatous vascular cylinder cells. In roots with older syncytia *LeEXPA4* mRNA was detected only in cells abutting syncytia, but not in the syncytia. In contrast, the expression pattern of *LeEXPA5* followed the processes of syncytium expansion: *LeEXPA5* transcripts were present in syncytia upon syncytium induction (1–5 DAI). Later, the expression was generally limited to distal parts of syncytia and to cells located between syncytia and vascular elements. Immunogold localisation studies showed the lack of expansin 5 in the

vicinity of formed cell wall openings thus excluding its involvement in cell wall opening formation. The observed expression pattern suggests that *LeEXPA5* might be involved in the cell wall relaxation accompanying hypertrophy of syncytial elements.

Recently, gene expression was investigated using Affymetrix Gene Chip in soybean roots infected with *H. glycines* (Ithal et al. 2007b). Among genes upregulated during feeding site induction and development (2, 5 and 10 DAI) eight putative α-expansin sequences were present (*AI759701, CD394837, CD417217, AF516880, CA785167, AW509184, CF805734* and *CF805822*). In addition, upregulation of another expansin, *EXPR3* (*BM091956*), was detected by qRT-PCR in 2, 5 and 10 DAI syncytia (Ithal et al. 2007a). At least three expansin genes *GmEXPA1, -3* and *-8* are upregulated in roots containing 8, 12 and 16 DAI syncytia (Puthoff et al. 2007; Tucker et al. 2007). Interestingly, these studies did not reveal specific activation of genes in infected roots, as all of the identified expansin isoforms were also expressed in uninfected roots.

Although there are many studies showing upregulation of expansin genes in nematode feeding sites, still little is known about mechanisms that trigger their expression. In addition, there are not enough data providing direct evidence linking a given expansin gene to particular cell wall modification processes occurring during development of giant-cells or syncytia (described in Sect. 19.7 in this Chapter).

Extensins are structural components of the cell wall and they are responsible for a variety of cell wall properties including strength and flexibility. They create a cross-linked scaffold interconnecting cellulose microfibrils. In contrast to expansins, they are not active inducers of cell wall relaxation. Extensins are characterised by a high content of glycosylated hydroxyproline and repetitive amino acid sequences that contain serine and hydroxyproline (Kaliszewski and Shpak 2001). They are encoded by multigene families and are expressed during growth, development and responses to abiotic and biotic stresses, for example during mechanical stress, lateral root development, root-hair growth and phyllotaxy (Kaliszewski and Lamport 1994).

Extensin mRNAs were found to be strongly induced in tobacco roots infected with *M. javanica* at 7 and 14 DAI (Niebel et al. 1993). Their expression was detected using *in situ* hybridisation and promoter:GUS lines in cortical cells of galls and in the dividing pericycle, but not in giant-cells or the vascular parenchyma. Immunogold labelling revealed that extensin is present in the cell wall of cortical and pericyclic cells of galls and in the intercellular spaces between pericyclic and vascular parenchyma cells that are in direct contact with nematodes or giant-cells (Niebel et al. 1993). In addition, in tomato roots parasitized with *M. incognita* two other extensin genes, *Lemmi8* and *Lemmi11*, were upregulated and their mRNA levels increased in 14 DAI galls (Van der Eycken et al. 1996). The role of extensins during development of NFS induced by root knot nematodes has not yet been determined. Based on the localisation of extensins it was concluded that they might play a role in the biosynthesis of the cell wall of newly derived cells in the gall tissues or their upregulation might be the response to the mechanical stress of the pressure created by enlarging galls (Niebel et al. 1993; Van der Eycken et al. 1996).

An early study showed that upregulation of extensins was not observed during development of syncytia in tobacco roots infected with *G. tabacum* spp. *solanacearum*, other than at early time points of infection representing the migration phase of the nematode and necrosis of root cells (Niebel et al. 1993). However, microarray analyses suggest that syncytium development is accompanied by differential expression of a set of extensin genes. In soybean infected with *H. glycines* three genes encoding putative proline-rich EXT-like proteins (*AW185750, BQ453262* and *BI497973*) and one proline-rich extensin (*CF807748*) were downregulated, while four other putative members of proline-rich EXT-like family (*BU577532, AW307368, BI971744* and *CF807746*) were upregulated in 2 DAI syncytia (Ithal et al. 2007b). In *A. thaliana* extensin genes also seem to be differentially expressed in syncytia after *H. schachtii* infection. Genes encoding three hydroxyproline-rich glycoproteins containing proline-rich extensin domains (*At5g49280, At1g14710* and *At3g45230*), four proline-rich proteins containing proline-rich extensin domains (*At5g45350, At2g40820, At1g63830* and *At4g16140*), two pollen Ole e I allergens, extensin proteins (*At1g78040* and *At4g08685*) and one leucine-rich repeat protein/extensin (*At2g19780*) were all upregulated. In addition, downregulation was observed for sequences coding for eight hydroxyproline-rich glycoproteins containing proline-rich extensin domains (*At5g09520, At4g38080, At5g09520, At5g09480, At2g47930, At3g52460, At5g51680* and *At5g19800*), four pollen Ole e I allergens and extensin proteins (*At4g02270, At5g05500, At1g28290* and *At2g47540*), 13 proline-rich extensin-like proteins containing proline-rich extensin domains (*At5g35190, At3g54590, At3g28550, At1g23720, At5g49080, At5g06640, At4g13390, At4g08410, At5g06630, At3g49840, At3g54580, At3g01560* and *At4g08380*), two proline-rich proteins, containing proline-rich extensin domains (*At5g14540* and *At2g27390*), one extensin-like protein (*At1912090*) and one leucine-rich repeat protein/extensin (LRX1) (*At1g12040*) (Szakasits et al. 2009).

19.4 Expression of Genes Involved in Cell Wall Degradation in NFS

Endo-β-1,4-glucanases (EGase, EC 3.2.1.4) are enzymes that modify the cellulose-hemicellulose network via hydrolysis of β-1,4 linkages between glucose residues (Brummell et al. 1994). Plant EGases belong to glycosyl hydrolase family 9 (GH9) and are grouped into three different subfamilies: members of the α- and β-subfamily are secretory proteins with a signal peptide at the N-terminus and a GH9 catalytic core while γ-subfamily members have an additional membrane spanning domain (Del Campillo 1999). When secreted to the wall, α- and β-subfamily EGases act on the outermost layers hydrolysing non-crystalline cellulose while γ-subfamily EGases are thought to act on the innermost amorphous cellulose but do not show any activity on crystalline cellulose, xyloglucan or xylans (Master et al. 2004; Mølhøj et al. 2001). Little is known about the physiological function of many EGases. They seem to be involved, similarly to expansins, in many processes requiring cell

wall modifications such as cell growth (Catala et al. 1997), fruit ripening (Rose and Bennett 1999) and organ abscission (Del Campillo and Bennett 1996).

The first indirect evidence for involvement of cell wall degrading enzymes within developing syncytia was provided by Grundler et al. (1998), who detected precipitation of liberated reducing sugars close to cell wall openings. Three years later, expression of plant EGases in tobacco roots infected with *G. tabacum* ssp. *solanacearum* or *M. incognita* was reported (Goellner et al. 2001). RT-PCR revealed that the same EGases (*NtCel2, -4, -5, -7* and *-8*) are expressed in roots containing syncytia or giant-cells, with *NtCel4* and *NtCel5* expressed at low level. Three of the EGases genes exhibiting the highest expression levels (*NtCel2, -7* and *-8*) were detected in giant-cells by *in situ* hybridization. In roots containing syncytia only *NtCel7* and *NtCel8* mRNAs, but not *NtCel2* mRNA were detected. In addition to giant-cells and syncytia, expression of *NtCel7* and *NtCel8* was also detected in meristematic tissues of the root tips and lateral root primordia. Interestingly, the closest homologues of *NtCel7* and *NtCel8* in tomato, named *SlCel7* and *SlCel9C1* (previously *TomCel8*, Catala and Bennett 1998) were upregulated in tomato roots infected with *G. rostochiensis* (Karczmarek et al. 2008). It seems that cyst nematodes induce homologous genes in different plants of the Solanaceae family. Recent studies using an approach combining cDNA-AFLP with the computer software GenEST confirmed that *SlCel7* is expressed in tomato roots infected with *G. rostochiensis*. In addition, *SlCel4* also appeared to be upregulated in parasitized roots (Swiecicka et al. 2009).

The *A. thaliana* gene *AtCel1* is an example of a gene differentially expressed in giant-cells and syncytia. The promoter of *AtCel1* fused to GUS was upregulated in giant-cells induced by *M. incognita* (Shani et al. 1997), but not within syncytia in either *A. thaliana* or tobacco (Mitchum et al. 2004; Sukno et al. 2006). Interestingly, the tobacco *NtCel2* gene, which is expressed in tobacco roots infected with root-knot nematodes and cyst nematodes, has 73% nucleotide identity to *AtCel1*. However, the expression level of *NtCel2* in roots containing syncytia is much lower than in roots containing giant-cells, as the mRNA of this gene can only be detected by *in situ* hybridisation on root sections containing giant cells (Goellner et al. 2001). Detailed studies were performed on the expression of all 25 members of the endoglucanase family in *A. thaliana* roots parasitized by *H. schachtii* (Wieczorek at al. 2008). It was demonstrated that seven members of the family *AtCel2, AtCel3, KOR, KOR3, At1g64390, At2g32990* and *At4g02290* are upregulated in 5–7 DAI syncytia. Two, *KOR3* and *AtCel2*, were expressed exclusively in syncytia and not in other parts of the roots. In addition, some EGases were differentially regulated during syncytium development: *KOR* was equally expressed during all developmental stages, while the highest expression level for *AtCel3* and *At2g32990* was observed at 5 DAI, for *AtCel2* and *At4g02290* at 10 DAI and for *KOR3* and *At1g64390* at 15 DAI.

In soybean expression of 11 EGases (*GmCel1, -2, -4, -5, -6, -7, -8, -9, -10, -11* and *-12*) in root fragments parasitized by *H. glycines* was investigated by real time RT-PCR (Tucker et al. 2007). *GmCel4, -6, -7, -8* and *-9* were strongly upregulated in root fragments containing syncytia and these changes in expression for *GmCel4* and *GmCel6* were also confirmed by Gene Chip analysis (Puthoff et al. 2007).

In the case of EGases, there is some information about possible factors triggering their expression in NFS and about regulatory elements responsible for their specific activation. The giant-cell specific *AtCel1* promoter is active in elongation zones of the root and shoot and in lateral root primordia. Wounding does not activate *AtCel1* (Sukno et al. 2006). Deletion analysis of the *AtCel1* promoter allowed identification of the region from −1,673 to −1,171 responsible for localised expression in giant-cells and roots. However, this fragment alone was not sufficient to elicit expression in giant-cells.

Due to the specific upregulation in syncytia, as compared to uninfected roots, the possible mechanism involved in regulation of *AtCel2* and *KOR3* expression was studied. The EGase promoters were analysed *in silico* but no specific patterns or motifs that might be responsible for triggering expression specifically in the syncytia could be identified. For example, the promoter of *KOR3*, a syncytium-specific gene, shares the same motifs with genes downregulated in syncytia such as *KOR2* and *At1g48930* (Wieczorek et al. 2008). In addition, potential factors inducing expression of *AtCel2* and *KOR3* were studied including sucrose and plant hormones. *AtCel2* expression was activated by sucrose, gibberellin and the synthetic auxin NAA, while ABA reduced expression of both *AtCel2* and *KOR3*. Neither NPA (inhibitor of auxin transport) nor ACC (precursor of ethylene biosynthesis) affected the expression of these two genes. Recently, the promoter of *NtCel7*, an EGase specifically expressed in syncytia and giant-cells, was isolated and its activity was analysed in soybean (*Glycine max*), tomato and *A. thaliana* using promoter:GUS fusions (Wang et al. 2007). The *NtCel7* promoter is auxin responsive, but ethylene, gibberellin or sucrose do not affect its activity.

The microfibril network of the cell wall is embedded in a pectin matrix that is responsible for wall porosity, charge density and microfibril spacing. Pectin is the only cell wall polysaccharide that is largely restricted to the primary wall and is reduced or absent in the secondary non-expandable cell wall (Willats et al. 2001). There are three major groups of pectins: homogalacturonans (HGAs), substituted galacturonans and rhamnogalacturonan (RGAs). These are synthesized in the Golgi apparatus together with other complex pectic polysaccharides that are substituted with distinct oligosaccharides. Some are bound by ester linkages to cell wall polymers and can only be released by enzymatic de-esterification. Pectins form a hydrated gel, thus they make polymer slippage during cell wall growth and elongation easier. Pectins can be degraded in two different ways. They can be demethoxylated by pectin esterases or depolymerised by polygalacturonases or lyases. Taking into account the massive changes of the cell wall architecture in that occue in the NFS it has seemed likely for some time that expression of these enzymes is changed upon nematode infection. Vercauteren et al. (2002) found a putative *A. thaliana* pectin acetylesterase homologue to be upregulated in the early stage of infection with both *M. incognita* and *H. schachtii*. A pectin methylesterase like-enzyme, *AtPME2*, and a pectin esterase, *At2g45220*, were shown to be downregulated in syncytia induced in *A. thaliana* by *H. schachtii* (Puthoff et al. 2003). Global analysis of gene expression during gall formation in *A. thaliana* showed

changes in abundance of five pectin lyase genes (Jammes et al. 2005). *At4g24780* was upregulated at 7–14 DAI, *At1g04680, At3g27400* and *At3g09540* were upregulated at later stages (14–21 DAI), whereas increased expression of *At1g67750* was detected only in galls at 21 DAI. In syncytia induced by *H. schachtii* seven pectate lyases were upregulated (*At3g27400, At4g24780, At3g54920, At5g04310, At3g55250, At1g67750* and *At3g55140*) and four downregulated (*At2g09540, At1g11920, At3g24670* and *At5g15110*) (Szakasits et al. 2009). Comparing these Gene Chip data with data obtained from galls (Jammes et al. 2005) some similarities can be observed as *At3g27400, At4g24780* and *At1g67750* are upregulated in both types of NFS. Recently, Barcala et al. (2009) presented new data from laser microdissected *A. thaliana* giant-cells at 3 DAI induced by *M. incognita*. This analysis showed that upregulation of the pectate lyase *At3g53190* was higher in giant-cells than in gall tissue. This gene, however, did not show any changes in expression in the previous reports (Jammes et al. 2005). Among 'gall distinctive' genes with the lowest fold change were two genes responsible for pectin degradation, pectin esterase *At2g45220* and pectate lyase *At3g07010*. In syncytia, pectin esterase *At2g45220* was similarly downregulated, whereas pectate lyase *At3g07010* did not show any changes in expression (Szakasits et al. 2009). Two other genes, pectin methylesterase (*At1g11580*) and pectin esterase (*At5g20860*), can be found among 'gall distinctive' genes with the highest fold change value. Expression of both genes in syncytia remains unchanged.

By use of differential display, two strongly enriched fragments with high homology to polygalacturonases, *GmPG1* and *GmPG2*, were found in soybean after infection with *H. glycines* (Mahalingam et al. 1999). During the last years extensive Gene Chip data from soybean infected with *H. glycines* have been published (Ithal et al. 2007a, b; Puthoff et al. 2007; Tucker et al. 2007). Four pectate lyases (*AW309146, AI442263, BI316021* and *CD397515*) were shown to be strongly upregulated in 8, 12 and 16 DAI syncytia (Puthoff et al. 2007). Two pectin esterases, *BE821923* and *AW30932*, were moderately upregulated at all examined developmental stages, whereas *BE475550* was downregulated in syncytia induced by *H. glycines* (Ithal et al. 2007b). Another pectin esterase gene, *BI970277*, showed reduced expression in 2, 5 and 10 DAI syncytia (Ithal et al. 2007a). Tucker et al. (2007) demonstrated by use of Gene Chip and RT-PCR that polygalacturonase (*PG1*) and two pectate lyases (*PL1* and *PL2*) were significantly upregulated in roots of *Glycine max* infected with *H. glycines*. In order to determine the function of the cellulose binding protein from *H. glycines* (Hg CBP) Hewezi et al. (2009) isolated its orthologous clone from *H. schachtii* (Hs CBP). It is expressed only at the early stages of syncytium formation and not during the migratory phase. Using a yeast two-hybrid approach they identified *A. thaliana* pectin methylesterase 3 (*AtPME3*) specifically interacting with the Hs CBP. They investigated lines overexpressing *AtPME3* and showed that these plants have longer roots and increased susceptibility to *H. schachtii*, while the mutant *pme3* exhibits opposite phenotypes. They also showed increased activity of *AtPME3* in the CBP overexpressing line. The authors conclude that CBP interacts and potentially targets the pectin methylesterase in order to aid the formation of the NFS and successful parasitism.

19.5 Expression of Genes Involved in Cell Wall Biosynthesis in NFS

The elaborate modifications of the cell wall structure in the NFSs are thought to be based on the activity of both cell wall loosening and cell wall biosynthetic enzymes. One of the first events occurring in the ISC is the deposition of callose around the inserted stylet tip and on adjacent parts of the cell wall (Hussey et al. 1992; Fig. 19.1f). This polysaccharide is an amorphous 1,3-β-glucan polymer. It is not abundant in the plant cell wall under normal conditions, but is present in the cell plate between dividing cells, in pollen tubes and in phloem sieve tubes. Additionally, callose can be deposited when plants are exposed to the abiotic or biotic stresses, including wounding or pathogen attack (Scheible and Pauly 2004). Twelve callose synthase genes (GLSs) have been found in the *A. thaliana* genome. Interestingly, Gene Chip analysis of microaspirated 5/15 DAI syncytia induced in *A. thaliana* by *H. schachtii* did not detect any of these genes to be upregulated. It showed however, the downregulation of three callose synthase genes, *AtGSL3*, *-6* and *-9* (Szakasits et al. 2009). It is likely that the upregulation of the callose synthase genes takes place only at the onset of the syncytium formation and this is the reason why no activation of GLS genes could be detected later.

The main component of the plant cell wall is cellulose, a linear polymer of 1,4-β-glucans that forms mechanically strong paracrystalline microfibrils. Cellulose biosynthesis takes place at rosette-like cellulose synthase complexes, which consist of at least three different monomers of the catalytic subunit (CesA). Genetic analyses revealed the presence of three subunits that are essential for the formation of the primary wall, CesA1, -3 and -6, and three subunits responsible for the secondary cell wall formation, CesA4, -7 and -8. Each of the CesA proteins possesses eight transmembrane domains that form a pore in the plasma membrane (Delmer 1999). Besides the *CesA* genes a group of cellulose-synthase-like genes (*CSL*s) has been identified. It includes six sub-groups: *CSLA, CSLB, CSLC, CSLD, CSLE* and *CSLG*. In *A. thaliana* ten cellulose synthase genes have been identified, in addition to 29 cellulose-synthase-like genes (Richmond and Somerville 2000).

The biosynthesis of the cell wall in NFS is still poorly understood. The wall morphology greatly resembles the structure of transfer cell walls, which is characterised by rapid expansion, extensive thickening (Fig. 19.1a) and formation of ingrowths (Jones and Northcote 1972a, b; Figs. 19.1c–e). There are very few studies on the expression of genes involved in cell wall biosynthesis in NFS. By use of laser microdissection and microarray analysis Ithal et al. (2007b) conducted transcriptome profiling of syncytia induced by *H. glycines* in soybean. They found two cellulose synthases, *CD409747* and *BI469627*, homologues of *A. thaliana AtCSLE1* and *CesA2*, respectively, to be downregulated at all examined developmental stages. A similar result for these genes was obtained in syncytia induced by *H. schachtii* in *A. thaliana* roots (Szakasits et al. 2009). Other members of the cellulose synthase family, responsible for the primary and secondary wall formation, as well as cellulose-synthase-like genes were also downregulated. Barcala et al. (2009) used

laser microdissection to capture giant-cells at early stages of development (3 DAI) and precursors of root vascular cells for Gene Chip analysis. They showed *CSLD5*, a cellulose synthase-like gene, to be upregulated with a higher fold-change value in giant-cells than in gall tissue. This analysis also revealed that two other cellulose synthases, *CesA2* and *At4g15290* (*AtCSLB5*), are downregulated in giant-cells. A more comprehensive analysis was performed for ten cellulose synthase genes in NFSs formed by *M. incognita* in *A. thaliana* roots (Hudson 2008). Based on promoter:GUS studies and developmental quantitative RT-PCR it was shown that *CesA* genes responsible for primary and secondary wall formation have a similar expression pattern in infection sites. The highest level of expression occurred at 5 DAI. Sections of the infection sites from plants carrying promoter:GUS constructs revealed that expression of secondary wall-related *CesA* genes was mainly localised within the giant-cells, whereas expression of primary wall-related *CesA* genes was found in surrounding hyperplastic gall tissue.

Our understanding of cell wall biosynthesis in nematode feeding sites and the genes involved in this process is still limited. Although the deposition of callose in the nematode feeding sites is well documented microscopically (Fig. 19.1f) and has been analysed with the aid of immunohistochemistry (Hussey et al. 1992), the genetic background of this process still remains to be elucidated. In contrast, the comprehensive genetic analysis performed by Hudson (2008) provides relatively detailed evidence regarding the expression of the *CesA* genes and their involvement in the formation of the feeding sites induced by *M. incognita* in *A. thaliana*. Interestingly, it seems that the composition of the cell wall greatly differs between giant-cells and syncytia. While most members of the cellulose synthase gene family were upregulated in either giant-cells or surrounding gall tissue, they were downregulated in syncytia. To elucidate the biological reason of this phenomenon and to identify factors responsible for syncytial cell wall thickening more focused studies are needed.

19.6 Ultrastructure of Feeding Site Wall in Resistant Interactions

Resistance against root-knot nematodes usually appears as an early hypersensitive response. Juveniles are able to enter plant roots, but they become surrounded by necrotised cells and no further development of the NFS or the juvenile takes place (Bleve-Zacheo et al. 1982; Kouassi et al. 2005; Paulson and Webster 1972). One of the few exceptions is the response of *S. sparsipilum* resistant to *M. fallax* (Kouassi et al. 2004). In this combination an NFS composed of several giant-cells is induced in the vascular cylinder. The giant-cell walls are thickened, plasmodesmata are absent and, at walls facing vessels, finger-like cell wall ingrowths are formed. Necrosis of parenchymatous cells next to this NFS leads to giant-cell degeneration. In contrast, cyst forming nematodes are usually able to induce

syncytia in resistant plants. These syncytia usually remain functional long enough to support the development of males. The ultrastructure of cell wall, cell wall ingrowths and cell wall openings differs strongly according to plant and nematode species (Sobczak and Golinowski 2008). In the case of *H. schachtii* infecting beet or radish, syncytial walls are only slightly thickened and cell wall openings are few and narrow. In addition, cell wall ingrowths are rare or completely absent (Grymaszewska and Golinowski 1998; Holtmann et al. 2000; Wyss et al. 1984). On the contrary, in a resistant cultivar of *Sinapis alba* infected with *H. schachtii* the syncytial wall is strongly thickened, cell wall openings are narrow but numerous, and cell wall ingrowths are formed at syncytial walls facing the xylem parenchyma (Golinowski and Magnusson 1991; Soliman et al. 2005). A thickened syncytial wall is also typical for syncytia induced by *H. glycines* in resistant soybean (Endo 1991; Kim et al. 1987; Riggs et al. 1973), and *G. rostochiensis* or *G. pallida* infecting resistant lines of different *Solanum* sp. (Bleve-Zacheo et al. 1990; Rice et al. 1985, 1987; Sobczak et al. 2005). In all these interactions narrow and few cell wall openings are formed and no development of cell wall ingrowths and plasmodesmata was reported.

19.7 Nematode Development and Cell Wall Modifications in Plants with Silenced Expression of Cell Wall-Related Genes

Several attempts have been made to unravel the functions of plant cell wall modifying proteins during plant-nematode interactions. Both knock-out mutants and transgenic lines with silenced genes have been used to investigate the function of various genes in feeding site development and their potential importance for successful parasitism. However, both approaches appear to have limitations, as in some cases no recognisable phenotype and no effects on nematode development could be observed. Analysis using knock-out T-DNA insertion mutants of *A. thaliana* showed that silencing of a single expansin gene has no effect on nematode development (Wieczorek unpubl.; Wieczorek and Grundler 2006). These results, however, are not surprising when single members of large gene families are studied and can be explained by functional redundancy. Similarly, *A. thaliana* plants transformed with antisense *AtCel1* mRNA did not differ from control plants in the number of induced galls or in giant-cell ultrastructure (Sukno et al. 2006). This may indicate that *AtCel1* is not essential for proper giant-cell development or that the effect of silencing is masked by functional redundancy.

By contrast, the importance of the induction of *AtCel2* and *KOR3* genes for successful feeding site induction by *H. schachtii* in *A. thaliana* was confirmed using T-DNA mutants. Although *cel2* and *kor3* mutants did not exhibit any visible phenotype, the number of developed females was reduced by 48% in *cel2* and 45% in *kor3* at 3 weeks post inoculation (Wieczorek et al. 2008). However, the effect on the anatomy and morphology of NFS was not presented in these studies.

Tomato hairy roots with silenced expression of *LeEXPA5* are characterised by a smaller diameter of galls when compared to control roots transformed with GFP. In addition, nematodes produce fewer egg masses with fewer eggs when reproducing on *LeEXPA5*-silenced roots. This confirms the importance of the *LeEXPA5* activation for plant-nematode interaction (Gal et al. 2006). Syncytia induced in *EXPA5*-silenced tomato plants follow a developmental pattern typical for syncytia associated with *Globodera* sp. (Fudali, Griesser, Janakowski, Sobczak, Golinowski-unpubl.). They are usually induced in the cortical parenchyma and spread towards and then along the vascular cylinder. However, they are composed of fewer and less hypertrophied elements, especially in the parts derived from the vascular cylinder (Fig. 19.3c). Syncytial elements are interconnected by fewer and narrower cell wall openings than are present in syncytia induced in control plants. The syncytial wall was uniformly thickened, but numerous vesicles, paramural bodies and cell wall appositions were present (Fig. 19.3d).

The silencing of genes homologous to *SlCel7* and *SlCel9C1* via RNAi in potato results in a reduction in the number of fully developed PCN females to 60% of controls in *cel7*-silenced plants and 30% of controls in *cel9C1*-silenced plants. Moreover, females developing on transgenic plants show morphological abnormalities. They are smaller, saccate and appear transparent because their bodies do not contain eggs (Karczmarek et al. 2008). Syncytia induced in transgenic potato lines with silenced expression of *Cel7* or *Cel9C1* endoglucanases develop normally but the syncytial hypertrophy is low and cell wall openings are few and narrow indicating that the syncytial wall is not able to expand properly when one of the genes crucial for syncytial wall rearrangement is silenced (Kurek, Janakowski, Fudali, Sobczak, Karczmarek, Helder, Goverse, Golinowski-unpubl.). Additionally, in transgenic plants with silenced expression of *Cel9C1* all parenchymatous vascular cylinder cells are incorporated into syncytia and no cells are left to divide and form the peridermis-like cover tissue surrounding syncytia (Sobczak and Golinowski 2008). Cell wall ingrowths were not observed in any of the transgenic lines, although syncytia were in direct contact with conductive xylem and phloem elements. These experiments indicate that the silenced isoforms cannot be functionally replaced by other EGases. A similar phenomenon was also observed in *A. thaliana kor3* and *cel2* mutants (Wieczorek et al. 2008). Nevertheless, the silencing of *AtCel1* did not affect development of root-knot nematodes, probably due to functional redundancy (Sukno et al. 2006).

Development of *M. incognita* is impaired on *A. thaliana* mutants in each of the eight *CesA* (*CesA1–8*) genes (Hudson 2008). A significant reduction in the number of root galls and a decreased size of the galls and females was observed on mutant plants. In addition, females did not develop normally or produce any eggs. Complementation with the corresponding gene expressed from the constitutive 35S promoter restored the wild type phenotype in the case of *CesA4, -5* and *-7* genes, which are involved in secondary cell wall synthesis. In addition, these mutants were complemented with the gene expressed from the giant-cell inducible *NtCel7* promoter. Limited effects on the mutant phenotype and nematode infection rate were observed but an increase in the sizes of females was seen when compared to

the non-complemented mutant lines. It was suggested that *CesA* genes involved in secondary cell wall formation are critical for the normal function of giant-cells as well as for their ability to supply the developing female with a sufficient amount of nutrients. This might be due to the increase in the number of cell wall ingrowths resulting in an increased flow of solutes that can be taken up by the feeding nematode.

19.8 Summary

The list of cell wall modifying enzymes and proteins of plant origin which exhibit changed expression patterns after nematode infection is long and constantly growing. Nematodes can modify existing developmental programmes of the roots, for example by adjusting expression patterns of root tip specific genes such as *NtCel7* (Goellner et al. 2001), or they can induce expression of genes that are not usually expressed in roots, such as *AtCel2* (Wieczorek et al. 2008). The upregulation of expansins, EGases, extensins and cell wall component synthesising proteins has been well documented by numerous studies. How the parasitic nematode is able to induce synchronised changes in the expression of plant genes still remains to be answered. Many authors speculate that this might be achieved by indirect means, via interfering with plant signalling pathways by locally changing the concentration of sugars such as sucrose (Hofmann et al. 2007) or plant hormones (Karczmarek et al. 2004; see Chap. 16). These two possible activation mechanisms were supported by recent studies demonstrating that sucrose might be the factor triggering expression of *AtCel2* (Wieczorek et al. 2008), while auxin may activate *NtCel7* (Wang et al. 2007). Nematode secretions may also contain peptides or proteins that directly activate plant genes (Davis et al. 2008). However, to our best knowledge such nematode effectors or corresponding plant genes involved in cell wall remodelling have not been identified. So far there are numerous plausible roles for cell wall modifying proteins during NFS development starting from the cell wall relaxation and degradation and ending with cell wall synthesis. Given the evidence that genes belonging to the same family might be involved in opposite processes it might be very difficult to predict their roles. However, studies using immunolocalisation could be helpful in elucidating the roles of genes of interest. Detailed immunogold localisation studies did not confirm specific involvement of Cel7 and Cel9C1 EGases or tomato expansin 5 in cell wall opening formation in syncytia induced by *G. rostochiensis* (Fudali et al. 2008; Karczmarek et al. 2008). The main limitation of gene silencing or knock-out plants is functional redundancy as observed in T-DNA *A. thaliana* mutants with knocked-out expansin genes (Wieczorek unpubl.). However, there are also examples of successful application of these functional genetic approaches, like the *A. thaliana cel2* mutant or potato with silenced expression of EGase family members (Wieczorek et al. 2008; Karczmarek et al. 2008). The findings of these studies demonstrate how important cell wall modifications are for feeding site induction and expansion as well as nematode development. Interestingly, they show also that the nematode's ability to manipulate plant gene expression is restricted, as

in some mutants or knock-out plants there is no functional redundancy leading to reduced susceptibility. This may have potential applications in breeding for nematode resistance.

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Chapter 20
Water and Nutrient Transport in Nematode Feeding Sites

Florian M. W. Grundler and Julia Hofmann

20.1 Nematodes as Obligate Parasites Depend on Plant Water and Solute Supply

Obligate plant parasitic nematodes are fully dependent on plant nutrients and water supply. Different nematode species developed multiple strategies to obtain the required resources from their hosts. Migratory ectoparasitic nematodes use simple strategies such as piercing root epidermal cells and withdrawing nutrients for short periods. As described in Chaps. 4 and 5, sedentary endoparasitic nematodes induce specialized feeding cells which establish a strong sink for water and nutrients. Accordingly, long-distance transport is crucial for supplying nematode-induced sink organs. Further, specific adaptations such as wall modifications, facilitated water transport, and active solute transport via transport proteins or symplasmic transport via plasmodesmata, assist short-distance transport. Modifications in molecular and physiological mechanisms assist the functioning of highly adopted feeding structures for plant parasitic nematodes.

20.2 Water Transport

Apart from the general need for water, the modified nematode-induced feeding cells may have an additional increased demand for water because of their specific physiological features. Further these cells fulfil a function which is unusual for plant cells: they feed another organism that is essentially dependent on them. This function is not trivial: females of *Heterodera schachtii* parasitizing *Arabidopsis* roots have been calculated to take up an amount of solutes that is equivalent to four times the syncytium volume per day (Sijmons et al. 1991). It is worth mentioning that

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these syncytia pose a fusion of several hundred root cells (Golinowski et al. 1996) and therefore a substantial amount of water is needed to compensate these continuous losses.

Another important fact is that feeding sites are metabolically highly active. For sedentary nematodes they are regarded as newly established plant organs which provide specifically tailored nutrients to the associated nematodes. An accumulation of solutes induces an increase in the osmotic value and an increased turgor pressure in feeding cells. Pressure probe measurements of syncytia of *H. schachtii* in *Arabidopsis* roots revealed turgor pressures around 0.9 MPa as compared to 0.4 MPa in root parenchyma cells (Böckenhoff 1995). The high differences in the osmotic potential together with nematode feeding induce a strong sink for water.

20.2.1 Long Distance Water Transport

Xylem vessels transport water from the root to the shoot and should, in theory, be mainly responsible for water supply of nematode feeding sites. However, the importance of the xylem for root nematodes has not been well studied making it difficult to evaluate the importance of long distance transport. However, after degeneration of the rhizodermis and the formation of the exodermis at the infection site, water transport via the xylem becomes essential. Nematode invasion into the central cylinder usually is either destructive or cells which are transformed into members of feeding sites are no longer available for xylem formation (Golinowski et al. 1996; Wyss et al. 1992), so that this process is often reduced in the affected root section forming a bottleneck for water transport. It has been shown recently that the formation of the xylem is controlled by finely tuned mechanisms which are based on the exchange of information between root parenchyma cells and the endodermis (Carslbecker et al. 2010). It is clear that these mechanisms will be disturbed by nematodes and nematode-induced feeding cells. Interference with xylem formation and the strong water sink of feeding sites may have adverse effects on plant development by causing a reduced water supply to the shoot and an increased sensitivity to drought. Root nematodes, however, have not been observed to change the structure of existing xylem vessels, whereas the pinewood nematode *Bursaphelenchus xylophilus* was found to induce increased cavitation of the xylem in pine seedlings leading to embolism and sudden wilting (Utsuzawa et al. 2005).

In order to monitor water transport from the xylem into giant cells induced by *Meloidogyne incognita* Dorhout et al. (1988) applied the fluorochrome Tinopal DBS to tomato roots: the dye was transported in the xylem but moved out only into giants cells indicating that there is a strong flow of water from the xylem into the giant cells. In some samples no transfer of dye could be observed. In these cases either the suction strength of the giant cells and the associated nematode was too low, or the nematode did not actively feed. In addition, this study showed that no water is taken up through the penetration canal along the nematodes' body.

20.2.2 Short Distance Water Transport

A remarkable feature of nematode induced giant cells and syncytia is the formation of cell wall protuberances protruding from the cell wall into the protoplast of the feeding cell at the interface to the xylem (Jones and Northcote 1972a, b). According to the concept of transfer cells, the protuberances were interpreted as sites of increased short-distance transport, which serve to facilitate water and nutrient import from the xylem into the feeding cells (Jones 1981; Jones and Northcote 1972a, b). Numerous studies on the anatomy of the cell wall of nematode feeding sites show that these structures always occur but both their location along the wall and their size and shape are highly variable although all studied feeding sites were taken from functional interactions (Jones 1981; Jones and Northcote 1972a, b; Golinowski et al. 1996; Sobczak et al. 1997). A specific ultrastructural analysis of syncytia in *Arabidopsis* roots associated with male cyst nematodes revealed that the protuberances—in contrast to syncytia associated with females—were small and not elaborate (Sobczak et al. 1997). Male nematodes have a markedly lower demand for food, their syncytia are generally much smaller. These nematodes may therefore induce a lower sink and this may be reflected by smaller protuberances.

Due to its physicochemical nature the plasmalemma has a limited capacity for water transport. As well as diffusion, short-distance water transport can further be facilitated by aquaporins which form water pores through the plasmalemma. Their relevance in syncytia and giant cells is difficult to determine. The first suggestion that aquaporins are specifically upregulated was provided by the increased promoter activity of *TobRB7* detected in giant cells (Opperman et al. 1994). A wealth of information on specifically regulated genes in nematode-induced feeding cells is now available but in no case has a distinctive up-regulation of aquaporins been observed. Aquaporins belong to the family of major intrinsic proteins (MIPs) and members of this family were found to be both up- and down-regulated in giant cells (Jammes et al. 2005; Barcala et al. 2010). Analysis of syncytia revealed that 9 of 35 MIPs in *Arabidopsis* were among the 100 genes with the strongest decrease in expression level (Szakasits et al. 2009). However, these plants were cultivated under sterile conditions on agar medium which could influence the necessity for aquaporin gene expression. Further detailed analyses are necessary to understand how plants orchestrate the different mechanisms to enable and control water transport to the feeding sites.

20.3 Solute Supply of Nematode-Induced Feeding Structures

20.3.1 Long-Distance Solute Transport

Plant nematodes are obligate parasites and rely on their hosts to supply nutrients and energy. Whereas migratory nematodes feed on many different cells, sedentary

nematodes remain close to the infection site and induce feeding structures. These feeding structures accumulate enough nutrients to allow the nematodes to complete their life cycle. Accordingly, metabolites produced in photosynthetically active plant source tissues or liberated from storage tissue have to be transported into and accumulated in the feeding structures in the roots. In plants, long-distance transport from source to sink tissues is performed in the phloem elements, the second long-distance route next to the xylem. The phloem is composed of two closely related cell types—the sieve elements and the companion cells—which are tightly connected by a high number of branched plasmodesmata (PD), forming the sieve element/companion cell complex (SE/CC). SE are specific transport tubes connected by an interface cell wall perforated by pit fields of PD (sieve plate) allowing extensive solute passage. As the cellular organisation of SE is drastically reduced and optimized for transport, control and metabolism is overtaken by the CC.

The sink character of nematode induced feeding structures has been shown using fluorescent dyes and isotope labelling. Radioactive signals accumulate in nematode-feeding structures after exposing shoots of infected plants to labelled CO_2 (Bird and Loveys 1975; McClure 1977) or sucrose (Böckenhoff et al. 1996). Root sections down-stream of the nematode infection site showed reduced levels of radioactivity underlining the strong sink character of syncytia. In order to visualise the up-take of phloem-derived solutes into nematode feeding sites the fluorescent dye carboxyfluoresceine-diacetate (CFDA) was loaded into green source leaves (Böckenhoff et al. 1996; Dorhout et al. 1993; Hofmann et al. 2007; Hoth et al. 2005). Due to its physicochemical nature CFDA enters the SE/CC where it dissociates and is trapped. The dye moves from the source leaves via the transport phloem into sink tissues such as root. In this way both phloem elements and symplasmically connected cells can be detected. Further, GFP was expressed under the control of the phloem-specific p*AtSUC2*-promoter, showing an increased number of phloem elements passing by the feeding cells, indicating extensive solute unloading and thus underlining the strong sink character of nematode feeding cells (Hoth et al. 2005).

20.3.2 Short-Distance Solute Transport

20.3.2.1 Apoplasmic Solute Transport During Early Stages of *H. schachtii* Development

Successful development of sedentary nematodes relies on the import of phloem-derived solutes and nutrients into nematode feeding cells. In general, cells of plant sink tissues may be supplied in two ways. In the first, phloem-derived solutes are transferred directly into cells of the sink tissue (e.g. root tips) following the symplasmic route via PD which form small cell wall channels and connect the protoplasts of adjacent cells. In the second way, solutes are transported apoplasmically across the plasmamembrane by active transport proteins (Kim and Zambryski 2005).

Presently, most studies on solute supply of nematode feeding structures have been conducted using the model interaction between *A. thaliana* and *H. schachtii*. Nematode induced syncytia have first been described to be symplasmically isolated due to cell wall depositions along PD in early stages of nematode development (Golinowski et al. 1996; Sobczak et al. 1997). Further observations showed that dyes microinjected into syncytia did not diffuse into adjacent cells nor into phloem elements (Böckenhoff and Grundler 1994). Later the concept of symplasmic isolation was challenged. Therefore, detailed analyses of syncytium solute up-take were performed applying fluorescent markers. When *Arabidopsis* scions expressing a phloem mobile p*AtSUC2*-GFP were grafted onto wild type root stocks no GFP could be detected in syncytia in the first days of nematode development (Hofmann and Grundler 2006). However, GFP is a rather large fluorochrome (27 kDa) and may therefore not correctly depict passage through small sized PD. Therefore, CFDA (0.46 kDa) was loaded into the source leaf phloem of infected plants (Hofmann et al. 2007). The dye moved in the transport phloem towards sink areas such as root tips, but was also not taken up by early-staged syncytia (Fig. 20.1a). These results confirmed the symplasmic isolation of syncytia in the first days of nematode development.

In order to analyse the background of this finding, the occurrence of PD was studied. Viral movement proteins (MP) are a powerful tool for the localisation of PD in plant cell walls. During virus pathogenesis, these proteins attach to and manipulate PD in order to facilitate movement of viral RNA or DNA between plant cells. Accordingly, the MP of the potato leaf roll polerovirus $MP17^{PLRV}$ was fused to GFP and expressed in *Arabidopsis* (Hofius et al. 2001) and the $MP30^{TMV}$-GFP fusion (tobacco mosaic virus) was expressed in tobacco (Kragler et al. 2003) to observe PD along plant cell walls. These transgenic plant lines were then inoculated with different plant-parasitic nematodes in order to study the occurrence of PD in walls of giant cells and syncytia (Hofmann et al. 2010a; Hoth et al. 2008). In syncytia of *H. schachtii*, no PD were observed during the first four days after infection, while at seven days after infection numerous GFP signals were found (Hofmann et al. 2010a).

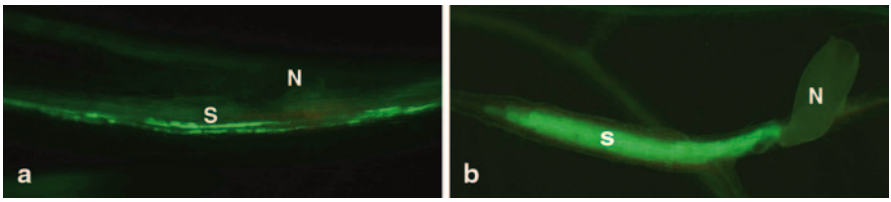

Fig. 20.1 Phloem-loading experiments using carboxyfluoresceine-diacetate (CFDA). Source leaves of nematode-infected plants were treated with CFDA that is transported into sink areas of the plant via the phloem elements. Early staged syncytia are frequently isolated from the phloem (**a**) and therefore do not take up the dye (five days after nematode inoculation). Phloem elements passing by the syncytium are transporting the green fluorescing dye. At later stages (**b**) plasmodesmata between the phloem and the syncytium open so that symplasmic transport of CFDA is facilitated (12 days after nematode inoculation). N = nematode, S = syncytium

The presence of PD however, does not necessarily lead to symplasmic solute transport. Plants may regulate transport through PD by callose deposition along the cell wall surrounding PD, which leads to an inwards compression and thus a reduction or eventually an interruption of solute passage (Wolf et al. 1991). In early-staged syncytia numerous local callose depositions were observed in the cell walls using an anti-callose antibody (Hofmann et al. 2010a). Thus, in this early period the feeding cells have to be supplied by the apoplasmic route.

The expression of various genes involved in transport and allocation in nematode-induced syncytia has been studied by transcriptome analyses (Puthoff et al. 2003; Szakasits et al. 2009). More detailed analyses were performed on sugar transporter genes (Hofmann et al. 2009a). Presently 90 sugar transporter genes are annotated in the *Arabidopsis* Membrane Protein Library data base. In syncytia, eleven of those genes were significantly up- and 19 genes were significantly down-regulated. Amongst those, the most strongly up-regulated genes were the membrane based hexose transporter *STP12* and the plastidal sugar transporter *ANTR2*. Furthermore, two sucrose transporters *AtSUC2* and *AtSUC4* have been found to be expressed and up-regulated in syncytia compared to non-infected control roots (Hofmann et al. 2007; Juergensen et al. 2003; Jürgensen 2001; Scholz-Starke 2002). Functional analyses using T-DNA insertion mutants and RNAi-based gene silencing showed the significant role of the studied sugar transporters for nematode development (Hofmann et al. 2009a; Hofmann et al. 2007).

In addition to up-regulation of sugar transporter genes, the amino acid permeases *AAP6* and *AAP8* were highly induced in syncytia compared to control roots (Szakasits et al. 2009). Down-regulation of the ammonium transporter gene *AtAMT1;2* (Fuller et al. 2007) indicates the preferential up-take of amino acids into syncytia rather than inorganic nitrogen forms. The role of these transporters for nematode development has not been studied to date.

Finally, the occurrence of active sugar transport in nematode-induced syncytia was studied in electrophysiological analyses (Hofmann et al. 2009a). Most sugar transporters operate by exploiting the proton-motive-force. They are primarily energised by the activity of a proton pump (H+-ATPase) carrying H^+ through the plasma membrane. This can be measured as a transient depolarization outside the membrane. Typical depolarisation and repolarisation profiles were detected in response to glucose, fructose, sucrose and raffinose application to 10–21 day old syncytia indicating active sugar import (Hofmann et al. 2009a). Furthermore, apoplasmic sugar import was shown by applying fluorochrome-labelled glucose (2-NBD-glc). In contrast to the symplasmically translocated CFDA, the 2-NBD-glc was imported into almost all syncytia and nematodes at 3 dai (Hofmann et al. 2009a), a time period at which no functional PD have been observed.

20.3.2.2 The Formation of Secondary PD in Cell Walls of Syncytia Induced by *H. schachtii*

During the first days after nematode infection syncytia are established, nematodes start feeding and growing and the syncytia proliferate and expand rapidly. Thus,

energy and nutrient demands of the expanding syncytium and growing nematodes rise, inducing increased solute supply. Nutrient demand increases are especially high during development of female nematodes. Even though symplasmic isolation may be advantageous to implement highly controlled import mechanisms and specific development, solute import by transporters is less efficient compared to influx through functional PD. In plants, PD frequently change their configuration between closed and opened to meet changing demands of specific tissues or during developmental processes as in embryos (Kim and Zambryski 2005), seeds (Asthir et al. 2001; Wilson et al. 2006), meristems (Gisel et al. 1999; Oparka et al. 1994; Rinne and van der Schoot 1998), fibers, and root nodules (Complainville et al. 2003), as well as during dormancy (Rinne et al. 2001).

Symplasmic phloem-unloading into developing syncytia was observed to be established gradually and was first detected at 4 dai by CFDA trafficking (Hofmann et al. 2007). Simultaneously, the first functional PD in cell walls of syncytia were observed using MP-GFP fusion constructs (Hofmann et al. 2010a; Hoth et al. 2008). Co-localisation of PD by the $MP17^{PLRV}$-GFP and of callose by immuno-localisation using a red-fluorescent secondary antibody (Alexa 594) discernible from GFP (Fig. 20.2) showed the deposition of callose on a number of PD, while other PD were free of callose. In the following days both the number of syncytia taking up the phloem-derived dye and the number of PD increased, while the frequency of callose deposition decreased. Finally, at 15 dai almost all syncytia attached to female nematodes were symplasmically connected to the phloem (Hofmann et al. 2007) (Fig. 20.1b). At this time syncytia are established as important sink organs

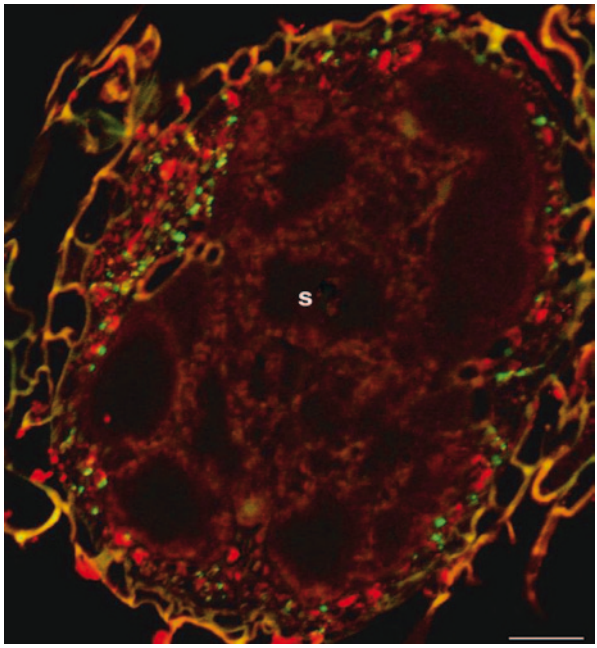

Fig. 20.2 Immunolocalisation using an anti-callose antibody was performed on cross sections of syncytia induced in an *Arabidopsis* line expressing the $MP17^{PLRV}$-GFP fusion construct. Signals for callose deposition are depicted in red, GFP expression in *green* and a co-localisation is indicated in *yellow*. S = syncytium, bar = 20 µm

that compete with other organs for energy and nutrients and therefore reduce the productive efficiency of crop plants.

While a clear symplasmic connection between syncytia and phloem elements at later stages of nematode development is evident, syncytia remain obviously isolated from neighbouring cells of the stele. When phloem-derived dyes enter syncytia they seem to be trapped and do not diffuse into neighbouring cells. In established young syncytia local callose deposition was observed along the outer cell walls of recently incorporated cells indicating a symplasmic borderline towards neighbouring cells (Hofmann et al. 2010a). These results suggest that syncytia form symplasmic domains, cell complexes connected amongst each other, but isolated from surrounding cells (Gisel et al. 1999). The isolation from neighbouring cells ensures that all inflowing solutes are available for syncytium metabolism and the feeding nematode, whereas surrounding cells do not obtain benefit from the accumulated assimilates.

An indication for the highly selective accumulation process for hexoses in syncytia could be demonstrated by regulation of the hexose transporter gene *STP12* in syncytium containing root segments. In samples of the infected root segments *STP12* was down-regulated whereas it was up-regulated in pure micro-aspirated syncytium content (Hofmann et al. 2009a) thus indicating massive *STP12* down-regulation in the tissue around the syncytium.

The role of callose deposition during syncytium formation was studied in an experiment using an *Arabidopsis* mutant with reduced ability to degrade callose. In the mutant plants nematode development was significantly affected (Hofmann et al. 2010a). Syncytia in mutants were smaller and could not expand as well as syncytia in the wild type. Two reasons may account for this finding: first, callose may still block PD that are supposed to connect to the phloem thus reducing the inflow of nutrients. Second, syncytial enlargement by incorporation of neighbouring cells starts by the formation of local cell wall openings at PD (Grundler et al. 1998). Reduced callose degradation along PD may therefore interfere with this process of cell wall degradation. In this context it should be mentioned that the expansion of syncytia through integration of neighbouring cells must be a complex process during which candidate cells must be pre-adapted before full integration. It is therefore not far fetched to postulate mobile signals transported from syncytia to adjacent cells.

20.4 Other Plant-Nematode Interactions

Apart from *Arabidopsis*, transporter gene expression in syncytia has been studied in other plant species using different transcriptome profiling approaches. A genome-wide expression profile of syncytia of *Heterodera glycines* in soybean roots revealed the significant up-regulation of two sugar and one amino acid transporter genes primarily in early stages of development (2 and 5 dai). Transcriptome analyses studying syncytia of *Globodera rostochiensis* in tomato roots revealed the up-regulation of genes involved in allocation such as hexose transporters (Swiecicka et al. 2009; Uehara et al. 2007). Further, gene expression in root-knots induced by

M. incognita was studied intensively in *Arabidopsis* showing several transporter genes to be differentially expressed compared to control roots (Hammes et al. 2005; Jammes et al. 2005). The ammonium transporter gene *AtAMT1;2* is highly downregulated at 21 dai in galls of *M. incognita* in Arabidopsis (Fuller et al. 2007). Jones et al. (1975) found indications for active sugar transport into giant cells in *Impatiens balsamina* roots by studying the transmembrane potential. Functional analyses silencing the sucrose transporter gene *AtSUC4* reduces *M. incognita* development thus underlining the importance of transporter gene expression (Hofmann et al. 2009b; Jürgensen 2001).

While all the studied nematode feeding structures show expression of genes involved in transport and allocation, there are different reports about the presence and functionality of PD. Giant cells induced by root-knot nematodes in *I. balsamina* were found to have pit fields of PD between giant cells, but no PD between giant cells and neighbouring cells (Jones and Dropkin 1976). Giant cells of *Meloidodera floridensis* and *Meloidodera belli* in loblolly pine, peony, and sage, respectively showed numerous pit fields with plasmodesmata in the cell walls, but wall ingrowths were not detected in a thorough examination of the entire wall (Mundo-Ocampo and Baldwin 1983a). Therefore, giant cells were believed to be symplasmically isolated from their surrounding. However, studies using fluorescent dyes showed contrasting results. Galls induced by *M. incognita* in *Arabidopsis* showed no clear or direct import processes while galls in tomato were found to be stained by phloem-derived dye (Dorhout et al. 1993; Hofmann et al. 2009b; Hoth et al. 2008). Studies on giant cells are difficult since they are embedded into the proliferating gall tissue. In order to localise potential PD in the cell walls of giant cells and neighbouring cells, the MP17^{PLRV}-GFP fusion construct was used. Contrasting results were once again observed. While Hoth et al. (2008) did not find GFP signal in the cell walls of 18 day old giant cells, Hofmann et al. (2010a) detected clear signals at 13 days after infection (Fig. 20.3). Presently, this contradiction can only be explained by the different age of sampled galls and the different age of the host plants in the two studies. In the experiments of Hoth et al. (2008) giant cells were five days older and plants were three weeks older than in the other study by Hofmann et al. (2010a). The advanced age of the inoculated plants may affect GFP gene expression (Kronberg et al. 2007) and could therefore have impeded microscopic detection of the MP17^{PLRV}-GFP signal.

The occurrence of PD was observed in feeding cells of different nematode species induced in different host plants. Syncytia of *Naccobus aberrans*, the false root-knot nematode, showed numerous PD towards phloem elements in tomato (Jones and Payne 1977) and in syncytia of *Atalodera ucri* pits and pit fields in the cell wall towards the vascular elements suggest symplasmic solute import (Mundo-Ocampo and Baldwin 1983b). However, in syncytia of *G. rostochiensis* in tomato no functional PD were found between syncytia cells and phloem elements as secondary cell wall material was deposited along PD openings (Fudali and Golinowski 2007). In syncytia induced by *G. tabacum* and *H. schachtii* in tobacco roots PD were observed in the cell walls but they were coupled with callose deposition questioning their functionality (Hofmann et al. 2010a). In syncytia of a resistant sugar beet

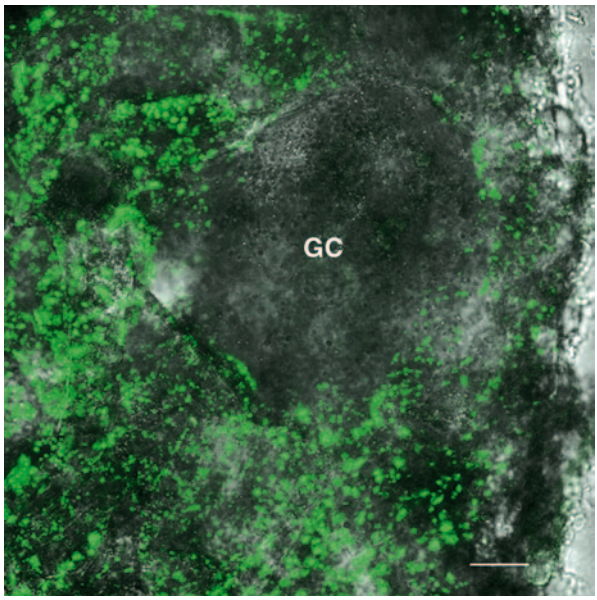

Fig. 20.3 Giant cells induced in an *Arabidopsis* line expressing the MP17^{PLRV}-GFP fusion construct. A large number of *green* GFP signals indicating the presence of functional plasmodesmata were detected along cell walls of giant and neighbouring cells (13 days after inoculation). GC = giant cell, bar = 20 µm

carrying HS1$^{pro\text{-}1}$, cell wall openings along PD were narrower than in the susceptible sugar beet (Holtmann et al. 2000).

Presently, it appears as if feeding cells of single nematode species in different host species are specifically structured. As shown for the interaction between *Arabidopsis* and *H. schachtii* the timing of analysis is very important so that early-staged syncytia may show no PD contrary to late-staged syncytia.

20.5 Nematode Feeding

All plant parasitic nematodes use their stylets to pierce the wall of host cells but withdrawal of nutrients from these cells is specific to the different types of nematodes. Nematodes of the genera *Trichodorus* and *Paratrichodorus* within the order Triplonchida are the only plant parasitic nematodes capable of ingesting cytosol of root cells together with its organelles. They have a solid ventrally curved stylet which is retracted after cell wall disruption. The cytoplasm of the host cells is sucked through a small tube formed by solidified secretions. If completed, this type of parasitism causes immediate cell death (Karanastasi et al. 2004).

Other plant parasitic nematodes belonging to the order Dorylaimida and Tylenchida have hollow stylets. After piercing the cell wall, secretions are released through the stylet into the affected cell. These secretions are thought to modify the cytoplasm and condition it for feeding. The secretions of dorylaimids seem to possess lytic activity so that components of the cytoplasm are pre-digested and prepared to be taken up through the stylet (Wyss et al. 1988). However, in tylenchid

nematodes conditioning of parasitized cell tends to be more subtle and often leads to a re-organisation of the host cell. The longer the nematodes remain at a single feeding site the more the affected cell seem to be modified into specific feeding structures.

In migratory tylenchids, e.g. *Tylenchorhynchus dubius*, feeding is relatively short and the main feature of re-organisation is a modified "clear zone" of cytoplasm around the site of stylet insertion (Wyss 1986). *Criconemella xenoplax* is a tylenchid species that feeds on a single root cell for several days. During parasitism the plasmalemma of the feeding cell becomes closely attached to the stylet orifice in a way that allows nutrient uptake unimpeded from callose depositions. Plasmodesmata between these feeding cells and adjacent cells are specifically modified suggesting facilitated solute transport (Hussey et al. 1992). *Helicotylenchus spp.*, another tylenchid nematode, feeds for many days on single root cells within the stele of cereals. Each single cell is surrounded by cells with dense cytoplasm indicating high metabolic activity. In this interaction so-called feeding tubes were observed (Jones 1978).

Feeding tubes are formed at the laterally located stylet orifice close to the stylet tip. Since the nematode stylet protrudes though the cell wall into the cytoplasm it is highly probably that it is sheeted by the plasmalemma after some time. Feeding tubes emerge from the stylet orifice, span the plasmalemma and extend into the cytoplasm often in close connection with the endomembrane network. Adult root-knot nematodes produce feeding tubes consisting of an electron-dense crystalline wall. After formation, feeding tubes become embedded in accumulated smooth endoplasmic reticulum (ER) and which is surrounded by rough ER (Hussey and Mims 1991). In cyst nematodes, the feeding tubes have an amorphous wall which is spanned by tubules of the ER (Sobczak et al. 1999). Although studied extensively, the nature, formation and function of feeding tubes is still not yet fully understood but is thought to be mainly of nematode origin (for review, see e.g. Hussey and Grundler 1998; Davis et al. 2004).

In *H. schachtii*, the first feeding tubes occur about 36 h after syncytium induction (Wyss 1992). A detailed *in vivo* analysis of the feeding behaviour of this nematode demonstrated that feeding tubes are stripped off the stylet and newly formed during recurring feeding phases. Detached feeding tubes float through the cytoplasm and are eventually degraded in the syncytium (Sobczak et al. 1999). Three phases could be discriminated: during phase I the nematodes insert the stylet into the syncytium, during phase II they feed from the syncytium by active pumping, and during phase III active feeding stops, the stylet is retracted, and the feeding tube is detached (Wyss 1992).

Feeding tubes seem to mediate direct solute transport from the cytosol of the feeding cell to the stylet orifice acting as molecular sieves with a defined size exclusion limit (SEL). However, the selective uptake of molecules by nematodes is not uniform across genera. Böckenhoff and Grundler (1994) determined an SEL between 20 and 40 kDa for *H. schachtii* by microinjection of differently sized fluorochrome-labelled dextrans. 28 kDa GFP was found to be taken up efficiently by the root knot-nematode *M. incognita*, less efficiently by the cyst nematode

G. rostochiensis and not at all by *H. schachtii*. Accordingly, the SEL of root-knot nematode feeding tubes appears to be larger than that of cyst nematodes (Urwin et al. 1997; Goverse et al. 1998). SEL of the feeding tubes does not only affect nutrient up-take of nematodes but is also important for efficacy of *in planta* expressed recombinant proteins, dsRNA and siRNA which are aimed to be used for the control of plant parasitic nematodes. If these compounds are unable to pass the molecular sieve of the feeding tube a strategy that is based on their up-take by the nematode will not be effective.

20.6 Nutrient Cycling and Limited Nutrient Supply

Nutrients and solutes imported into nematode-feeding structures are used on the one hand for nematode feeding and on the other hand as an energy supply for the (in some cases) substantial re-organisation of the affected host cells. Isotope labelling revealed that in *Arabidopsis* plants most of the incoming ^{14}C-sucrose remained inside the syncytia induced by *H. schachtii*, while smaller amounts were taken up by the nematode (Böckenhoff et al. 1996).

The availability of nutrients in nematode-feeding sites in plant roots depend on phloem transport flux rates and solute composition. The most abundant sugars in the phloem are sucrose and raffinose and most abundant amino acids are glutamine and asparagine (Winter et al. 1992). Where there is a symplasmic connection between the phloem and the feeding cells these are the major nutrients expected to be available for plant cell metabolism and nematode nutrition. In the case of sedentary endoparasitic nematodes syncytia and giant cells may act as intermediate structures synthesising nutrients specific for the nematode's diet.

Presently, there is only limited information about the metabolic processes in nematode affected plant cells. Transcriptome analyses suggested changes of the primary metabolism of nematode feeding sites based on gene expression results (Bar-Or et al. 2005; Ithal et al. 2007; Jammes et al. 2005; Puthoff et al. 2003; Szakasits et al. 2009). First metabolite analyses showed changes in amino acid and sugar levels in nematode-induced syncytia (Betka et al. 1991; Hofmann et al. 2007; Hofmann et al. 2009a). Recently, a detailed metabolite profiling study provided the first broad insight into metabolite levels and putative metabolic processes in syncytia (Hofmann et al. 2010b). This study revealed on the one hand a global increase of amino acid levels and on the other hand increased levels of very specific sugars such as 1-kestose. Further, integrated analysis of metabolite and transcript levels indicated a transcriptional regulation of specific pathways of the primary metabolism in nematode-induced syncytia (Hofmann et al. 2010b). The first functional evidence of the importance of a specific metabolic pathway was provided as nematode development was reduced on a *starch synthase 1* T-DNA insertion mutant (Hofmann et al. 2008).

Limitations in nutrient supply are not only critical for nematode survival but have been suggested to determine sexual differentiation. Juveniles of *Meloidogyne*

floridensis were observed to develop as females under normal conditions but as males under food stress conditions (Triantaphyllou and Hirschmann 1973). Similarly, higher nutrient availability was described to promote female nematode development of *H. schachtii* while scarcity increases male development (Betka et al. 1991; Grundler et al. 1991). An almost exclusive female development was induced under an optimal sucrose supply in the plant growth medium of axenically cultivated *Brassica rapa* (Grundler et al. 1991). Betka et al. (1991) suggested a relationship between different amino acids in syncytia and sex determination: higher concentrations of lysine, methonine, phenylalanine and tryptophane favoured male development while glutamine was related to female development. Furthermore, juveniles developing into females were shown to require about 40 times more food than male juveniles (Mueller et al. 1981).

The high nutrient demand of juveniles developing into females will induce stronger sinks in plant roots. This may lead to symplasmic connection of syncytia of female juveniles, while those juveniles developing male mostly feed on syncytia that remain symplasmically isolated from the phloem (Hofmann and Grundler 2006). During grafting experiments of p*AtSUC2-gfp* scions onto wild type rootstocks, syncytia of male juveniles never took up GFP, whereas it was translocated into syncytia induced by female juveniles (Hofmann and Grundler 2006). Therefore, the expression and activity of plasma membrane-bound transport proteins has to be more important for male development. Accordingly, the expression of the sugar transporter gene *STP12* is in fact higher in syncytia of male juveniles than in syncytia of female juveniles (Hofmann et al. 2009a). Furthermore, silencing *STP12* had a stronger effect on male compare to female nematode development (Hofmann et al. 2009a).

It is presently unclear if the formation and maintenance of the syncytium determines sexual differentiation or if juveniles developing different sexes induce the formation of distinct feeding structures. Microscopic observations showed that syncytia of nematodes developing into males expand into the procambial and cambial cells of the vascular cylinder (Sobczak et al. 1997) while, syncytia of those nematodes developing into females are usually induced in the procambial or cambial root cells (Golinowski et al. 1996). It is unclear if the lack of PD formation allows only male development or if syncytia of male developing juveniles do not require PD for a successful development.

20.7 Conclusions

Plant parasitic nematodes rely on water and nutrient supply from their host plants. How nutrients need to be supplied depends on the feeding strategy of the nematodes. The most sophisticated interactions are induced by sedentary endoparasitic nematodes that induce the formation of specific feeding structures in plant roots. At present there is some information on solute supply of syncytia induced by *H. schachtii* in *Arabidopsis* roots that has been studied in more detail in recent years. However, many questions remain open. Presently it is far from clear what signals

are employed to provoke the formation of a new sink structure and of numerous phloem elements along this sink. It is unclear what finally forces the plant to supply nematode feeding structures with high amounts of nutrients, how the switch from apoplasmic to symplasmic solute supply is regulated and what triggers the gating of PD. The formation of the sink could be based on the nematode feeding activity leading to a permanent loss of plant derived solutes or an evolutionary highly adopted signal secreted by nematode saliva into the affected plant cells.

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Part IV
Applied Aspects of Molecular Plant Nematology: Exploiting Genomics for Practical Outputs

Chapter 21
Molecular Tools for Diagnostics

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21.1 Introduction

Over 26,600 species of nematodes have been described, of which more than 4,000 are parasites of plants (Hugot et al. 2001). However, estimates of the total number of nematode species on the planet vary from 100,000 to several million (Hugot et al. 2001; Blaxter 2003), and thus the actual total number of plant-parasitic nematode (PPN) species is likely to be several-fold higher than is currently known. Because of the homogeneity of their morphological characters, nematodes are amongst the most difficult animals to identify. In addition, their small size and soil distribution make most PPN species difficult to detect. Traditional diagnostics rely on the delineation of morphological features, but the intraspecific variability of these frequently obscure characters renders reliable identification a formidable task, even for well-qualified taxonomists. Nonetheless, correct identification to species level is an absolute prerequisite for the implementation of successful management strategies against PPNs. Because they are independent of phenotypic variation, DNA-based technologies have provided avenues to overcome such limitations. Indeed, the "DNA revolution" started in plant nematology 25 years ago, with the first use of DNA restriction fragment length polymorphisms as diagnostic characters (Curran et al. 1985). A major contribution was provided a few years later, with the first demonstration that the polymerase chain reaction (PCR) could efficiently be used to identify single individuals from clonal lineages of root-knot nematodes (Harris et al. 1990). Since then, plant nematologists have increasingly employed molecular techniques for diagnostic purposes (see for review Powers 2004; Blok 2005). In the present chapter, we will briefly (1) review the DNA technological approaches and advances made in the discovery and use of molecular markers for the specific identification of PPNs, and (2) explore the fast-evolving methodologies that are

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currently being developed to provide diagnostics with greater robustness, precision and speed.

21.2 Markers for PCR Diagnostics

The study of genes and their functions is rapidly having an effect on many aspects of life, from health care to food safety and plant quarantine issues. An extensive range of techniques and genetic markers have significantly advanced the study of nematodes. For diagnostics, the most frequently used markers are those based on ribosomal, mitochondrial and satellite DNA regions, each of which have advantages and disadvantages.

21.2.1 rDNA

Ribosomal DNA (rDNA) markers, based on the ribosomal DNA repeating unit which includes the small subunit (SSU) 18S, large subunit (LSU) 28S, and 5.8S coding genes, the internal transcribed spacer (ITS), external transcribed spacer (ETS) and the intergenic spacer (IGS) regions, have been used extensively for diagnostic purposes and more recently for phylogenetic studies. The majority of sequence variation in rDNA occurs in the transcribed and non-transcribed intergenic regions in comparison with the more conserved structural RNA coding regions. Ribosomal DNA markers have been widely used as genetic markers in most living organisms including plants, insects, fish and nematodes (Hajibabaei et al. 2007). The main advantage of rDNA based markers is that they are present as multicopies, providing ample material for study with sufficient variation and stability. The 18S rDNA has been invaluable for broad phylogenetic reconstruction of the Nematoda (Blaxter et al. 1998; Holterman et al. 2006; Van Megen et al. 2009), for analyzing family relationships (Waeyenberge et al. 2000; Oliveira et al. 2004; Subbotin et al. 2008), and for barcoding nematode communities (Powers et al. 2009). As a result, characterization of new PPN species or populations very often includes analysis of 18S sequences (Chizhov et al. 2006; Palomares-Ruis et al. 2009). The ITS rDNA is most frequently used for diagnosis of *Heterodera* and *Globodera* spp. of cyst nematodes (Subbotin et al. 2001) and for *Ditylenchus* spp. of stem nematodes (Subbotin et al. 2005). Species-specific multiplex PCR primers based on ITS sequences have been validated for detection of virus-vectoring nematodes, including four *Xiphinema* species (Hübschen et al. 2004) and two species of *Paratrichodorus* (Riga et al. 2009). Diagnosis or characterization of lesion nematodes has more often relied upon the D2-D3 expansion segment of 28S rDNA (Subbotin et al. 2008; Yan et al. 2008; Múnera et al. 2009).

For some nematodes, these rDNA markers may not be reliable for species discrimination. This is the case for some *Meloidogyne* spp., for which intraspecific

and intra-individual variations have been described (Blok et al. 1997a; Hugall et al. 1999; Adam et al. 2007). However, the IGS region between 5S and 18S (IGS2) has diagnostic value for distinguishing *M. mayaguensis* (senior synonym = *M. enterolobii*) from the other tropical RKN species (Blok et al. 1997a) and for diagnosis of *M. chitwoodi, M. fallax*, and *M. hapla* (Wishart et al. 2002).

21.2.2 mtDNA

Metazoan mitochondrial DNA (mtDNA) genomes are relatively small circular molecules ranging from 12–20 kb in size, although larger mtDNA genomes have been described (Tang and Hyman 2007). These extrachromosomal genomes contain approximately 37 genes coding for tRNAs, rRNAs and mRNAs, the latter encoding proteins primarily involved in the electron transport chain and oxidative phosphorylation of mitochondria (Boore 1999). Studies of vertebrate and invertebrate mtDNA markers have shown that sequence divergence accumulates more rapidly in mitochondrial than in nuclear DNA (Brown 1985). This divergence, which includes insertions and deletions, can be attributed to the accelerated rate of mutation in mtDNA as compared to nuclear DNA and most likely arises from the lack of effective repair mechanisms during replication (Wilson et al. 1985). Currently, more than 700 mt genomes have been sequenced for a range of metazoan organisms. However, despite the obvious socioeconomic importance of PPNs, little information exists regarding the importance of mt genomes in nematode genetics, systematics and ecology (Hu and Gasser 2006). However, one notable exception concerns studies of the mtDNA genomes of potato cyst nematodes, *Globodera* spp. (Armstrong et al. 2007; Gibson et al. 2007; Riepsamen et al. 2008). This research revealed that in contrast with the single mt genomes found in many metazoans, those of *G. pallida* and *G. rostochiensis* are multipartite, consisting of six or more small circular genomes of varying size.

Some species of *Meloidogyne*, including *M. mayaguensis*, can be differentiated on the basis of size differences in the region between the cytochrome oxidase II (COII) and 16S rRNA genes on the mitochondrial genome (Stanton et al. 1997; Blok et al. 2002; Jeyaprakash et al. 2006).

21.2.3 satDNA and Other Variable Number Tandem Repeat Regions

Satellite DNA (satDNA) markers are highly repeated tandem arrays of short sequences ranging anywhere from 70–2,000 bp in length. SatDNA is highly repetitive in nature, as is mtDNA, and as such provides easily detectable targets, and the discovery that some satDNAs are divergent between different species has been

exploited to develop various diagnostic probes. The majority of the satDNA literature relevant to plant nematology is centred on satDNA markers in root-knot nematodes. In *Meloidogyne* species, satDNA contain different signature sequences and differ in copy number, length and polymorphic regions (Meštrović et al. 2006, 2009). For example, satDNA represents 2.5% of the genome in the case of *M. incognita* (Piotte et al. 1994) and 20% in the case of *M. fallax* (Castagnone-Sereno et al. 1999). Examples of the use of satDNA as a diagnostic probe include repeat sequences from *M. hapla*, which did not cross react with either *M. chitwoodi* or *M. incognita*. The probe used in these studies has sufficient sensitivity to be able to detect DNA from individual females, including females within infected root tissue (Piotte et al. 1995). The development of satDNA probes and their subsequent use in PCR-based detection systems provides an alternative approach for the sensitive detection of the major *Meloidogyne* spp. (Castagnone-Sereno et al. 1995; Randig et al. 2002a, 2009), as is the case for the pine wood nematode *B. xylophilus* (Castagnone et al. 2005) and the cereal and legume root lesion nematode *Pratylenchus thornei* (Carrasco-Ballesteros et al. 2007).

Repeated segments have been found in the mtDNA molecules of a number of metazoan species. Tandemly as well as non-tandemly arranged repeated sequences occur in the control regions of several vertebrate species. Almost all of these repeat copy number variations take place between individuals of a species, even, in some cases, within individuals (heteroplasmy) and in several cases nucleotide sequence information can be obtained from them (Okimoto et al. 1991). Also, repeated segments have been detected in the mtDNA molecules of invertebrates as for the parasitic nematode *Romanomermis culicivorax* (Hyman et al. 1988) and for root-knot nematodes (Okimoto et al. 1991). This latter study not only reported the discovery of three sets of repeat sequences (102, 63 and 8 ntp, respectively) in mtDNA molecules of the species *M. javanica*, but also that differences in the 102 nt repeat mtDNA sequence of *M. javanica* and different host races of *M. incognita*, *M. hapla* and *M. arenaria* were sufficient to distinguish the different host races of each species. The use of VNTR *loci* in population genetic and evolutionary studies has been criticized because these markers mutate via stepwise mechanisms (alleles of a given size are not identical by descent) (Shriver et al. 1995). Hyman and Whipple (1996) evaluated the utility of a PCR-based assay of 63 bp mtDNA VNTR to analyse root-knot nematode population differentiation and demonstrated that PCR results obtained from individual nematodes are consistent with results obtained from pooled nematodes of the same isolate. Later studies carried out on *M. incognita* revealed low, but qualitatively significant diversity among populations, although most of the differences lie at the individual level even within the same isolate (Whipple et al. 1998). In spite of probable mutation of this *locus*, the associated homoplasy could be reduced considering the level of taxonomic or genetic resolution in which the isolates are enclosed (Estoup et al. 1995). Therefore, this kind of data may allow correlations to be made between the geographic and genetic population structure of the apomictic *Meloidogyne* species and their epidemiological incidence; this knowledge will have value for the design of rational control programmes regarding these nematodes.

21.2.4 Protein Encoding Genes

21.2.4.1 Choosing a Suitable Marker

There are several factors that influence selection of a marker (protein encoding) for nematode diagnostics. The gene of interest should have a sufficient number of polymorphisms between different species to avoid primer cross-reactivity or false positives. The presence of insertions or deletions (indels) can greatly facilitate the design of diagnostic primers or probes, although these features may create alignment problems that may hinder phylogenetic utility. Both introns and exons in protein encoding genes provide useful variation for the development of primers and probes. Conserved intron-exon junctions greatly facilitate sequence alignments needed for primer design. Primers designed to span introns ensure that contaminating genomic DNA does not interfere with RT-PCR. Because introns tend to be less constrained than exons, diagnostically useful variation may still be present among closely related species or populations. The mixture of conserved exons and variable introns present in many protein-coding genes can be exploited for the design of diagnostics that cover different taxonomic scales.

21.2.4.2 Examples of Protein-Coding Genes Used for Diagnostics

Nematode phylogenies based on dense taxon sampling, multiple populations, and broad geographic representation provide an invaluable yet challenging molecular framework for diagnostic marker selection and validation. While nuclear protein encoding genes are commonly employed for phylogenies of bacteria, fungi, and other eukaryotes, relatively few have been used for nematodes. Among these are RNA polymerase II (Baldwin et al. 1997), major sperm protein (Setterquist et al. 1996; Lunt 2008), dystrophin and EF1a (Lunt 2008). While to date none of these protein-coding genes have been used for nematode diagnostics, the gene for the heat shock protein *Hsp70* has been employed successfully for detection of the pine wood nematode, *B. xylophilus* from wood samples using both conventional (Leal et al. 2005) and real time PCR (Leal et al. 2007). Alleles associated with virulence or avirulence of *B. xylophilus* isolates have also been detected by PCR-RFLP of *Hsp70* (Takemoto and Futai 2007).

The chorismate mutase (CM) gene represents a rare case where a marker associated with nematode virulence encodes a protein that has been functionally characterized. CM is a secreted enzyme produced in the nematode oesophageal glands; it has been suggested that its function is to manipulate the host plant shikimate pathway that controls cell growth, development, and pathogen defenses (Lambert et al. 1999; Doyle and Lambert 2003). In the soybean cyst nematode *H. glycines*, the *Hg-cm-1* gene has been shown to contain polymorphisms that correlate with virulence on soybean cultivars carrying different genetic sources of nematode resistance (Bekal et al. 2003, 2008; Lambert et al. 2005). *Hg-cm-1* was used to characterize

Table 21.1 Protein-coding genes for nematode diagnostics

Locus	Primers	5′ to 3′ sequence	Reference for PCR conditions
Hsp70	Bx701F	AACACCGTATTTGGTAAGCTTTTGACTGAG	Leal et al. (2005)
	Bx701R	CTTCGGCCTGGACGACCTTGAAT	
	Bm701F	AAAACACTGTATTTGGTGAGATTGACTGAA	
	Bm701R	TCGGCCTGGATGACCTTGAAG	
Hsp70	Forward[a]	TAAGATGTC+TTTT+AC+AGATGC+CAAG	Leal et al. (2007)
	Reverse[a]	GCC+TGGACGAC+CTTGAAT	
	Probe[a]	FAM-AT+TGG+CCGCAAATT+CGA+TGAA +CC-IAblkFQ[b]	
Hg-cm-1	Hg-cm-1-TM-F	CCAAGGACGTGGTCAATTACA	Lambert et al. (2005)
	Hg-cm-1-TM-R	CCCTGCGCCGAAACACT	
	Hg-cm-1B-TM	VIC-TTCATAACAACATCTCAATCG-MGBNFQ[b]	
	Hg-cm-1A-TM	FAM-TTCATCAAAACATCTCAATCG-MGBNFQ[b]	
Hsp90	U288	GAYACVGGVATYGGNATGACYAA	Skantar and Carta (2004)
	L1110	TCRCARTTVTCCATGATRAAVAC	

[a] The + sign indicates and follows the nucleotide that is modified by a locked nucleic acid (LNA)
[b] Probes were labeled at the 5′ end with fluorescent reporter dyes VIC or FAM and at the 3′ end with a dark quencher dye IAblkFQ or MGBNFQ

several *H. glycines* inbred lines; this marker may be useful for genotyping field populations to predict virulence.

In Scholl and Bird (2005), a bioinformatic screen of nematode EST (expressed sequence tag) datasets was employed to select for orthologous, single-copy genes that were conserved across the phylum. Each of the 47 genes selected had a homolog in *Caenorhabditis elegans*, reflecting evolutionary constraint across a large phylogenetic distance. Phylogenetic reconstruction for five root knot and three cyst nematodes was then performed for the multi-gene alignments, and selective pressure tests for various data sub-partitions revealed the presence of purifying selection for most genes. The *loci* identified by this analysis may pave the way for future development of diagnostic assays or microarrays based upon robust multi-gene phylogenies.

Adoption of single copy protein-coding genes for PPN molecular identification has been hindered by low amplification due to the small amount of DNA in individual nematodes. This limitation was overcome for the single copy *Hsp90* gene through the use of degenerate primers and a novel PCR cycling method called RAN-PCR (Skantar and Carta 2000). A method of whole genome amplification known as multiple displacement amplification (MDA) increased the amount of template available from individuals, improving the strength of *Hsp90*-based PCR reactions (Skantar and Carta 2005). It is notable that *Hsp90* (known as *daf-21* in *C. elegans*) was one of the 47 orthologs selected from the bioinformatic screen

of ESTs (Scholl and Bird 2005), giving further support to the phylogenetic and diagnostic potential of this marker. To date, *Hsp90* has been used to construct nematode phylogenies (Skantar and Carta 2004; Mundo-Ocampo et al. 2008; Palomares-Rius et al. 2009) and has been included in molecular descriptions of unusual *M. hapla* (Handoo et al. 2005) and *M. arenaria* populations (Skantar et al. 2008). *Hsp90*-based diagnostic assays are in development for *H. glycines* and *H. schachtii* (Skantar and Niblack, unpublished) and for root-knot nematodes (Skantar, unpublished). Continuing improvements in DNA preparation and PCR methods and a growing database of nematode *Hsp90* sequences should facilitate the future development of diagnostics based on this gene. Table 21.1 summarizes the protein-coding genes that have been evaluated for PPN diagnostics using a PCR/qPCR approach.

21.3 Other Diagnostic Methods

21.3.1 Restriction Fragment Length Polymorphisms (RFLP)

In combination with PCR, restriction fragment length polymorphism (RFLP) is undoubtedly the most used technique in the absence of species-specific primers. In this method, target DNA is amplified by PCR and subsequently digested with restriction enzymes and the resulting restriction fragments are analyzed by gel electrophoresis. There are many examples of the use of PCR-RFLP in both animal and plant parasitic nematode diagnostics, with a range of DNA targets available including rDNA and mtDNA sequences. One of the best examples is that of *Bursaphelenchus* spp. in which amplified ribosomal ITS sequences have provided species-specific restriction fragment patterns for 44 *Bursaphelenchus* species, including two intraspecific types in both *B. mucronatus* and *B. leoni*, providing genetically distinct provenances of several *Bursaphelenchus* species (Burgermeister et al. 2009). ITS RFLP has also been used extensively for diagnosis and characterization of the *Heterodera* spp. and *Globodera* spp. of cyst nematodes (Thiéry and Mugniéry 1996; Subbotin et al. 2000; Madani et al. 2004, 2007; Skantar et al. 2007), and RFLP profiles have supported synonymization of *Afenestrata* spp. within the *Heterodera* (Mundo-Ocampo et al. 2008). ITS-RFLPs were used for discrimination of cereal cyst nematodes *Heterodera filipjevi* and *H. avenae* from wheat fields in the Pacific Northwest (Yan and Smiley 2010) and for diagnosis of the false root-knot nematode *Nacobbus* spp. (Reid et al. 2003; Vovlas et al. 2007). RFLP profiles were recently described for some relatively understudied species, including sheath nematodes, *Hemicycliophora* spp. (Van Den Berg et al. 2010) and lance nematodes (*Hoplolaimus* spp.; Bae et al. 2009a, b). For the root-knot nematodes, digestion of mtDNA amplified fragments was shown to distinguish the quarantine nematode *M. chitwoodi* from the closely related *M. hapla* (Powers and Harris 1993).

21.3.2 Random Amplified Polymorphic DNA (RAPD)

RAPD is a powerful PCR-based technique that has been successfully applied to the identification and estimation of molecular genetic diversity in various nematode species including *Meloidogyne* spp. (Cenis 1993; Castagnone-Sereno et al. 1994; Blok et al. 1997b). More recently it has been applied to *Bursaphelenchus* spp. (Metge and Burgermeister 2008) to determine the genetic relationships among 30 *B. xylophilus* isolates from USA, Canada, Japan, China, South Korea and Portugal. This work demonstrated the reduced genetic variation of introduced isolates as compared to native North American isolates. Intraspecific genetic variation within some species and the difficulty in scoring banding patterns makes this technique less useful in nematode diagnostics; however the technique is extremely useful for the development of sequence characterized amplified region (SCAR) markers.

Zijlstra et al. (2000) studied the identification of *M. incognita, M. javanica* and *M. arenaria* using SCARs based on a PCR assay. Three randomly amplified polymorphic DNA (RAPD) markers, OPA-12, OPB-06 and OPA-01, species-specific to the root-knot nematode species *M. arenaria, M. incognita* and *M. javanica*, respectively, were identified. After sequencing these RAPD-PCR products, longer primers of 18–23 nucleotides were designed to complement the terminal DNA sequences of the DNA fragments. This resulted in three pairs of species-specific primers (SCAR primers) that were successfully used in straightforward, fast and reliable PCR assays to identify *M. incognita, M. javanica* and *M. arenaria*. The variant SCAR markers can be amplified with DNA from egg masses, second-stage juveniles and females. This technique was also used to develop SCAR markers for *Meloidogyne* species causing damage on coffee in Brazil and used in multiplex PCR reactions allowed the detection of *Meloidogyne* mixtures (Randig et al. 2002b). SCAR markers also exist for *Heterodera glycines* and other cyst forming nematodes (Ou et al. 2008).

21.3.3 Non-PCR Methods

Apart from methodologies based on the amplification of a target DNA sequence, other molecular methods that are based on the hybridization between a specific DNA probe and the genome of the target nematode have also been investigated. In particular, the squash-blot procedure has been developed, where the nematode sample is simply crushed onto a membrane (without the need for preliminary DNA purification) and then hybridized with a specific probe. For example, using satDNA-based specific probes, this approach allowed discrimination of *M. chitwoodi* or *M. fallax* from *M. hapla*, or the detection of single *M. exigua* individuals. Identification by this method was possible using nematodes at any developmental stage, and even within root tissues (Castagnone-Sereno et al. 1999; Randig et al. 2002a). Interestingly, comparable results were obtained using either radioactive or digoxigenin-labeled probes, with no loss of detection sensitivity. Some refinements of

the approach were introduced with the reverse-dot-blot hybridization technique. Indeed, coupling PCR amplification of a target fragment and its further hybridization with membrane-bound species-specific oligonucleotide probes enabled unequivocal identification and simultaneously discrimination of various nematode species, e.g., seven *Pratylenchus* spp. (Uehara et al. 1999), or 13 common species of animal-parasitic strongyles (Traversa et al. 2007). However, although successfully applied to several nematode species, hybridization-based methods remain poorly used compared to PCR-based methods, most likely because the number of practical steps required are less user-friendly for diagnostics on a routine basis.

21.4 Soil PCR

Molecular diagnosis of PPNs is usually performed in combination with morphological examination, and methods are often designed for individual nematodes, typically juveniles or adults. Molecular diagnostics that do not require isolation of plant pathogens from soil are often used for the quantification or identification of fungi and bacteria. In recent years, there has been a growing interest in the development of similar methods for nematodes. Soil ecologists have designed molecular strategies for analysis of nematodes within soil faunal communities (Waite et al. 2003; Griffiths et al. 2006; Simmons et al. 2008). Unlike many bacteria and fungi, nematodes tend to be unevenly distributed in the soil, both spatially and temporally. This presents challenges in terms of the sampling strategy needed for detection, as well as the time and high cost of sample processing. Limitations on the amount of soil that can be processed by either commercial kits or laboratory methods put additional constraint on the size of samples that can be easily processed. Pooling of soil cores can be used to reduce the cost of processing while maintaining the desired sampling density (Griffiths et al. 2006).

Another strategy is to isolate the nematodes *en masse* from soil and then prepare the bulk DNA for PCR. Diagnostics were developed for root-knot nematodes using this approach. Genomic DNA was purified from nematodes that had been isolated from soil by Baermann funnel extraction, followed by centrifugal flotation (Qiu et al. 2006). Baermann extraction and the Whitehead tray methods rely on nematode motility and therefore only recover live nematodes. Conversely, passive methods such as sugar flotation do not discriminate against slower moving or dead nematodes. These factors should be taken into account when choosing an extraction method, since the selectivity of different recovery methods can influence the results (McSorley and Frederick 2004).

Hamilton et al. (2009) performed a direct comparison between DNA sequence-based identification and direct extraction and microscopy methods. In this study, DNA was extracted from soil using a commercial kit, followed by amplification of the small subunit 18S RNA with metazoan-specific primers. Sequence analysis of multiple cloned amplicons was used to identify the species present and their relative abundance. When compared with traditional sieving and counting, the molecular

approach gave a roughly similar assessment of nematode communities in different soil types, but with some important differences. Although much larger amounts of soil can be processed using the direct extraction method than with soil DNA purification kits, the recovery of individual taxa may not be uniform for all soil types. Moreover, while the sequence-based approach allows ready comparison of data across different studies, rare taxa may be missed if the number of clones analyzed is too small. Despite its broader emphasis on soil community structure, this study exemplified many of the technical challenges and experimental biases that are also relevant to the development of soil-based nematode diagnostics. It is likely that declining costs associated with next generation sequencing technologies will further expand the options for genetic characterization of nematode communities from soil and their molecular diagnostics.

Examples of truly soil-dependent molecular methods for PPN diagnosis are few, because the circumstances in which such analysis is practical or economical are relatively rare. However, the case of lesion nematodes on wheat in the Pacific Northwest represented a nearly ideal scenario in which to test the utility of soil PCR for nematodes (Yan et al. 2008). This study was motivated by the need for a reliable method to distinguish *Pratylenchus thornei* from *P. neglectus*. Rotation of wheat with biofuel crops such as canola led to increased populations of *P. neglectus* but not *P. thornei*, so accurate identification of these species was needed for implementation of rotation schemes that would keep both lesion nematodes at low levels. Even in the hands of a trained taxonomist, there are few reliable morphological characters to set these species apart. In this study, the PCR performance of DNA template produced from two commercially-available soil DNA extraction kits was compared with DNA extracted using less expensive in-house reagents and glass beads for mechanical disruption of nematode tissue. DNA template extracted from soil using 1 mm glass beads and an SDS-phosphate buffer, followed by treatment with polyvinylpyrrolidone (PVPP) to remove humic substances, performed as well in PCR tests as the commercial kits and at significantly reduced cost ($ 0.30 per reaction for the in-house reagents, versus $ 4.16 for the kits). The sensitivity and specificity of species-specific 28s rDNA primers for detecting *P. thornei* or *P. neglectus* was demonstrated in PCR reactions with template extracted from either sterile soil spiked with known amounts of each nematode or from soil samples taken from naturally-infested wheat fields. One reason for the success of this approach is that the threshold of detection for these nematodes (as low as 126 *P. thornei*/kg soil) was significantly less than the economic damage threshold for lesion nematodes in the Pacific Northwest (2,000 nematodes/kg soil). In this circumstance, the assay was sensitive enough to be used for disease forecasting and management. A comprehensive DNA-based testing service for nematodes of wheat, barley, and rotation crops was developed in Australia (Ophel-Keller et al. 2008). Procedures were designed to accommodate relatively large soil samples (500g) processed at high capacity (>160 samples/day). Diagnostics were developed for *H. avenae, P. neglectus, P. thornei,* and *Ditylenchus* spp., as well as for several fungal species. Unfortunately, specific details of the soil extraction and PCR methods are protected by confidentiality agreements, and therefore not publicly available.

In another study *Nacobbus* spp. was detected in soil using species-specific ITS rDNA primers (Atkins et al. 2005). After DNA extraction using a commercial kit, this method was sensitive enough to detect 15 J2 added to 0.5 g sterile soil. This equates to 3,000 J2/100 g soil, reportedly within the range of nematode densities encountered in South American soils. Detection of *Nacobbus* from several naturally infested potato fields was also successful, although the number of nematodes actually present was not confirmed by conventional extraction methods.

For some species that affect leaves, stems, bulbs or tubers, it is desirable to identify nematodes directly from infected plant parts. An assay based upon ITS1 rDNA was developed for detection and identification of *Aphelenchoides fragariae* from host plant tissues (McCuiston et al. 2007); the *Nacobbus* primers described above were also used to detect this nematode from potato tubers (Atkins et al. 2005). There have also been diagnostic assays developed for detection and identification of *Bursaphelenchus* spp. from the wood of infected trees (Leal et al. 2005, 2007).

21.5 Validation and Troubleshooting

Assay validation remains one of the most challenging aspects of nematode molecular diagnosis. The specificity of primers and probes should be tested against as many populations of the target species as possible, and against closely related species that could result in false positives. The sensitivity of the assay must be appropriate for the diagnostic situation and the intended purpose. Appropriate controls must be run along with test specimens in order to ensure that an assay is performing as intended. Unfortunately, restrictions on the movement of quarantined species can make it difficult for labs to obtain the control specimens they need, especially populations from other countries. This problem can sometimes be solved by transporting dead nematodes in ethanol or DESS, a fixative that preserves morphological features of nematodes and is compatible with PCR (Yoder et al. 2006). Additionally, some rare or unculturable populations of great diagnostic interest may be available only in limited quantities. To the extent possible, integrated morphological and molecular archives of such specimens should be preserved (De Ley et al. 2005).

In some instances, specimens arrive for diagnosis in less than optimal condition, which can affect the success of DNA-based diagnostic methods. It may be possible to increase the amount of genomic DNA using whole genome amplification; however, this approach may not be successful if contamination by fungi or bacteria is present. Ideally, an assay should be validated using independent biological and experimental replicates. This is an essential aspect of assay development. In actual practice, however, there may be times when the available material is minimal. In cases when results are weak or cannot be replicated for lack of material, it may be preferable to classify a diagnosis as “inconclusive” rather than “negative”. Despite careful assay validation and controls, the diagnostic environment for nematodes is constantly evolving. We cannot predict the occurrence of new species or evolution of population variants that do not fit with established protocols. It pays to remain

vigilant of any results that fall outside the norm, and to recognize that diagnostics are only as good as the foundation of knowledge built from prior identifications.

21.6 Future Research and Perspectives

21.6.1 New Developments in Real-Time PCR, qPCR

Standard techniques for counting and identifying nematodes are time-consuming, difficult, at times inconclusive and reliant on qualified experts. For a number of years, real-time quantitative PCR has been applied in animal parasitology for the detection of infectious organisms such as *Plasmodium*, *Toxoplasma*, *Leishmania* and *Neospora*. Real-time qPCR is advantageous in that it is quick to perform and requires no post-amplification manipulations. qPCR can be used to count genome numbers and to study levels of gene expression (Bell and Ranford-Cartwright 2002). Real time qPCR probes have been developed for a number of plant-parasitic and quarantine nematodes such as the pinewood nematode, *B. xylophilus*, based on the ribosomal DNA internal transcribed spacer region. The assay was highly specific and sensitive, detecting as little as 0.01 ng of *B. xylophilus* DNA. The real-time PCR assay also successfully detected *B. xylophilus* in field samples, and it should be very useful for quarantine purposes (Cao et al. 2005). More recently, a real-time PCR approach based on the Taqman chemistry has been developed using satDNA as a probe, that could unambiguously detect as few as one single nematode or 1 pg of target DNA (François et al. 2007). In addition, the procedure allowed the detection of *B. xylophilus* directly in infested wood samples without the need for a laborious DNA extraction method (François et al. 2007), which opens new perspectives for the management of this invasive pest.

qPCR techniques have been used extensively for the quantitative detection of *Globodera* spp. Based upon conventional multiplex PCR amplification of ITS rDNA (Bulman and Marshall 1997), Bates et al. (2002) developed semi-quantitiative real time PCR assays for detection of the potato cyst nematodes (PCN) *Globodera pallida* and *G. rostochiensis*. Proportions of the two species in mixed samples were determined through comparison of melting peak heights with standard peaks from known species ratios. Using a modified DNA extraction method from cysts with multiplex real-time PCR, Quader et al. (2008) further demonstrated robustness of the ITS rDNA assays for identification of several worldwide PCN populations. A similar approach was also applied by Bačić et al. (2008) for detection of PCN populations from Serbia. *Globodera pallida* and the beet cyst nematode *Heterodera schachtii* were successfully identified using real time PCR with SYBR green dye and melting peak detection (Madani et al. 2005). In another study, EvaGreen dye and melting curve analysis were used to simultaneously identify *G. pallida*, *G. rostochiensis* and the tobacco cyst nematode (TCN) *G. tabacum* (Madani et al. 2008). This study employed locked nucleic acid (LNA)-modified primers and TaqMan probes to increase hybridization affinity and efficiency. Nakhla et al. (2010) achieved discrimination of PCN and TCN using somewhat different mul-

tiplex primers and TaqMan probes in a two-tube real-time PCR assay. The assay was validated with a wide range of PPN species and populations to demonstrate specificity; reaction sensitivity was confirmed for one or many juveniles or single cysts. In another study, TaqMan probes were used to distinguish *G. rostochiensis*, a quarantined pest, from *G. artemisiae,* a non-regulated cyst nematode of the *Asteraceae* (Nowaczyk et al. 2008). These recent developments provide valuable new options for detection and identification of PCN that are urgently needed for surveys and regulatory purposes.

Other studies have applied fluorogenic probes and real-time PCR for detection of root-knot nematodes (Ciancio et al. 2005; Zijlstra and Van Hoof 2006), and virus-vector trichodorid nematodes (Holeva et al. 2006). In an ambitious project, Berry et al. (2008) attempted to simultaneously detect three PPNs, *M. javanica*, *P. zeae* and *X. elongatum* associated with sugarcane in South Africa using real time qPCR. However, when using three primer sets in multiplex reactions, competition between DNA species was found to interfere with reaction specificity, with *M. javanica* competing with *P. zeae* and *X. elongatum*. This study highlights the importance of primer specificity and potential limitations of the technique for detecting nematodes in mixtures.

21.6.2 Pathogen Microarrays

A number of DNA diagnostic microarrays exist for plant pathogens including viruses (Boonham et al. 2007), fungi (Szemes et al. 2005), and bacteria (Frédérique et al. 2010). While microarray technology has been used frequently for examination of PPN gene expression in the context of nematode interactions with their host plants (see reviews by Caillaud et al. 2008; Klink and Matthews 2009), to date few nematode DNA microarrays have been developed for diagnostics of PPNs. The use of padlock probes in multiplex pathogen arrays is discussed in Szemes et al. (2005), in which the multiplex diagnostic system was validated using genomic DNAs of characterized isolates and artificial mixtures which included the temperate *Meloidogyne* species *M. hapla*. In order to investigate the potential of microarray-based methods for diagnostics of PPNs, François et al. (2006) developed a DNA microarray to detect the quarantine nematode *M. chitwoodi*. Capture probes were designed from nematode SCAR and satellite DNA sequences. The method allowed the specific detection of *M. chitwoodi* in pure and spiked samples. The results highlight the potential of DNA microarray technology for nematode diagnostics. However with any DNA microarray technology the main obstacle is the design of high quality probes.

21.6.3 Nematode Bioindicator Microarrays

Nematodes are an important component of the soil ecosystem, exerting profound effects on organic matter decomposition, nutrient transformation and energy transfer (Yeates and Bongers 1999; Neher 2001; Coleman et al. 2004). The indicator value

of nematodes in relation to soil functioning or soil properties is well illustrated, with omnivore–predator nematodes being most sensitive to environmental disturbance (Bongers and Bongers 1998; Georgieva et al. 2002). In recent years, the use of nematodes as bioindicators has attracted increasing attention in relation to the remediation of contaminated soils by industrial pollutants, heavy metals and the excessive use of pesticides and fertilizers. Due to the recent interest in DNA barcoding (see below) and nematode genomics, the number of DNA sequences available has expanded tremendously. In the future it should be possible to create DNA microarrays from bioindicator species. Nematode species of the Orders *Dorylaimida* and *Monochida* such as *Aporcelaimellus obtusicaudatus*, *Clarkus papillatus*, *Ecumenicus monohystera*, *Mylonchulus brachyuris* and *Anatonchus tridentatus*, are frequently found in cultivated and natural soils, and the presence or absence of these nematodes can indicate alterations in the soil system.

21.6.4 Barcoding

DNA barcoding entails the use of a short DNA sequence from a particular region of the genome to provide a signature, or 'barcode', for identifying species. Although molecular markers have been successfully used in molecular systematics for decades, the concept of DNA barcoding as a mean to provide a universal identification for all eukaryotic forms of life on Earth has emerged only recently (Hebert et al. 2003a). A desirable *locus* for DNA barcoding should be standardized (so that large databases of sequences for that *locus* can be developed), present in most of the taxa of interest and provide large variation between species yet a relatively small amount of variation within a species. Although several *loci* have been suggested, a 648-bp region of the mitochondrial cytochrome c oxidase subunit I (COI) gene was proposed as a potential barcode for animals and many other eukaryotes. *COI* was experimentally validated for routine species-level diagnoses across broad divisions of the animal kingdom, including more than 13,000 congeneric species pairs representing 11 phyla (Hebert et al. 2003b). Indeed, as of 2009, the Barcode of Life Database (http://www.barcodinglife.org) has collected COI sequences from over 620,000 specimens from over 58,000 animal species (Ausubel 2009). However, there has been no systematic survey of COI sequences among nematodes, due to a lack of phylum-wide primers and poor PCR success rates (De Ley et al. 2005). Alternatively, sequences from nuclear ribosomal DNA genes have proven to bear sufficient information for efficient diagnosis in taxonomic groups with very different lifestyles and habitats, including PPNs (Powers 2004; Holterman et al. 2009), soil nematodes (Floyd et al. 2002) or marine nematodes (De Ley et al. 2005). Nevertheless, DNA barcoding using COI sequences was shown to be a reliable tool for specific identification of filaroid nematode species (Ferri et al. 2009). Clearly, a universal system of barcoding for the phylum Nematoda has yet to be developed (and further accepted among nematologists). Moreover, DNA barcoding has been criticized for both theoretical and practical reasons, and it has recently been suggested that the development and maintenance of DNA barcoding as a taxonomic

tool for nematodes (at least) requires a permanent interaction between traditional and molecular approaches, according to the principles of integrated taxonomy (e.g., Ferri et al. 2009).

21.7 Conclusions

There will soon be increasing demand for diagnostic assays that can be used "on site" by extension nematologists and technicians from plant protection services. At present, the most practical format for such an assay would likely consist of a quantitative or semi-quantitative real-time PCR procedure, based on a set of genus- or species-specific primers, that has been implemented into portable equipment. As mentioned above, some studies already provided promising results, although they still require rigorous appraisal and evaluation, concerning both the isolation of minute amounts of DNA from biological samples and the removal of substances inhibitory to the amplification process. Finally, the cost for equipment and consumables will probably be the deciding factor in acceptance of any molecular diagnostic tool for field application.

High-throughput sequencing technologies are currently giving access to the whole-genome sequences of an expanding panel of organisms, including PPNs. In particular, the genomes of the root-knot nematodes *M. incognita* and *M. hapla* have recently become available (Abad et al. 2008; Opperman et al. 2008), and other sequencing projects are in progress. Together with the increase in computation capacities and methodological improvements, this accumulation of sequence data now offers new opportunities for comparative genomics. From a practical point of view, such an approach will no doubt aid the discovery of DNA sequences specific for any taxonomic level of interest (i.e. genus, species, population), that would become new targets for molecular identification/diagnostic purposes.

Although DNA-based taxonomy of PPNs indeed represents a powerful approach, it would nevertheless be misleading to consider it as the panacea for achieving the goal of species identification. In particular, considering the huge amount of still undescribed nematode species, strong expertise in traditional (morphological) taxonomy will be needed to delineate species boundaries and to assign a taxonomic binomial to a molecular signature. Enhancing the linkage between molecular and morphological data to provide a more integrated taxonomy (De Ley et al. 2005; Ferri et al. 2009) will undoubtedly strengthen the systematic exploration of the phylum Nematoda.

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Chapter 22
Breeding for Nematode Resistance: Use of Genomic Information

Bernard Caromel and Christiane Gebhardt

22.1 Introduction

Plant parasitic nematodes are an important threat to cultivated plants, in addition to pathogenic viruses, bacteria, fungi, oomycetes and chewing insects. Sedentary nematodes, belonging to the genera *Meloidogyne* (root-knot nematodes) and *Heterodera* or *Globodera* (cyst nematodes), are the most damaging plant nematodes. These soil borne sedentary nematodes invade the plant's roots, where each animal establishes a feeding site. This is an intricate developmental process, which leads to major changes in root structure and metabolism, for example, the formation of syncytia (Jones and Northcote 1972) or root galls ('knots') containing giant cells (see Chaps. 4 and 5). Damage is caused to the plant by the loss of nutrients diverted to the nematode over several weeks for the completion of its own life cycle. Cyst nematodes can survive for many years in the soil without the appropriate host plant, due to the formation of cysts (the remains of the female nematode's body surrounded by a hardened cuticle), which encapsulate the next generation of juveniles. The juveniles hatch in response to host cues and begin a new life cycle. Chemical control of nematodes is difficult, due to the limited effectiveness of nematicides and their toxicity to other soil organisms. Infested soils cannot be cultivated for long time with susceptible crops. Nematodes are therefore frequently quarantine pathogens.

Harnessing crops with genetic resistance against nematodes is therefore an important component in plant breeding programs. This requires the phenotypic screening of germplasm to identify sources of resistance, the introgression of resistance factors, which are often found in the crop plant's wild relatives, into adapted genetic backgrounds and then the combination of resistance with other agronomic traits such as yield and quality. This is a very time consuming process, particularly for nematode resistance. Resistance to nematodes is assessed by inoculation of test plants with specific pathotypes or by planting in nematode infested soil. After four

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to twelve weeks, the newly formed cysts or galls are observed and eventually quantified. This phenotypic evaluation is time consuming, costly and often ambiguous. Alternative methods for handling nematode resistance in breeding programs are therefore of interest.

Research on the genetic and molecular basis of nematode resistance over the last 20 years, which is reviewed in this chapter, has opened two new options, which can facilitate breeding for resistance. First, the genetic dissection of qualitative and quantitative nematode resistance based on molecular linkage maps has resulted in DNA-based markers, which are closely linked with resistance loci of various origins. Such markers can assist the introgression, combination and maintenance of specific resistance genes in breeding materials, thereby reducing the number of phenotypic tests and increasing the precision of selection. Second, a small number of genes conferring resistance to nematodes have been cloned and characterized at the molecular level. These genes can be transferred to adapted, susceptible cultivars by genetic transformation, thereby avoiding the lengthy cycles of introgression breeding from wild species. The DNA sequence of cloned nematode resistance genes can also be used to develop gene specific markers, the diagnostic value of which is no longer limited by recombination events.

22.2 Mapped Nematode Resistance Genes and QTLs

Since the development of molecular markers, numerous major genes and Quantitative Trait Loci (QTL) involved in nematode resistance have been located on genetic maps from several crop species. The number of resistance genes or QTLs mapped in a particular species reveals both the importance of nematodes as a threat to that species and the research effort dedicated to this species. The highest number of publications concerning the genetics of resistance to nematodes are available for soybean, cereals, and species from the *Prunus* genus and the Solanaceae family (potato, tomato, pepper, etc.).

22.2.1 Mapped Genes and QTLs for Resistance to Nematodes in Solanaceae

22.2.1.1 Genes and QTLs Acting on Resistance to Potato Cyst Nematodes

Cyst nematodes (*Globodera* sp.) are a major pest of potato (*Solanum tuberosum* ssp. *tuberosum*) in temperate climates. Two species in particular are a threat for potato production: *G. rostochiensis* and *G. pallida*. Several major genes conferring complete resistance to *G. rostochiensis* have been described whereas resistance to *G. pallida* is mainly quantitative due to oligogenic inheritance. The first nematode resistance gene mapped in potato was the *Gro1* gene. The *Gro1* locus on chromo-

some VII originates from the wild potato species *S. spegazzinii* (Barone et al. 1990). This was followed by the mapping of the *H1* gene from *S. tuberosum* ssp. *andigena* on to chromosome V (Gebhardt et al. 1993; Pineda et al. 1993) and of the *GroV1* gene from *S. vernei* to a similar position on the same chromosome arm (Jacobs et al. 1996). *Gro1, H1* and *GroV1* all confer resistance to *G. rostochiensis*. *H1* was first introduced in the potato cultivar 'Maris Piper' in 1966 and many cultivars now carry this gene (Cook 2004; Ross 1986). As these major genes proved efficient and were not been readily overcome by *G. rostochiensis* populations, very little work was done on the genetic dissection of quantitative resistance to *G. rostochiensis*, with the exception of Kreike et al. (1993), and quantitative resistance was not used in breeding schemes. In 2000, half of the potato area in Britain was planted with cultivars resistant to *G. rostochiensis* (Trudgill et al. 2003), most of them carrying the *H1* gene. The *H1* gene is one of few success stories for introgression of durable genetic resistance against a pathogen in the cultivated potato. However, repeated use of cultivars containing *H1* has led to selection for *G. pallida* in many parts of the UK.

Breeding for resistance to *G. pallida* is much more difficult than breeding for resistance to *G. rostochiensis*. The genetic determinism of resistance, originating from five relatives of potato, *S. tuberosum* ssp. *andigena, S. spegazzinii, S. vernei, S. sparsipilum* and *S. tarijense* has been investigated. *Gpa2*, originating from *S. tuberosum* ssp *andigena* is the only major gene for resistance to *G. pallida* and was located to potato chromosome XII (Rouppe van der Voort et al. 1999). This gene, which confers resistance to a very restricted range of *G. pallida* populations, was introgressed in *S. tuberosum* ssp *tuberosum* due to its close vicinity with the *Rx* gene, which confers extreme resistance to *Potato Virus X* (Rouppe van der Voort et al. 1999). Because of its narrow pathotype spectrum, the utility of the *Gpa2* gene in potato growing areas is low. High level and broad spectrum resistances to *G. pallida* in potato are oligogenic and are determined by one major effect QTL and one or few minor effect QTLs. Major effect QTLs have been mapped on the short arm of chromosome IV in *S. tuberosum* ssp. *andigena* ($GpaIV^{s}_{adg}$, Bradshaw et al. 1998; Bryan et al. 2004; Moloney et al. 2010), on the long arm of chromosome XI in *S. tarijense* ($GpaXI^{l}_{tar}$, Tan et al. 2009), and on chromosome V in the three species *S. spegazzinii* (*Gpa*, Kreike et al. 1994, *GpaM1*, Caromel et al. 2003), *S. vernei* (*Gpa5*, Bryan et al. 2002; Rouppe van der Voort et al. 2000, *Grp1*, Rouppe van der Voort et al. 1998) and *S. sparsipilum* ($GpaV_{spl}$, Caromel et al. 2005). *Grp1* also provides resistance to *G. rostochiensis*. Minor effect QTLs acting on *G. pallida* resistance mapped on chromosomes IV, VI, VIII and XII in *S. spegazzinii* (Caromel et al. 2003; Kreike et al. 1994), on chromosome XI in *S. sparsipilum* (Caromel et al. 2005), and in *S. andigena* (Bryan et al. 2004) and on chromosome IX in *S. vernei* (Bryan et al. 2002; Rouppe van der Voort et al. 2000) and/or *S. tarijense* (Tan et al. 2009). Epistatic interactions between major effect and minor effect QTLs have been detected in *S. tarijense* and *S. sparsipilum*. In *S. sparsipilum*, the joint presence of resistance alleles at both QTLs boosts the resistance reaction which takes the form of a strong necrosis around the head of the nematode (Caromel et al. 2005).

Many new sources of resistances to potato cyst nematodes have recently been discovered in the Potato Commonwealth Collection (Castelli et al. 2003). The genetic determinism of these new sources has not been published to date.

Potato cyst nematodes also attack tomato. The *Hero* gene, introgressed from the wild tomato species *Solanum pimpinellifolium*, confers a high level of resistance to all pathotypes of *G. rostochiensis* and partial resistance to *G. pallida*. This gene was mapped on the short arm of tomato chromosome T4 (Ganal et al. 1995). This genomic area is collinear with the potato chromosome IV region where the $GpaIV^{s}_{adg}$ QTL has been mapped (Bradshaw et al. 1998; Bryan et al. 2004; Moloney et al. 2010).

22.2.1.2 Genes and QTLs Acting on Resistance to Root-Knot Nematodes

The root-knot nematode species most frequently encountered on Solanaceous crops are *Meloidogyne incognita, M. arenaria* and *M. javanica* in Mediterranean, tropical and equatorial climates, and *M. hapla, M. fallax* and *M. chitwoodi* in temperate climates. Resistance to root-knot nematodes originates from the wild relatives, *S. arcanum* and *S. peruvianum* (both formerly belonging to the *Lycopersicon peruvianum* complex) for tomato (Ammati et al. 1985; Veremis et al. 1999; Veremis and Roberts 1996a, b, c, 2000), and *S. bulbocastanum* and *S. sparsipilum* for potato (Brown et al. 1996; Kouassi et al. 2006). In pepper, resistance originates from the most cultivated species *Capsicum annuum* as well as from related species (Djian-Caporalino et al. 2006). Six *Me* genes from *C. annuum* clustered in a 28 cM region on pepper chromosome 9 (Djian-Caporalino et al. 2007). The three *Mi* genes mapped in tomato are located on chromosome 6 (Mi-1 and Mi-9; Ammiraju et al. 2003; Klein-Lankhorst et al. 1991; Messeguer et al. 1991) and on chromosome 12 (*Mi-3*; Yaghoobi et al. 1995). In potato, resistance genes to *M. fallax* ($MfaXII^{s}_{spl}$; Kouassi et al. 2006) and to *M. chitwoodi* (*Rmc1*; Brown et al. 1996) are located on chromosomes XII and XI respectively. Interestingly, the genomic regions to which nematode resistance genes have been mapped on potato chromosome XII, tomato chromosome T12 and pepper chromosome P9 are collinear (Djian-Caporalino et al. 2007). Thus, the majority of genes conferring resistance to root-knot nematodes in Solanaceous crops could have descended from a common ancestor.

All currently available tomato cultivars resistant to root-knot nematodes (*M. incognita, M. arenaria* and *M. javanica*) possess the *Mi-1* gene from *S. peruvianum*. However, the resistance conferred by *Mi-1* is broken at temperatures above 28°C (Williamson 1998). The *Mi-9* gene from *S. arcanum*, located in the same genomic region as *Mi-1*, has the same spectrum of action than *Mi-1* but is temperature-insensitive. The repeated use of *Mi-1* in tomato breeding has selected *Meloidogyne* sp. populations, which are able to develop on plants carrying this resistance gene (Castagnone-Sereno et al. 2001; Jacquet et al. 2005; Kaloshian et al. 1996; Tzortzakakis et al. 2005). Other *Mi* resistance genes are difficult to introgress into cultivars due to the sexual barrier between the wild and the cultivated species (Williamson 1998). Of the other *Mi* genes, *Mi-3* is particularly interesting because it is effective at high

temperature against *M. incognita* strains virulent on *Mi-1* (Yaghoobi et al. 1995). Interspecific crosses followed by embryo rescue methods have recently allowed the introgression of *Mi-3* resistance into a *S. lycopersicum* genetic background (Moretti et al. 2002).

22.2.2 Mapped Genes and QTLs for Resistance to Nematodes in Soybean

22.2.2.1 Genes and QTLs Acting on Resistance to Soybean Cyst Nematodes

The soybean cyst nematode (SCN), *Heterodera glycines* (HG), is the most damaging pest of soybean (*Glycine max*). *H. glycines* populations are classified into HG groups, depending on their multiplication rates on soybean indicator lines (Niblack et al. 2002). Hundreds of soybean cultivars carry some resistance to SCN, but none are highly resistant to all HG groups. The genetic bases of the resistance are narrow. Two accessions, Peking and PI88788, are in the pedigree of most resistant cultivars bred in the United States (Concibido et al. 2004; Klink and Matthews 2009). As a review on mapping of SCN resistance has recently been published (Concibido et al. 2004), this report will outline only the major characteristics of soybean resistance to SCN.

The genetic architecture of the analysed SCN resistance sources is mainly polygenic, involving dominant and recessive additive QTLs and epistatic interactions between QTLs and/or genetic background. Several mapping studies have been published over the last fifteen years localizing additive or epistatic SCN resistance QTLs on 19 of the 20 soybean linkage groups (Concibido et al. 2004; Wu et al. 2009a). Two loci are particularly noticeable: the *rhg1* locus on linkage group G and the *Rhg4* locus on linkage group A2. Resistance conferred by *rhg1* is recessive and resistance at this locus was found in six soybean accessions. Resistance conferred by *Rhg4* is dominant and resistance at this locus was found in at least three soybean accessions. Epistatic interactions between both loci and between *rhg1* and another QTL contribute significantly to resistance (Meksem et al. 2001a; Wu et al. 2009a). Resistance QTLs originating from the wild ancestor of domesticated soybean, *G. soja*, mapped to different locations than the QTL originating from *G. max*. Introgression of QTLs of *G. soja* would probably improve the genetic diversity of resistance QTLs in soybean germplasm (Kabelka et al. 2005; Wang et al. 2001; Winter et al. 2007).

22.2.2.2 QTLs Acting on Resistance to Root-Knot Nematodes

Resistance to root-knot nematodes (RKN) in soybean is quantitative. Major effect QTLs conferring resistance to *M. incognita* are located on linkage groups O and M, and minor effect QTLs have been mapped on linkage groups G and C2 (Fourie et al. 2008; Ha et al. 2007a; Li et al. 2001; Shearin et al. 2009; Tamulonis et al. 1997a). The effect of the major QTL on linkage group O is reduced on race 2 of *M. incog-*

nita (Fourie et al. 2008). In contrast to the quite broad spectrum of the majority of RKN resistance genes in tomato (Williamson 1998), soybean resistance QTLs to *M. arenaria* and *M. javanica* mapped on different chromosomes to the QTLs acting on resistance to *M. incognita* (Tamulonis et al. 1997b, c).

22.2.2.3 QTLs Acting on Resistance to the Reniform Nematode

The reniform nematode (*Rotylenchulus reniformis*) attacks many species of cultivated plants including cotton and soybean. In cultivated soybean (*G. max*), resistance to the reniform nematode is quantitative. Three QTLs on linkage groups B1, L and G are involved in resistance to *R. reniformis* (Ha et al. 2007b). The QTLs on linkage groups B1 and G act in an epistatic manner.

22.2.3 Mapped Genes and QTLs for Resistance to Nematodes in Cotton

Despite the high commercial value of cotton, papers reporting mapped genes or QTLs acting on nematode resistance in cotton have only been published recently. The root-knot nematode *M. incognita* and the reniform nematode *R. reniformis* are the most damaging nematodes in cotton. A review by Starr et al. (2007) summarises the knowledge on nematode management in this species.

22.2.3.1 Genes and QTLs Acting on Resistance to Root-Knot Nematodes

Resistance to *M. incognita* in cotton (*Gossypium hirsutum*) originates mostly from a small number of unrelated *G. hirsutum* sources. The first source of resistance was the upland cotton accession Auburn 623 RNR. Resistance from this accession is oligogenic, with a major effect QTL always detected on chromosome 11 and minor effect QTLs on chromosomes 14 or 7 (Shen et al. 2006; Ynturi et al. 2006). In a second, unrelated resistance source, the Acala accession NeemX, the resistance is conferred by a major recessive gene *rkn1* (Wang et al. 2006; Wang and Roberts 2006). The effect of *rkn1* is enhanced by an epistatic interaction with a linked locus, in an interspecific cross involving G. *barbadense* (Wang et al. 2008). Thus, for all resistance sources identified to date, high resistance levels to *M. incognita* in cotton require a genetic factor located on chromosome 11 (Niu et al. 2007).

22.2.3.2 QTLs Acting on Resistance to the Reniform Nematode

In cotton, no resistance to the reniform nematode, *R. reniformis*, was found in the tetraploid cultivated species *G. hirsutum*. Resistance was therefore introgressed

from the wild diploid species *G. longicalyx* and *G. aridum*. The resistance gene from *G. aridum* mapped to a region on chromosome 21 that is duplicated on chromosome 11 (Romano et al. 2009), where a resistance gene from *G. longicalyx* has also been mapped (Dighe et al. 2009). Interestingly, this region of cotton chromosome 11 also carries RKN resistance genes and QTLs (Niu et al. 2007; Shen et al. 2006; Wang et al. 2006; Ynturi et al. 2006).

22.2.4 Mapped Genes and QTLs for Resistance to Nematodes in Grasses

Several cyst nematodes attack cereals around the world, including *Heterodera avenae, H. filipjevi* and *H. latipons*. A review on the importance of damage caused by nematodes in temperate and Mediterranean climates has recently been published (Nicol and Rivoal 2007). A lot of breeding effort has been put into producing wheat and barley cultivars resistant to *H. avenae*. *H. avenae* is the most widely distributed cereal cyst nematode (CCN) under temperate and Mediterranean climates. It causes severe yield losses in wheat and barley. Several pathotypes of *H. avenae* have been described (Andersen and Andersen 1982).

22.2.4.1 Genes and QTLs Acting on Resistance to Cyst Nematodes

Several genes (*Cre* genes) and QTLs acting on resistance to *H. avenae* have been mapped in wheat. Two of these (*Cre1* and *Cre8*) originate from hexaploid wheat (Safari et al. 2005). The others originate from related *Aegilops* species and were introgressed via chromosome addition or substitution lines (Barloy et al. 2007; Delibes et al. 1993; Eastwood et al. 1994; Jahier et al. 2001; Ogbonnaya et al. 2001a; Romero et al. 1998). Even though these genes have been mapped as major dominant genes, they express partial resistance to *H. avenae*. Moreover, the effectiveness of these genes depends on the nematode pathotype. Thus, against Australian pathothypes, *Cre3* is the most efficient gene in reducing the number of cysts, followed by *Cre1* and then *Cre8* (Ogbonnaya et al. 2001a; Safari et al. 2005), whereas *Cre3* exhibits a lower resistance than *Cre1* against a Spanish pathotype (Montes et al. 2008). *Cre1* and *Cre3* map to homeologous loci (de Majnik et al. 2003).

In barley, most genes conferring resistance to *H. avenae* mapped to the *Ha2* locus on chromosome 2H (Kretschmer et al. 1997), which is collinear to the *Cre1/Cre3* locus in wheat. A single additional resistance locus (*Ha4*) has been mapped on another barley chromosome (Barr et al. 1998) allowing pyramiding of *Ha2* and *Ha4* in the same cultivar.

No resistance to nematodes has been found in the widely cultivated rice species *Oryza sativa*, but accessions of the cultivated African rice species, *O. glaberrima*, have been described as resistant to cyst and root-knot nematodes (Plowright et al. 1999). *H. sacchari* is a cyst nematode which attacks sugarcane and rice. Lorieux

et al. (2003) mapped a major gene (*Hsa-1*^{Og}) on chromosome 11, which confers resistance to *H. sacchari* in a *O. sativa* x *O. glaberrima* interspecific progeny. The resistance gene, originating from *O. glaberrima* has been introgressed into the *O. sativa* genetic background.

22.2.4.2 Genes and QTLs Acting on Resistance to Root-Knot Nematodes

The cereal root-knot nematode, *Meloidogyne naasi*, can reduce yield of wheat and barley. It is widely distributed in temperate climates. No resistance has been found in cultivated species (Person-Dedryver and Jahier 1985). Resistance has been introgressed into wheat from the wild relative *Aegilops variabilis* (Yu et al. 1995). Wheat translocation lines carry the resistance gene *Rkn-mn1* on chromosome 3BL, and molecular markers flanking and cosegregating with *Rkn-mn1* have been designed (Barloy et al. 2000).

In rice, *Meloidogyne graminicola* is the most damaging root-knot nematode. A high level of resistance to *M. graminicola* has been found in the cultivated African rice species, *O. glaberrima* (Plowright et al. 1999), and partial resistance has been found in some *O. sativa* cultivars. No resistance gene from *O. glaberrima* has been mapped to date. Six low effect QTLs ($R^2 < 11\%$), that confer tolerance to *M. graminicola*, have been mapped on rice chromosomes 1, 2, 6, 7, 9, 11 (Shrestha et al. 2007). Plants carrying these QTLs support *M. graminicola* reproduction without significant loss of yield. The QTL on chromosome 11 is not collinear to the *Hsa-1*^{Og} from *O. glaberrima*, conferring resistance to *H. sacchari*.

22.2.4.3 QTLs Acting on Resistance to Root-Lesion Nematodes

Resistance to the root-lesion nematodes *Pratylenchus thornei* and *P. neglectus* is a quantitative trait in wheat. QTLs acting on resistance have been mapped on the six wheat chromosomes 1B, 2B, 3B, 4D, 6D and 7A (Schmidt et al. 2005; Toktay et al. 2006; Williams et al. 2002; Zwart et al. 2005, 2006). QTLs located on chromosomes 2B, 6D and 7A have been reported to confer resistance to both root-lesion nematode species *P. thornei* and *P. neglectus*.

22.2.5 Mapped Genes for Resistance to Root-Knot Nematodes in Prunus Species

Several nematode genera attack *Prunus* species, the most damaging of these belong to the *Meloidogyne* genus. Several genes acting on resistance to one or more RKN species have been mapped in several *Prunus* species, including Myrobolan plum (*P. cerasifera*), Japanese plum (*P. salicina*), peach (*P. persica*) and almonds (*P. dulcis*).

RKN resistance genes originating from plum (*Ma* and *Rjap*) mapped to a collinear region of LG7 (Claverie et al. 2004a; Lecouls et al. 1999; Yamamoto and Hayashi 2002). They confer resistance to *M. incognita, M. javanica, M. arenaria*, and *M. floridensis*. In almond, the *RMja* gene, conferring resistance to *M. javanica* and *M. arenaria* but not to *M. incognita* nor to *M. floridensis*, also mapped to LG7, in a collinear position to the plum resistance genes (Van Ghelder et al. 2010).

RKN resistance genes originating from peach (*RMiaNem* and *RMia557*) mapped to linkage group 2 (Claverie et al. 2004a; Gillen and Bliss 2005; Lu et al. 1999). These genes confer resistance only to *M. incognita* and *M. arenaria* and to some isolates of *M. javanica* (Claverie et al. 2004a; Esmenjaud et al. 2009). Resistance to RKN in apricot appears to be polygenic (Esmenjaud et al. 2009).

Most *Prunus* species (peach, almond, apricot, and plum) are sexually compatible, and fruit-producing cultivars are grafted on rootstocks. Thus, RKN resistance genes found in plum, peach and almonds can be combined in rootstock cultivars for each fruit-producing species (Esmenjaud et al. 2009; Nyczepir and Esmenjaud 2008).

22.2.6 Mapped Genes and QTLs for Resistance to Nematodes in Sugar Beet

Heterodera schachtii, the beet cyst nematode (BCN), is widely distributed in temperate climates. It has a broad host range and attacks many species from the Chenopodiaceae and Brassicaceae families. Monogenic resistance to *H. schachtii* was introgressed in sugar beet (*Beta vulgaris*) from the wild relatives *B. procumbens* and *B. webbiana*, in monosomic addition lines. RFLP mapping studies identified nematode-resistant beet lines resulting from translocation events between the wild chromosome segment carrying the nematode resistance genes $Hs1^{pro\text{-}1}$, $Hs1^{web\text{-}1}$ and $Hs2^{web\text{-}7}$, and the cultivated beet chromosome IV (Heller et al. 1996). Further studies indicated that the translocated alien chromosome fragment carrying the $Hs1^{pro\text{-}1}$ gene, may carry additional genes involved in BCN resistance (Sandal et al. 1997).

Sugar beet is also attacked by several species of RKN. An accession of the wild beet *B. vulgaris* ssp. *maritima*, resistant to six species of RKN, was used to introgress resistance in cultivated sugar beet (Yu et al. 1999). Molecular markers linked to the RKN resistance gene have been designed (Weiland and Yu 2003). A review on the use of genetic resistance to control pests in sugar beet has recently been published (Zhang et al. 2008).

22.2.7 Mapped genes and QTLs for Resistance to Nematodes in Other Species

Resistance to a range of RKN species has been mapped in a small number of other crop species. Resistance to *M. incognita* has been mapped in sweetpotato

(Cervantes-Flores et al. 2008; McHaro et al. 2005) while resistance to *M. arenaria* has been mapped in peanuts (Burow et al. 1996; Chu et al. 2007) and resistance to *M. javanica* in carrot (Boiteux et al. 2000). Finally, resistance to two less widespread RKN species has been mapped: to *M. exigua* in coffee (Noir et al. 2003), and to *M. trifoliophila* in *Trifolium* (Barrett et al. 2005).

The citrus nematode *Tylenchulus semipenetrans* is distributed worldwide in citrus growing areas. Resistance to *T. semipenetrans* was introgressed into a citrus rootstock (Swingle Citrumelo) via intergeneric hybridization between grapefruit (*Citrus paradisi*) and *Poncirus trifoliata*, a close relative of *Citrus* (Hutchison 1974). A major effect QTL originating from Swingle Citrumelo, accounts for more than 50% of the resistance to the citrus nematode (Ling et al. 2000).

Xiphinema index is a migratory root ectoparasite which belongs to the Dorylaimida. In addition to causing direct damage to the root system, it is the vector of Grapevine Fanleaf Virus, the most severe viral disease of grape. Resistance to *X. index* was introgressed from *Vitis arizonica* into the cultivated species, *V. vinifera*. A major effect QTL on chromosome 19 of grape is responsible for almost 60% of the resistance to *X. index* (Xu et al. 2008).

22.3 Molecular Marker-Assisted Breeding for Resistance to Nematodes

With the publication of papers describing DNA-based markers linked to nematode resistance genes or QTL, marker-assisted breeding (MAB) for nematode resistance has become possible. However, compared to the number of publications reporting markers linked to nematode resistance genes or QTLs, there are few publications reporting the use of these markers in breeding programs. One explanation for this disparity might be that many new nematode resistance genes have only been mapped in experimental populations, often in progeny of wild species or in interspecific crosses, which are quite remote from advanced breeding materials. In such 'exotic' materials, the linked markers are diagnostic for resistance only in the descendants of the cross used for mapping but lack general diagnostic value in multiparental advanced breeding populations. Moreover, where a molecular marker indeed proves valuable in commercial breeding programs, this fact is usually not reported in the scientific literature. However, MAB for nematode resistance has many potential advantages compared to conventional bioassays. First, the cost of a bioassay (up to 400 € per genotype for a test of quantitative resistance against a quarantine nematode) is much higher than the cost of a marker based assay. Second, bioassays require more time than MAB, taking somewhere between a few weeks when testing monogenic resistance, or up to several months when testing for quantitative resistance. Finally, bioassays for quantitative resistance require sufficient plant material to perform several replications. For example, a standard resistance bioassay for *G. pallida* in potato requires at least five tubers. These five tubers are usually only available at least two years after sowing of the seeds issued from a

cross. During this time, the first steps of selection occur, mainly on tuber maturity or tuber appearance traits. If a resistance QTL is unfavorably linked to genes or QTLs involved in these tuber traits, genotypes carrying the resistance allele at the QTL will be discarded before the resistance bioassay is performed.

The first nematode resistance gene tracked with a marker was the *Mi-1* gene, which confers resistance to RKN in tomato. *Mi-1* was selected using the linked isozyme acid phosphatase marker (APS-1) (Medina-Filho and Stevens 1980; Rick and Fobes 1974). This isozyme marker was then converted to a DNA-based marker (Aarts et al. 1991) and a more closely linked DNA marker, REX-1, was developed and used in MAB (Williamson 1994, 1998). Despite the fact that *Mi-1* was introgressed from *S. peruvianum* into a *S. lycopersicum* background in the 1940s, the molecular marker REX-1 was found to be diagnostic in advanced breeding lines fifty years later. The absence of recombination between REX-1 and *Mi-1* is due to the inversion of a chromosomal segment of 650 kb between *S. lycopersicum* and *S. peruvianum*, allowing a nearly perfect association between REX-1 and *Mi-1* (Seah et al. 2004). With the molecular characterization of the *Mi* gene (Milligan et al. 1998; Vos et al. 1998, see below) new markers were developed from the gene sequence.

Further examples of MAB have been reported in potato for resistance to cyst nematodes (Achenbach et al. 2009; Gebhardt et al. 2006; Moloney et al. 2010; Sattarzadeh et al. 2006) and to root-knot nematodes (Zhang et al. 2007). In soybean (Concibido et al. 1996; da Silva et al. 2007; Ha et al. 2007a; Li et al. 2009; Meksem et al. 2001b; Noel 2004) and wheat (Barloy et al. 2007; Ogbonnaya et al. 2001b; William et al. 2007), MAB is used to select lines carrying resistance to cyst nematodes. In *Prunus*, markers detected in the vicinity of root-knot nematode resistance genes have been used as diagnostic tools in subsequent crosses (Esmenjaud 2009; Lecouls et al. 2004).

22.4 Genes Underlying Resistance to Nematodes

22.4.1 Six Nematode Resistance Genes Molecularly Characterized to Date

Plant genes conferring qualitative resistance to pathogens (*R* genes) respond to specific determinants of an invading pathogen, via direct or indirect recognition of effector molecules encoded by pathogen avirulence (*Avr*) genes (see also Chaps. 13 and 15). After recognition of the *Avr* gene product, the *R* gene activates signaling pathways that result in disease resistance (Jones and Dangl 2006). A common feature of receptors that recognize pathogen effectors is the leucine rich repeat (LRR) domain. The majority of cloned resistance genes encode proteins carrying a LRR in their C-terminal part and also containing a central nucleotide binding site (NBS). Depending on their N-terminal region, NBS-LRR proteins can be subdivided in

TIR-NBS-LRR proteins if they contain a domain sharing homology to the Drosophila Toll and Mammalian Interleukin-1 receptor, or CC-NBS-LRR proteins if they contain a putative coiled-coil or leucine zipper region. Other classes of resistance proteins do not contain the CC, TIR nor NBS domain and possess an extracellular LRR domain at their N terminus, a transmembrane domain, and a cytoplasmic tail (Martin et al. 2003). The structure and function of resistance proteins have recently been reviewed (Caplan et al. 2008; Tameling and Takken 2008; van Ooijen et al. 2007). At the beginning of 2010 six genes conferring resistance to nematodes have been characterized at the molecular level.

The first cloned nematode resistance gene, *Hs1*$^{pro\text{-}1}$, was isolated in sugar beet and acts against the cyst nematode *H. schachtii* (Cai et al. 1997). This was followed by several genes from plants belonging to the Solanaceae family: *Mi-1.2*, conferring resistance to *Meloidogyne* species, was isolated from tomato (Milligan et al. 1998; Vos et al. 1998), its homologue, *CaMi*, was isolated from pepper (Chen et al. 2007), *Gpa2*, conferring resistance to *G. pallida*, was isolated from potato (van der Vossen et al. 2000), *Hero*, conferring resistance to *G. rostochiensis* and *G. pallida*, was isolated from tomato (Ernst et al. 2002) and *Gro1-4*, conferring resistance to the cyst nematode *G. rostochiensis*, was isolated from potato (Paal et al. 2004). *Mi-1.2, CaMi, Gpa2, Hero* and *Gro1-4* belong to the NBS-LRR class of resistance genes, whereas *Hs1*$^{pro\text{-}1}$ has a more unusual structure.

The *Hs1*$^{pro\text{-}1}$ gene was introgressed into sugar beet as an alien chromosomal segment from the wild species *B. procumbens* (Heller et al. 1996). *Hs1*$^{pro\text{-}1}$ was cloned using a positional cloning approach. The protein encoded by *Hs1*$^{pro\text{-}1}$ contains a putative N-terminal extracellular LRR region and a transmembrane domain. It does not have obvious similarities with other resistance genes. *Hs1*$^{pro\text{-}1}$ has been functionally validated using *Agrobacterium rhizogenes*. Hairy roots, regenerated from susceptible beet and expressing *Hs1*$^{pro\text{-}1}$ under the control of the strong, constitutive CaMV35S promoter, expressed the same resistance level to *H. schachtii* as resistant beet lines (Cai et al. 1997). *Hs1*$^{pro\text{-}1}$ is specifically expressed in syncytia of *H. schachtii* and is induced following the formation of the nematode feeding site (Thurau et al. 2003). McLean et al. (2007) reported that the primary published sequence of *Hs1*$^{pro\text{-}1}$ was truncated. Heterologous transformation of susceptible soybean lines with full length *Hs1*$^{pro\text{-}1}$ cDNA enhanced the resistance of the soybean host against the soybean cyst nematode *H. glycines* (McLean et al. 2007).

The tomato *Mi-1.2* gene was cloned simultaneously by two teams, using a positional cloning approach (Milligan et al. 1998; Vos et al. 1998). It belongs to the CC-NBS-LRR class of resistance genes. Interestingly, the *Mi-1.2* gene not only confers resistance to several root-knot nematode species, but also to the potato aphid *Macrosiphum euphorbiae* (Rossi et al. 1998), the tomato psyllid *Bactericerca cockerelli* (Casteel et al. 2006) and to the whitefly *Bemisia tabaci* (Nombela et al. 2003). *Mi-1.2* is constitutively expressed throughout the whole plant and this expression does not vary after inoculation by one of the target pathogens (Goggin et al. 2004; Martinez de Ilarduya and Kaloshian 2001). *Mi-1.2* is one member of a cluster of seven homologues, within 650 kb of the genome (Seah et al. 2004). Expression of *Mi-1* in tobacco or *Arabidopsis* does not confer resistance to *Meloidogyne* species

(Williamson and Kumar 2006), whereas expression of this gene in the more closely related eggplant (*Solanum melongena*) confers resistance to *M. javanica* but not to potato aphids (Goggin et al. 2006). The resistance conferred by *Mi-1.2* is ineffective at high temperatures (Dropkin 1969). Jablonska et al. (2007) demonstrated that *Mi-9*, a nematode resistance gene which is efficient at high temperature, is a homologue of *Mi-1.2*.

A *Mi-1.2* homologue has been cloned from a resistant accession of pepper, by a candidate gene approach, using degenerate primers based on the sequences of *Mi-1.2* and other resistance genes (Chen et al. 2007). This homologue, named *CaMi*, shares 99% identity with *Mi-1.2 at* the amino acid level. Because pepper is a species recalcitrant to genetic transformation, *CaMi* has been functionally validated by transforming susceptible tomato lines with the genomic fragment isolated from pepper: several independent transformed tomato plants exhibited high levels of resistance to *M. incognita*, confirming that *CaMi* is sufficient to confer resistance to this nematode species in tomato. The resistance spectrum of *CaMi* has not yet been investigated and it is not known whether this gene also confers resistance to potato aphid, tomato psyllids or whitefly. As the mapping of *CaMi* has not been reported, its location remains unknown. It would be interesting to know if *CaMi* is located in the pepper nematode resistance gene cluster on chromosome P9 (Djian-Caporalino et al. 2007), which is collinear to tomato chromosome T12, or whether it is located on chromosome P6, collinear to the *Mi-1.2* position on tomato chromosome T6 (Wu et al. 2009b). In potato, another *Mi-1.2* homologue (with 81% identity at the the amino acid level), located in the collinear region on the *S. bulbocastanum* genome, confers resistance to the oomycete *Phytophthora infestans* (van der Vossen et al. 2005).

The *Gpa2* gene, originating from *S. tuberosum* ssp. *andigena*, also belongs to the CC-NBS-LRR class of resistance genes. While *Mi-1.2* exhibits a broad resistance spectrum, resistance conferred by *Gpa2* in potato is restricted to a few populations of the potato cyst nematode *G. pallida*. Interestingly, *Gpa2* is highly similar (88% identity at the amino acid level) and is closely linked to the potato resistance gene *Rx* which confers resistance to *Potato Virus X* (PVX) (van der Vossen et al. 2000). Eight *Gpa2/Rx* homologues are present in an interval of less than 200 kb on chromosome XII in the diploid resistant parent from which *Gpa2* was cloned (Bakker et al. 2003). In *S. accaule*, a *Gpa2* homologue (named *Rx2*), with the same specificity as *Rx*, mapped on chromosome V (Bendahmane et al. 2000). The main differences between *Gpa2*, on one hand and *Rx* and *Rx2* on the other hand, reside in the LRR domain, which is a major determinant of specificity in NBS-LRR proteins (Caplan et al. 2008; Ellis et al. 1999). Due to its narrow pathotype spectrum, *Gpa2* is not a target for breeding or for creation of transgenic plants.

The *Hero* gene, characterized in tomato, confers resistance to both potato cyst nematode species, *G. rostochiensis* and *G. pallida*. It encodes a CC-NBS-LRR protein, with an unusual stretch of 22 negatively charged amino acids in the LRR domain. *Hero* is a member of a cluster of 14 paralogues distributed within 118 kb (Ernst et al. 2002). It is constitutively expressed in all plant tissues, but the expression level increases in roots following inoculation with cyst nematodes (Sobczak et al. 2005). The *Hero* expression level reaches a peak as the syncytium begins to

degenerate. Because *G. rostochiensis* and *G. pallida* are more damaging in potato than in tomato cultivation, transgenic potato lines carrying the *Hero* gene have been created. Unfortunately, the *Hero* gene was unable to confer resistance to cyst nematodes in potato (Sobczak et al. 2005).

The *Gro1-4* gene, originating from the potato relative *S. spegazzinii*, has been cloned following a candidate gene approach. It belongs to the second NBS-LRR subfamily, carrying a TIR domain at its N-terminus and is a member of a gene family of nine homologues. Eight of these homologues, including the functional *Gro1-4* gene are spread over a region of more than 450 kb on chromosome VII. The ninth homologue is located in a similar region to *Hero* on chromosome IV (Paal et al. 2004). The *Gro1* gene family was identified using a probe derived from the sequence of the NBS domain of the *N* gene (Leister et al. 1996). The *N* gene confers resistance to *Tobacco Mosaic Virus* (TMV) in tobacco and is located in a region collinear to potato chromosome XI. Therefore, the *Gro1-4* gene is more related to *N* (38% sequence identity) than to other nematode resistance genes (Paal et al. 2004). *Gro1-4* is constitutively expressed in uninfected roots, and expression of *Gro1* family members has been detected in all plant tissues.,The plant source of the *Gro1-4* gene exhibits a broad spectrum resistance to all known pathotypes of *G. rostochiensis*. Cloned *Gro1-4* confers resistance to the Ro1 pathotype of *G. rostochiensis* but its effectiveness on pathotypes other than Ro1 has not been tested.

22.4.2 New Resistance Genes

Progress has been made towards the identification of other nematode resistance genes in several plant species. High resolution mapping studies and/or candidate gene approaches have been reported to characterize resistance genes, in tomato and Myrobolan plum, for genes conferring resistance to root-knot nematodes, and in potato, soybean and wheat, for genes conferring resistance to cyst nematodes.

In tomato, Jablonska et al. (2007) demonstrated that the *Mi-9* gene, originating from *S. arcanum* and conferring resistance to *Meloidogyne* sp., is a *Mi-1* homologue located on chromosome 6. The identification of the functional homologue has not yet been reported. Once this has been achieved, a comparison of the sequences of both genes and the generation of chimeras between the homologues may explain why one gene is temperature-sensitive whereas the other one is not. The *Mi-3* gene, which maps onto tomato chromosome 12, originates from *S. peruvianum*. This gene is temperature-insensitive and also confers resistance to *Meloidogyne* strains which are virulent on plants carrying *Mi-1* (Yaghoobi et al. 1995). Yaghoobi et al. (2005) mapped *Mi-3* in a genetic interval of less than 0.25 cM. The authors estimated the physical distance corresponding to this interval to be 25–30 kb. However, as the physical mapping was performed on a BAC library from *S. lycopersicum*, a new BAC library from *S. peruvianum* will have to be used to isolate the *Mi-3* resistance allele.

In Myrobolan plum, a high resolution mapping study allowed chromosome landing on a single BAC clone carrying the *Ma* gene for resistance to several *Meloido-*

gyne species (Claverie et al. 2004b). Further recombinant analysis and BAC sequencing identified a cluster of three TIR-NBS-LRR genes, one of which is probably the *Ma* gene (Esmenjaud 2009). Functional validation of these three candidate genes is in progress. Assuming these experiments are successful, the *Ma* gene will be the second nematode resistance gene belonging to the TIR-NBS-LRR class.

In potato, most of the major genes or major effect QTLs involved in nematode resistance have been mapped onto chromosome V. Cloning of several of these is in progress. A recent study (Achenbach et al. 2010) demonstrated that this chromosome was previously misoriented (Dong et al. 2000) and here we use the new orientation as defined by Achenbach et al. (2010). The *H1* gene, mapped on the short arm of chromosome V, confers resistance to the cyst nematode *G. rostochiensis*. Using a progeny of 1,209 genotypes, and information from an ultra high density map of potato (van Os et al. 2006), Bakker et al. (2004) mapped the *H1* gene to an interval less than 1 cM. On the long arm of chromosome V, major effect QTLs acting on resistance to *G. pallida* only (Bryan et al. 2002; Caromel et al. 2003, 2005; Kreike et al. 1994; Rouppe van der Voort et al. 2000) or to *G. pallida* and *G. rostochiensis* (Rouppe van der Voort et al. 1998) have been mapped. Using a progeny of 1,536 individuals, Finkers-Tomczak et al. (2009) mapped the *Grp1* major effect QTL in an interval of 1.08 cM. Even with such huge progeny, the authors were not able to separate the resistance to the two *Globodera* species, conferred by the *Grp1* locus. Thus, this dual specificity may be conferred by a single gene or by two closely linked genes. The $GpaV_{spl}$ major effect QTL, in combination with the $GpaXI_{spl}$ low effect QTL, confers almost complete resistance to *G. pallida* (Caromel et al. 2005). $GpaV_{spl}$ and *Grp1* are collinear. Taking into account the effect of both $GpaV_{spl}$ and $GpaXI_{spl}$ *QTLs,* it has been possible to map the $GpaV_{spl}$ QTL as a major gene, in a 0.8 cM interval in the original progeny of 239 genotypes (B. Caromel, unpublished results). By increasing the size of the progeny to 1,632 genotypes $GpaV_{spl}$ was mapped to an interval of 0.12 cM. The sizes of the progenies used to map *Grp1* and $GpaV_{spl}$ were similar, but the resolution obtained for the $GpaV_{spl}$ map was higher. This better resolution was due to higher recombination rates resulting from meiosis in the pure *S. sparsipilum* resistant clone, compared to the recombination rates occurring in the complex interspecific clone used as resistance source by Finkers-Tomczak et al. (2009).

The strategy used to characterize cyst nematode resistance genes in wheat has been based on a candidate gene approach, using a NBS-LRR coding sequence. Sequences have been isolated from the *Cre3* locus (Lagudah et al. 1997). Derived sequences were further used by the same team to tag other nematode resistance genes in wheat and barley (de Majnik et al. 2003; Seah et al. 1998, 2000). Functional demonstration of the role of these NBS-LRR sequences in nematode resistance has not yet been reported.

In soybean, the *rhg1* and *Rhg4* locus, acting on resistance to *Heterodera glycines*, have been extensively studied. Receptor-like kinase (RLK) sequences have been patented as candidate genes for both loci (Hauge et al. 2001; Lightfoot and Meksem 2000), but functional evidence for the role of RLK in nematode resistance has not yet been reported. In fact, further studies suggest that *rhg1* is a "multigenic" QTL, comprising an RLK, an unusual laccase, and a 46.1 kDa hypothetical transporter protein (Iqbal et al. 2009; Lightfoot et al. 2008; Ruben et al. 2006).

22.5 Breeding for Durable Resistance to Nematodes

There are many practical issues that need to be considered when breeding for nematode resistance. Even for major genes, differences in resistance levels have been noticed depending on the genetic background of the host (Jacquet et al. 2005; Mugniéry, personal communication). These differences could be explained by unmapped genetic factors acting additively or epistatically on resistance (unmapped QTLs). Several genes and QTLs have been overcome by certain nematode populations or are population-specific (Kaloshian et al. 1996; Montes et al. 2008; Rouppe van der Voort et al. 1997). Thus, cultivars with resistance to all populations of a given nematode species are more likely able to control this species over long period of time.

It is important to accumulate several QTLs or one major gene and QTLs in order to broaden the resistance spectrum of resistance genes or major effect QTLs and to increase durability. Indeed, the pathotype spectrum or the durability of resistance to nematodes, in plants carrying single genes or major effect QTLs, are usually weaker than those of the resistance sources (Turner et al. 2006). This reflects a partial transfer of genetic factors involved in resistance in the selected plants. Evaluation of the spectrum of resistance in individuals carrying different QTL combinations gives an indication of the potential durability of such QTL combinations. In the wild potato relative *S. sparsipilum*, resistance to *G. pallida* is conferred by one major ($R^2 = 76\%$) and one minor ($R^2 = 12.7\%$) effect QTL (Caromel et al. 2005). We evaluated the resistance level conferred by the four QTL combinations on eight populations of *G. pallida* originating from four European countries and from New Zealand. In plants carrying the resistance allele at the single major effect QTL the number of newly formed cysts that developed varied between two and fifty, depending on the nematode population, while in plants carrying resistance alleles at both QTL, this number never exceeded four cysts (Caromel 2004; Caromel et al. 2008).

In other pathosystems, Brun et al. (2009) and Palloix et al. (2009) demonstrated that the durability of major genes is higher in a genetic background carrying minor resistance QTLs than in a fully susceptible genetic background. Furthermore, Palloix et al. (2009) showed that growing cultivars with monogenic resistance promote further evolution of pathogens allowing adaptation to complex resistances combining the major gene and QTLs.

Even though quantitative phenotyping is more labour intensive than qualitative phenotyping, the resistance to nematodes can be evaluated by counting the numbers of galls or eggs or the numbers of newly formed cysts, in individuals of a plant progeny. Together with a genetic map, these quantitative data allow QTL detection. In several species, evenly distributed markers are available to build extensive genetic maps and the sequences of whole genomes, for the most studied species, will also help in designing new makers for mapping experiments. With a progeny of 150–300 individuals and appropriate detection methods, low effect QTLs can be detected even in the presence of major effect QTL (Caromel et al. 2005; Tan et al. 2009; Wu et al. 2009a). Markers flanking major and minor QTLs can further be used to assist the breeding process.

Another alternative to select for durable resistance is to select for plant resistance genes recognising nematode effectors encoded by genes which are under high selective pressure. Mutation in such genes would probably affect the fitness of the new nematode isolate, which would be counter-selected. In plant-virus interactions, Janzac et al. (2009) demonstrated that durability of resistance of major genes is a function of the selective constraints applied on the corresponding avirulence factors. Nematode avirulence genes are probably secreted or excreted into the plant tissue. With the increasing characterization of nematode-secreted molecules (Adam et al. 2009; Bellafiore et al. 2008; Davis et al. 2008; Jones et al. 2009; Patel et al. 2008; Roze et al. 2008; Sacco et al. 2007), selective pressure on the corresponding genes can be evaluated (Sacco et al. 2009). Transient expression of the products of the constrained nematode secreted genes, in plant tissues originating from a collection of plant genetic resources, would allow the identification of the corresponding resistance genes, as it has been shown for *Phytophthora infestans* avirulence products in potato (Vleeshouwers et al. 2008).

22.6 Conclusions

The past twenty years have seen substantial progress in the genetic dissection and molecular characterization of plant resistance to nematodes, thanks to the molecular genetic tools that became available to plant geneticists around 30 years ago (Tanksley 1983). Some of the results of this research have been translated in commercial breeding programs. We are confident that this process will continue.

Accumulating evidence suggests that major resistance genes or QTLs need to be introgressed together with low effect QTLs to build cultivars with durable resistance. Our next challenge is to accurately detect such low effect QTLs. This implies the need to consider all resistance as quantitative (by counting nematodes or galls), to genotype and phenotype larger progenies (typically 150–300 individuals) and to use enough replicates in the resistance assay to obtain a high heritability for the trait.

Combining accurate phenotyping on large progenies with high density marker coverage will allow detection and tagging of large and low effect QTLs involved in nematode resistance. The availability of whole genome sequences of the most important crop plants, a goal which is likely to be achieved in the near future, will provide new opportunities to identify, localize, diagnose and clone nematode resistance genes, providing breeders with a versatile instrument for precision resistance breeding.

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Chapter 23
Biological Control of Plant-Parasitic Nematodes: Towards Understanding Field Variation Through Molecular Mechanisms

Keith G. Davies and Yitzhak Spiegel

23.1 Introduction

Biological control is a subject that covers a huge area of application, bugs to control other bugs for application in medical, animal husbandry and agricultural situations. Stirling (1991) defined biological control of plant- parasitic nematodes as, 'A reduction of nematode populations which is accomplished through the action of living organisms other than nematode-resistant plants, which occurs naturally or through the manipulation of the environment or the introduction of antagonists'. In all these situations the underlying biology rests on the generic science pertaining to an interaction of one organism with another organism, either directly or indirectly, and it is sufficiently well understood that it can be used and be exploited to reduce an unwanted organism, in this case a nematode pest, to a number whereby it no longer has a detrimental effect on crop yield.

23.1.1 Historical Context

Prior to the use of nematicides, biological control was always viewed as a possible way to control plant-parasitic nematodes and as early as 1920 Cobb had suggested the use of predatory nematodes in sugar beet fields to control *Heterodera schachtii* (Cobb 1920). Further research suggested this approach was not economically viable (Thorne 1927). Several years later the focus changed to the use of predatory fungi and in particular the use of nematode trapping fungi (Linford 1937; Linford et al. 1938). However, these promising results were eclipsed by the spectacular results that began to be obtained by the newly developed nematicides (Stirling 1991). Throughout the later part of the twentieth century plant-parasitic nematodes have successfully been controlled by the use of these compounds, which were

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manufactured through the petro-chemical industry. More recently, with the increasing recognition of their toxicity and their harmful effects to both the environment and human health, legislation has been implemented to reduce their use. Although biological control has always tended to be in the shadow of plant resistance as an alternative to chemical control, it is again back on the agenda.

The focus of this renewed research effort into biological control was built on the observation that although mono-cropping can lead to an increase in plant-parasitic nematodes, with a concomitant reduction in crop yield, continued mono-cropping leads to the production of nematode suppressive soils (Gair et al. 1969). Subsequent research has shown that predatory fungi were responsible for this suppression (Kerry 1975; Kerry and Crump 1977; Kerry et al. 1982). The recognition that parasites were responsible for the development of nematode suppressive soils lead to the 'classical' approach to biological control where parasites were identified in nematode suppressive soils, mass produced and introduced into areas were nematode pests were abundant (Stirling 1991). Despite the best efforts of biological control scientists over the last 20–30 years, and although impressive results have been obtained using this approach, generally speaking this has not produced a robust method of nematode control which is consistent and broadly applicable. To date, there are relatively few commercially available products (Whipps and Davies 2000; Hallmann et al. 2009).

23.1.2 Technical Developments and Twenty-First Century Science

During the last decades of the twentieth century most research groups focused on attempting to isolate soil microorganisms, mainly fungi and bacteria, without devoting any special concern as to the mode-of-action of these potential microbial control agents. Early research that explored the mechanistic interactions between plant-parasitic nematodes and trapping fungi initially started out as fundamentally morphological in nature. This research later developed to examine biochemical aspects of infection processes and this approach continues today with an emphasis on molecular and genomic aspects (Tunlid and Ahrén 2010; Davies and Spiegel 2010). Similar trends can be seen within other research groups and this emphasises the interaction between the growth of biological control knowledge through research and its dependent relationship with technological development. For example, initial morphological studies exploited the use of light- and electron microscopy, which in turn expanded into investigations on 'ecological' aspects based on observations made using microbiological techniques such as selective media for the quantification of microbial populations in soil (Kerry and Hirsch 2010). The first decade of the twenty-first Century witnessed the use of a combination of approaches (molecular biology, biochemistry, genetics/genomic and developmental biology) aimed at getting a deeper understanding of such interactions (Davies and Spiegel 2010) and the challenge is therefore to bring a coherence to these approaches in an effort to develop robust control strategies that can work in the field situation.

23.2 Ecological Context

The most economically important plant-parasitic nematodes are root parasites of crops, where they inhabit the water films on soil particles and can feed on plant roots. This environment is highly fragmented and subject to continual wetting and drying. Because nematode movement is only possible between a water tension of 4.2 (a level at which plants are wilting) and 4.45 pF (Jones and Jones 1974), the hydraulic properties of the soil are critical and these are continually changing both spatially and temporally (Avendano et al. 2004).

23.2.1 The Soil as a Heterogeneous Environment

As large amounts of carbon are exuded by plant roots (Bekku et al. 1997) this carbon source is an important driver for determining the population dynamics of organisms occurring in the rhizosphere where nematodes reside as part of the community. Roots that permeate the soil are not homogeneous in their distribution but exploit nutrient rich patches which lead to changes in root morphology (Hodge 2006; Watt et al. 2006). Plant-parasitic nematodes, which are sensitive to the changing chemical properties of the rhizosphere, use these to locate plant host roots and therefore they are also patchily distributed (Costa et al. 2010).

23.2.2 Food-Webs and Multitrophic Interactions

Plant-parasitic nematodes form a part of a below-ground tritrophic interaction between plant roots and the soil/root microbial community in which the populations of phytonematodes are controlled by a number of different processes that include bottom up, horizontal, and top down control.

23.2.2.1 Bottom up Control

Bottom up control is where a population of nematodes is determined by the level of nutrients available. In the case of plant parasitic nematodes, which are obligate parasites of plants, this has involved a co-evolving arms race between host plants and parasitic nematodes. It is interesting that different species of plant-parasitic nematodes have adopted different life-cycle strategies that arguably form an evolutionary continuum from migratory ectoparasites, through to migratory endoparasites and on to sedentary endoparasites (Wood 1973; Yeates et al. 1993) exhibiting increasing specialisation. Interestingly not all plants are equally susceptible to nematode parasitism and different plants exhibit differing degrees of resistance and/or tolerance (Cook

and Starr 2006; Trudgill 1991) and of the most economically important nematodes, the cyst nematodes are regarded as considerably more host specific than the polyphagous root-knot nematodes. However, due care needs to be taken when investigating such parameters because the susceptibility of a particular plant to a particular nematode may be due to endophic fungi and or arbuscular mycorrhizal fungi (Hallmann and Sikora 2010) that may be playing an important role in bottom up control. The role of rhizobia on legumes and their ability to reduce nematode populations is much less clear with reports of some strains of rhizobia capable of inducing resistance in the root against plant-parasitic nematodes (Reitz et al. 2000; Mitra et al. 2004).

23.2.2.2 Horizontal Control

The maximum number of nematodes that can be sustained on a given root system represents its carrying capacity and the population increase can be modelled as a curve in which there is increased intra-specific competition for the available niches present on the root as the number of individual nematodes increases (Varley et al. 1973). Two species of nematode that reproduce continually and have overlapping ecological niches would also be subjected to intra-specific competition at low population densities. However, as populations of each species became larger they would increasingly be subjected to inter-specific competition, the outcome of which would be that one nematode species would be reducing the number of the other species (McSorley and Duncan 2004; Van den Berg and Rossing 2005). This control of one species of nematode by another represents horizontal control (Van der Putten et al. 2001). Competition experiments have been undertaken to evaluate the extent to which horizontal control plays a role in determining the population densities of *Meloidogyne maritima, Heterodera arenaria* and *Pratylenchus penetrans* in marram grass on costal sand dunes and it appeared that the outcomes were mediated by the host plant (Brinkman et al. 2005; de la Pena et al. 2008).

23.2.2.3 Top Down Control

The use of top down control is the strategy employed by most scientists envisaging the use of inundation of a selected natural enemy, usually a fungus or a bacterium, to control a particular plant-parasitic nematode. This method is based on the isolation of a nematode natural enemy that is parasitic on a particular nematode pest from a field that is nematode suppressive, mass produced and then deployed back to a field where the nematode is the cause of yield decline. Although this method has proved useful in pot tests and glasshouse studies, scaling up this approach to the field scale has been problematic leading to variable results. Analysis of the problem from an ecological perspective may therefore provide useful insights.

Field populations of nematodes usually occur as mixtures, both mixtures of different species and mixtures of different sub-species. Therefore in a nematode suppressive soil one would imagine that the natural microbial enemies of such a com-

munity would also contain a mixture of different species of microbial enemies and sub-species of microbial enemies. What follows *a priori* from the Red Queen Hypothesis (Van Valen 1973, 1976) suggests that a co-evolutionary arms race between hosts and parasites will lead to genetic diversity in which hosts are driven to produce genetic combinations which decrease parasite virulence, whereas parasites are driven to produce genetic combinations to maintain virulence. The results of such an arms race over extended time would be to allow infection and multiplication of the parasite without a detrimental effect on the host (Ebert and Hamilton 1996).

Co-evolutionary mathematical models predict that parasites that have a broad host range will tend to diverge, generating sub-populations with different host specializations. Because of the smaller size and quicker generation times of microbial parasites over their hosts each microbial subpopulation will co-evolve faster with its host, than a generalist population can evolve with different and variable hosts (Kawecki 1998). Therefore, at the initiation of such a host-parasitic arms race, it is not surprising that boom and bust population dynamics are the norm, and that the success of any selected microbial enemy from a particular pest nematode, deployed back into a field will produce inconsistent results. Investigations looking at microbial diversity and parasitism seem to support this view (see Sect. 23.4).

23.3 Molecular Approaches for Assessing Field Biodiversity

To gain insights into nematode suppression for the development of biological control strategies it is becoming increasingly obvious that knowledge of the diversity of the nematodes and their natural enemies, together with their temporal and spatial distribution, will play a key role. Conventional methods of identifying and quantifying both nematodes and their microbial enemies is time consuming and labour intensive and requires a high level of technical expertise, which, when all taken together, compromises the number of samples that can be processed (Costa et al. 2010). In addition, because of the heterogeneous spatial distribution of nematodes and their microbial enemies, and the limitations of soil sampling, most sampling techniques usually average the distribution of organisms and vary in their efficiency of their extraction (Ettema and Wardle 2002; McSorley and Frederick 2004). The population dynamics of host-parasite interactions occurring in the soil are therefore notoriously difficult to ascertain. The ability to quantify relationships between hosts and their parasites will be important in the development of biological control strategies and new molecular approaches have an important role to play.

23.3.1 Antibodies

Antibodies are a class of globular proteins produced by the lymphatic system of animals in response to its invasion by a foreign compound or organism. The function

of antibodies is to label these foreign bodies for destruction by the immune system. Antibody binding is very specific and this specificity can be used as a tool in the identification and diagnosis of pests and other organisms (Clark 1994). Antibodies have been shown to be useful in the identification and quantification of both plant-parasitic nematodes and their microbial enemies (Davies 1994). Antibodies can be made with variable degrees of specificity and theoretically can be made into a diagnostic tool for any level of molecular operational taxonomic unit (MOTU) required. Once produced, they are generally easy to use in a number of different formats from dip stick type assays and enzyme linked immune-assays through to fluorescence microscopy (Clark 1994). They can be used qualitatively as well as quantitatively and are therefore highly flexible (Davies et al. 1996). Monoclonal antibodies have been used to differentiate species of both root-knot nematodes (Davies and Lander 1992) and potato cyst nematode (Robinson et al. 1993) and can be used to quantify nematodes in soil samples (Davies and Carter 1995). Antibodies have also been successful in monitoring the nematophagus fungus *Pochonia chlamydosporia* and the bacterial parasite *Pasteuria penetrans* in the rhizosphere using immunofluorescence microscopy (Hirsch et al. 2001; Costa et al. 2006).

23.3.2 Phospholipid Fatty Acid Analysis (PFLA)

Fatty acids can be used in the classification and identification of microorganisms (O'Donnell 1994) and the interaction of nematodes with their fungal and bacterial communities has been studied using PFLA profiles (Bardgett et al. 1996; Denton et al. 1999). Changes in PLFA profiles reflect changes in the microbial community structure and are indicative of the biomass of the various groups of bacteria and fungi present (Bardgett et al. 1996). However, although the method is sensitive enough to monitor changes to some different bacterial groups, all fungi are measured by one particular fatty acid and this therefore limits their application (Denton et al. 1999). Thus, although PFLAs have been useful in monitoring changes in the microbial communities in relationship to environmental conditions (O'Donnell et al. 2005), they have limited use in assessing microbial diversity in any detail. However, PFLAs have been influential in studying the interactions and inter-connectedness of different trophic groups of nematode populations in a below ground grassland ecosystem (Bardgett et al. 1999).

23.3.3 DNA

Identification of organisms using DNA-based techniques on a routine basis dates back to the 1980s but was revolutionised by the invention of the Polymerase Chain Reaction (PCR) which relies on enzymatic amplification of a given DNA sequence (Saiki et al. 1985). A PCR-based method called denaturing gradient gel electrophoresis (DGGE) has enabled community fingerprints of large groups of microorgan-

isms to be obtained that provides a measure of their genetic diversity (Muyzer et al. 1993). Originally this technique was applied to bacterial communities in soil by amplifying the 16s rDNA gene and this combined with the sequencing of excised bands from the original gel can provide further taxonomic information (De Mesel et al. 2004). Similar approaches, using 18s rDNA have been applied to fungal communities but their quantification is complicated by the inability of the method to distinguish fungal spores from vegetatively growing hyphal fragments (van Elsas et al. 2000). DGGE analysis has been used to show that plant defence against nematodes was not mediated by changes in the soil microbial community (Wurst et al. 2009). Similar approaches have also been used to study nematode communities (Waite et al. 2003), but, because the number of MOTUs that can be used for describing nematodes is still very small the technique is currently only of limited value (Foucher et al. 2004; Wu et al. 2009).

Another PCR-based approach for characterising nematode communities has been the use of molecular barcodes using the 18s rDNA subunit which are designed to relate to MOTUs (Floyd et al. 2002; Blaxter et al. 2005). Systems are being constructed where it will be possible to use molecular barcodes to obtain sequences that can be used to interrogate online databases to obtain nematode identifications (Powers 2004). Indeed, using these approaches it has been possible not only to identify nematodes but also to construct phylum-wide evolutionary trees (Blaxter et al. 1998; Holterman et al. 2006) that recently have shed light on the evolution of parasitism amongst plant parasitic nematodes (Holterman et al. 2009). Taking these approaches forward it should be possible to elucidate and help understand nematode parasitic interactions such that light will be shed on the functional interactions between the organisms within the rhizosphere community.

23.3.4 Ecological Genomics

Studies of nematode control in their ecological context, particularly top down control which will probably give the most insights into organisms with biological control potential, will need all these molecular approaches to understand the necessary population dynamics that will underpin the development of nematode biological control strategies. The challenge for the biological control scientist is to integrate research in such a way that a model of the rhizosphere community can be developed. From the perspective of controlling plant -parasitic nematodes the new genome sequences (Abad et al. 2008; Opperman et al. 2008), and very shortly those of other nematode species, will be invaluable. This, together with the sequencing of both their plant hosts, and followed by the sequencing of their parasites, will allow the possibility of a multitrophic ecological genomic approach. Rhizospheric ecological studies will then allow the integration of different levels of organisation in which changes in the genetic makeup of a host with its parasites at the population level will be integrated with the specific mechanistic host-parasite interactions at the biochemical and molecular level (Zheng and Dicke 2008).

23.4 Towards Understanding Field Variation Through Molecular Mechanisms: Three Models

If the knowledge gained from sequencing genomes is to be integrated into understanding the population genetics of host-parasitic interactions, the identification of the functional genes involved in compatible and incompatible host—parasite interactions at the molecular level becomes indispensible. In microbial populations, as discussed above, such genes are likely to be involved in arms races that conform to the Red Queen Hypothesis which would lead to the prediction that these genes will have diversified (Van Valen 1973, 1976). Within the context of the soil environment, which we know is likely to be highly heterogeneous with respect to both hosts and parasites, this produces the challenge of species designation or MOTU identification within the context of the metapopulation structure (Fraser et al. 2009) present in the soil habitat. Therefore, understanding the functional mechanisms will be a necessary key to structuring the host-parasite metapopulations in soil and how these interact. This knowledge will be important for the applied biological control scientist to determine in which situations biological control as a management strategy will be successful. Three models are used to explore functional mechanisms of the interactions between potential biological control agents and plant-parasitic nematodes.

23.4.1 Arthrobotrys

Fungi have long been known to be parasitic on nematodes (Barron 1977) and more than 200 species of nematophagous fungi have been described (Tunlid and Ahrén 2010). One group of these are the nematode trapping fungi which, under conditions of limited nutrient availability and in the presence of nematodes, produce specialised organs that can capture nematodes (Barron 1977). The trapping of nematodes by fungi can be broken up into three distinct stages, starting with trap production, followed by recognition and capture and finally penetration and digestion. These three stages overlap to some extent and are not totally separate.

Many trapping fungi produce traps spontaneously, but as early as the 1950s it was shown that “nemin”, a mixture of compounds produced by the nematode, stimulated the formation of traps in *A. conoides* (Pramer and Stoll 1959; Pramer and Kuyama 1963). One particular group of trapping fungi, *Arthrobotrys* spp., have been studied in detail and microscopic studies of *A. oligospora* have shown that the production of traps and subsequent infection of nematodes occurs in a sequence of events, as outlined above, that is completed within 48–60 hours (Dijksterhuis et al. 1994). When a nematode makes contact with a trap a layer of extracellular fibrils that surround the traps become directed perpendicularly to the nematode cuticle and the fungus produces a penetration tube that is thought to be involved in production of hydrolytic enzymes that help to solubilise the cuticle. Then the nematode

becomes paralysed and the penetration tube forms an infection bulb from which hyphae develop and colonise the captured nematode (Tunlid and Ahrén 2010).

23.4.1.1 Lectin-Carbohydrate Interactions

Lectins are proteins that bind carbohydrates and function as recognition molecules (Sharon and Lis 2004). Early experiments based on sugar inhibition and red blood cell (RBC) assays suggested that a carbohydrate present on the surface of the nematode cuticle was interacting with an N-acetygalactosamine (GalNac)-specific lectin present on the surface of the traps in *A. oligospora* (Nordbring-Hertz and Mattiasson 1979). This specificity was confirmed with the finding that Type A RBCs, which contain a terminal GalNac, bound more easily than did Type B and O (Borrebaeck et al. 1984; Premachandran and Pramer 1984). However, the situation is complex, with different species of fungi revealing different binding specificities (Nordbring-Hertz and Chet 1988). Purification of these lectins (Rosén et al. 1992), and more recently the development of specific deletion mutants to see if they played and important role in nematode infection processes (Tunlid et al. 1999; Balogh et al. 2003), suggests they are part of a growing family of lectins that are specific to fungi (Tunlid and Ahrén 2010).

23.4.1.2 *Caenorhabdits elegans*, Genomics and Innate Immunity

Caenorhabditis elegans is a free-living nematode that has been used as a model organism for studying aspects of developmental biology and was the first multicellular animal to be sequenced. *Caenorhabditis elegans* is currently being used to study aspects of pathogenesis and innate immunity (Millet and Ewbank 2004; Darby 2005; Gravato-Nobre and Hodgkin 2005; Fuchs and Mylonakis 2006). The production of strains of *C. elegans* with cuticles that have different surface properties makes it particularly amenable as an experimental tool for dissecting the interactions between the cuticle and microbial pathogens. Many bacteria and fungi have been shown to be pathogens of *C. elegans* (Hodgkin and Partridge 2008). *Arthrobotrys* spp. are regarded as predators of *C. elegans* and bioassays have shown that these nematode trapping fungi differ in their ability to trap mutants of *C. elegans* that show altered lectin staining to the cuticle surface (Mendoza et al. 1999).

More recently, in a study of the infection of *C. elegans* with *Monacrosporium haptotylum*, the most highly up-regulated fungal gene was a subtilisin that was similar in sequence and expression profile to *PII* of *A. oligospora* and designated *spr1* (Fekete et al. 2008). However, another subtilisin *spr2*, although up-regulated at one hour, was then subsequently down- regulated at four and 16 hours only to be up-regulated again at 24 hours when the fungus was digesting the killed nematode (Tunlid and Ahrén 2010). Clearly, although these two genes were from the same family of subtilisins, their expression profiles would suggest they have very different functions.

Upon infection, *C. elegans* responds by the activation of several intracellular signalling pathways which led to the expression of defensive gene products or effector molecules (Millet and Ewbank 2004; Gravato-Nobre and Hodgkin 2005). There are several distinct signal transduction cascades including DAF2/DAF16, MAP kinase and the TGF-β pathway as well as several others none of which appear totally independent but form an integrated signalling network. However, analysis of the expression profiles of the worm showed no distinct shifts which could be detected between one and four hours after infection (Fekete et al. 2008; Tunlid and Ahrén 2010). The most responsive up-regulated gene in this study was *dod-3* which is regulated by the transcription factor DAF-16. Other genes that responded to *M. haptotylum* infection included *cnc-4* that codes for one of eleven caenacin peptides that are secreted in response to *Drechmeria coniospora* infection, and *lec-8* and *lec-10* that encode galactose binding proteins (Fekete et al. 2008) which are also up-regulated during bacterial infection (Mallo et al. 2002; Gravato-Nobre and Hodgkin 2005). Interestingly, C-type lectins which have also been implicated in the response of *C. elegans* to infection (O'Rourke et al. 2006) were all down-regulated.

23.4.2 Trichoderma

Trichoderma spp. are a group of free-living fungi that are common in the rhizosphere. They can colonise root-surfaces and even establish themselves in the root epidemis. Roots colonized by *Trichoderma* grow better and confer resistance to abiotic (Yedidia et al. 2001, 2003; Harman et al. 2004) and biotic stresses (Herrera-Estrella and Chet 1998; Harman 2006). Various mechanisms have been proposed for their biological control activity against root pathogens which include both direct interactions such as parasitism, antibiosis and competition and indirect mechanisms such as plant systemic induced resistance (Harman et al. 2004, 2006; Viterbo et al. 2007). It is thought that in situations where biological control occurs it is a result of a multi-mechanism action (Sharon et al. 2010). *Trichoderma* spp. have been tested for their ability to control plant- parasitic nematodes (Windham et al. 1989; Reddy et al. 1996; Khan and Saxena 1997; Rao et al. 1998; Meyer et al. 2000, 2001; Sharon et al. 2001; Spiegel et al. 2007) but with mixed success. Clearly, if multiple modes of action are involved, understanding the importance of each of these, from attachment and infection processes through to antibiosis, will help in developing control agents with improved potential.

23.4.2.1 Conidial Attachment

Conidial attachment to the nematode surface is a key step in the infection process and appears to show a degree of specificity as conidia will attach to gelatinous egg matrix (gm) and nematodes that had had contact with the gm but not with gm-free J2s and eggs (Sharon et al. 2007). Application of antibodies to the surface coat of in-

fective juveniles increased attachment of conidia to the nematodes. This increase in parasitism possibly occurs through the process of bilateral binding in which the antibody forms a bridge between the conidium and the juvenile and thereby increases the rates of infection (Sharon et al. 2010). Further experiments have suggested that a Ca^{2+}-dependent lectin-carbohydrate interaction may be involved (Sharon et al. 2007, 2009).

23.4.2.2 Lytic Enzymes

Trichoderma spp. produce a whole array of different enzymes that are involved in plant defense and in biological control processes (Viterbo et al. 2002a, b; Markovich and Kononova 2003; Steyaert et al. 2003). Most research has focused on enzymes that degrade carbohydrates such as chitin, and the chitinolytic system of *Trichoderma* has been studied in detail (Kubicek et al. 2001). Chitinases are a family of enzymes, including exochitinases and endochitinases, that degrade chitin (Sharon et al. 2010). Nutrient starvation of the fungus and the products of chitin breakdown induce the chitinolytic enzyme expression system, whereas glucose and other simple sugars tend to suppress chitinolytic expression (Viterbo et al. 2002a). Although proteolytic enzymes have been less studied, a serine protease (Prb-1) produced by *T. atroviride* strain IMI 206040 inserted as multiple copies exhibited biological control potential against *R. solani* and also exhibited improved control potential against root-knot nematodes (Sharon et al. 2007). It is perhaps not surprising that fungal egg parasites that need to disrupt the eggshell probably require a combination of both chitinolytic and proteolytic enzymes (Morton et al. 2004).

23.4.2.3 Antibiotics

Trichoderma species can produce a variety of antibiotic compounds, which may contribute to the biological control processes. The nature and roles of antibiotic peptides that belong to the peptaibol group have been intensively studied (Szekeres et al. 2005). Peptaibols generally exhibit antimicrobial activity, which is thought to arise from their ability to form pores in lipid membranes. A peptaibol synthetase gene has been cloned (Wiest et al. 2002) and further studies suggested that peptaibols are critical in the chemical communication between *Trichoderma* and plants as triggers of non-cultivar-specific defence responses (Viterbo et al. 2007). *Trichoderma virens* produces gliotoxin and gliovirin and also peptaibols (Howell 2003). The antifungal action of enzymes reinforced by synergism with antibiotics was comprehensively reviewed by Kubicek et al. (2001) and this again points to multiple modes of action combining to generate functionality. Nematicidal activity against *M. javanica* J2s was detected in *T. atroviride* culture filtrates (CFs) and the active component/s had low molecular weight (MW) and heat sensitivity. Immature eggs, exposed to CFs exhibited reduced hatching rates, whereas hatching of mature eggs was enhanced. The effect of CF on eggs was caused by both the enzymatic fraction, which contained

proteases and chitinases, and by the low molecular mass component/s. (Sharon, Chet and Spiegel unpublished). Appropriate candidates responsible for such nematicidal activity might be antibiotic peptides, such as peptaibols (Sharon et al. 2007).

23.4.2.4 Fungal Genomics

Utilizing genomic and metabolomic means to understand *Trichoderma*-plant disease interactions (Shoresh and Harman 2008; Woo et al. 2009) can further enhance the efficacy of *Trichoderma* as a biocontrol agent against nematode pests. Comparative genomics and maximum likelihood based methods showed that three different chitinase subgroups have expanded in copy number in *Trichoderma* species, indicating an important role of these chitinases during the mycoparasitism process (Ihrmark et al. 2010). Several regions and amino acids have been identified in four chitinase genes from different *Trichoderma* species, that are likely to determine functional properties playing an important role in the interaction between plant/pathogens and mycoparasites, such as substrate-specificity, processivity or pH-optima. These approaches can help lead to a better understanding of multitrophic nematode/plant/*Trichoderma* interactions and improvement of the biocontrol activity.

23.4.3 Pasteuria penetrans Genomics Applied to Culturing and Host Specificity

Pasteuria penetrans is an endospore-forming Gram positive bacterium that is part of a group of invertebrate parasites that infect nematodes and water fleas (*Daphnia* spp). One of the members of this group, *P. ramosa*, which is a parasite of *Daphnia*, has become a model for studying host-parasite co-evolution (Decaestecker et al. 2007) and innate immunity (Little et al. 2003; Kurtz 2005). Within the plant-parasitic nematode context, research over the last twenty years has focused on *P. penetrans* and its potential to be developed into a biological control agent of plant-parasitic nematode pests (Davies 2009). The *Pasteuria* group is phylogenetically closely related to *Bacillus* and *Clostridium* spp. (Charles et al. 2005) and there are a number of different species, the designations of which are determined by descriptions of morphology, host-range, life-cycle and, more recently, 16s rDNA sequence (Table 23.1). The two main features of this bacterium that have prohibited its commercial development are its host specificity and the inability to mass produce it *in vitro.*

23.4.3.1 Genomic insights for *In Vitro* Culturing

Mass production of *P. penetrans* has had to rely on *in vivo* culturing methods of which the majority are adaptations of the method developed by Stirling and Wachtel

Table 23.1 Species of *Pasteuria* so far described and their invertebrate host

Species designation	Host	Reference
P. penetrans	*Meloidogyne* spp.	Sayre and Starr (1985)
P. thornei	*Pratylenchus brachyurus*	Starr and Sayre (1988)
P. nishizawae	*Heterodera and Globodera* spp.	Sayre et al. (1991)
P. ramosa	*Daphnia* spp.	Ebert et al. (1996)
P. usage	*Belonolaimus longicaudatus*	Giblin-Davis et al. (2003)
P. hartismeri	*M. ardenensis*	Bishop et al. (2007)

(1980). Briefly, infective root-knot nematode juveniles are exposed to endospores so that each juvenile is encumbered with 5–10 endospores. These encumbered second-stage juveniles are then placed around the roots of a tomato plant at 25°C. After 6–8 weeks the tomato roots containing nematodes infected with spores are washed free of soil and air dried. The roots are then milled and can be used as inoculum for application to soil. Such milled tomato root can contain as many as 1.3×10^9 endospores per gram of root powder (Pembroke and Gowen, personal communication). Early attempts to grow *Pasteuria in vitro* produced very limited success. However, more recently, Pasteuria Bioscience LLC, Florida, has developed a system for *in vitro* mass culture of *Pasteuria* (Hewlett et al. 2004). Their system can mass produce different species of *Pasteuria* and *Pasteuria* usage has been cleared by the Environmental Protection Agency (2009) for the control of sting nematode (*Belonolaimus longicaudatus*) on golf courses in the United States.

One of the problems encountered in culturing *Pasteuria in vitro* has been getting vegetative colonies to sporulate and comparative genomics has helped to shed light onto this problem. Sporulation in *Bacillus subtilis* has been extensively studied and is dependent on a phosphorelay pathway (Burbulys et al. 1991) in which a phosphoryl group is transferred to the regulator Spo0F through a group of five kinases that are under environmental regulation. Like all known regulators of this type, Spo0F requires a divalent metal ion to be present in the conserved aspartic acid pocket in order for phosphorylation to occur (Grimshaw et al. 1998) and Mg^{2+} has been shown to be important for this process (Zapf et al. 1996). More recently it has been suggested that metal cations may play a role in the structure and function of Spo0F and its involvement in the initiation of sporulation (Mukhopadhyay et al. 2004). Investigations of the effects of the divalent cations Ca^{2+}, Cu^{2+}, Mg^{2+}, and Mn^{2+} on the structure and function of *B. subtilis* Spo0F showed that they bound to the aspartic acid pocket and that while Mg^{2+} supports phosphotransfer from the kinase KinA to Spo0F the copper cation Cu^{2+} inhibited their phosphotransfer (Kojetin et al. 2005).

Interrogation of the *Pasteuria* survey sequence revealed genes with a high degree of similarity to genes involved in sporulation (Bird et al. 2003) and this included Spo0F. Alignment of Spo0F between *B. subtilis, B. anthracis and B. thuringiensis* and *P. penetrans* showed that key amino acids are conserved across these species. From the results discussed above it was hypothesised that the presence of Cu^{2+}, at non-lethal concentrations in the sporulation media for *B. subtilis* and the related bacterium *P. penetrans*, might inhibit endospore formation while continuing to permit vegetative growth. Indeed, subsequent experiments revealed that the absence

of Cu^{2+} in the media resulted in an increased number of sporulating cells (Kojetin et al. 2005). This result suggests that the availability of Cu^{2+} could be used to induce vegetative cells to enter sporulation.

23.4.3.2 Host Specificity and Endospore Attachment

The first stage of infection of a nematode by *Pasteuria* is when an endospore adheres to the surface of the nematode cuticle, and as discussed above, the Red Queen Hypothesis (Van Valen 1973, 1976) would suggest that within the context of an arms race it might be expected that functional diversity would co-evolve. Indeed, one isolate of *P. penetrans* does not adhere equally well to all populations of root-knot nematodes and the process of initial attachment is not linked to the phylogenetic diversity of the nematode populations tested (Davies et al. 2001). Five different monoclonal antibodies (Mabs) were raised to *Pasteuria* strain PP1 endospores from a single host female. Studies using these Mabs showed that there was a diversity of surface types as different sub-populations of the endospores of strain PP1 were recognised by each of the five different Mabs (Davies et al. 1994). Baiting the PP1 population of endospores with different species and races of root-knot nematode and using the Mabs to characterise the endospores that were adhering to each of the different populations of nematode, showed that different sub-populations of the endospores were adhering to the different nematode populations. This indicates that immunological heterogeneity in the surface of the endospore was related to heterogeneity present in the outer surface coat of the different nematode populations (Davies et al. 1994). Similar differences were also observed in the recognition of the surface of endospores between isolates of *Pasteuria* from different geographical regions by the different Mabs. One particular Mab, PP1/117, appeared to recognise the concave surface of the endospore to a greater extent than the upper surface, revealing that the density of the antigen was greater on the concave surface; pretreatment of these endospores with sugars or glycolytic enzymes reduced the ability of the Mab to bind, suggesting the Mab was recognising a carbohydrate epitope (Davies and Redden 1997).

The biochemical mechanism by which endospores of *Pasteuria* adhere to the nematode cuticle is poorly understood; however, it has been proposed that carbohydrate-lectin type interactions may be involved (Davies and Danks 1993). In a genomic survey sequence of *Pasteuria*, collagen-like genes were identified (Davies and Opperman 2006) which were initially thought to be contaminating genes from the nematode itself. However, subsequent analysis showed them to be similar to collagen-like genes identified in other closely related *Bacillus* species (Sylvestre et al. 2002, 2003, 2005) and similar genes, that appear to be highly polymorphic, have also been found in *P. ramosa* (Mouton et al. 2009). It has been suggested that these genes form fibres on the surface of the endospore and are important in attachment to the nematode cuticle (Davies 2009). If collagen-like fibres on the surface of the endospore are involved in attachment, what are the molecules involved on the surface of the nematode? Mucins are a family of polypeptides associated with both

the innate and adapted immune systems (Strous and Dekker 1992), which possess a polypeptide backbone that is highly glycosylated with sugar side chains. Glycosylation is predominantly *O*-linked through *N*-acetylgalactosamine (GalNAc) to serine and threonine residues within a variable number of tandem repeats (VNTR) regions of the polypeptide core (Hicks et al. 2000; Theodoropoulos et al. 2001). Although there is no information on the role of mucins in plant-parasitic nematodes, it is interesting that the genes *muc-2, muc-3* and *muc-4*, that are members of the TES-120 family of proteins present in *Toxocara canis* (Tetteh et al. 1999) and are responsible for surface coat variation and have homologues in *C. elegans*, are also all present in various species of plant-parasitic nematodes (Davies 2009).

The investigations outlined above have been brought together in a hypothesis which suggests that collagen-like proteins on the surface of the endospore are interacting with mucin-like molecules present on the surface coat of the infective juvenile in what has been described as a 'Velcro'-like mechanism (Davies 2009). Although the details of this system are yet to be elucidated, it is clear that both collagen-like molecules in respect to the endospore and mucin-like molecules with respect to the surface coat of the nematode would perfectly fit within the context of Van Valen's (1976) Red Queen Hypothesis.

23.5 Future Developments

Over the last two decades since the publication of Stirling's (1991) book on biological control, biology has undergone a revolution that combines the technologies of inexpensive and rapid DNA sequencing together with '*in silico*' computing developments in the growing field of genomics. The purpose of this chapter has been to try and clarify some of the ecological constraints that have prohibited the development of biological control from being a robust strategy to control nematode pests, by exploring some of the molecular and biochemical mechanisms that underpin host-parasite interactions. These areas can be brought together in the new and rapidly developing field of *ecological genomics*, which is multidisciplinary and integrates a number of disciplines (Fig. 23.1). By taking such an approach genomics can act as a catalyst for enabling ecology to become an increasingly hypothesis-driven area of research, and bring unity to these formally disparate areas of study. Whereas formerly gene function was analysed through knocking out genes, and was a fairly blunt tool, comparative genomics can look at natural genetic variation across ecoclines and examine how this plays a role in the functional ecology of host-parasite interactions. Therefore, from following a simple reductionist approach that runs from the gene, up through proteins and biochemical pathways to cells, tissues and organisms, a more integrated, holistic approach can be undertaken in which downward causation from communities of populations selected by the environment, lead to the structuring of particular genotypes engaged in multitrophic interactions between plant-hosts, nematode-pests and their parasites (Davies and Spiegel 2010).

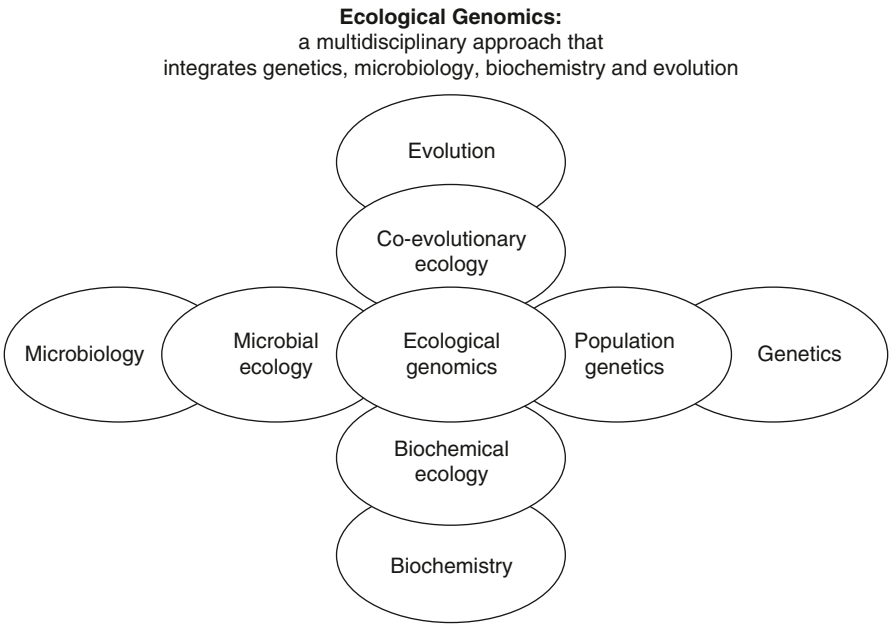

Fig. 23.1 Disciplines that need to be integrated within the context of ecological genomics as a strategy for the development of an understanding of biological control of nematodes. (Adapted from Straalen and Roelofs 2006)

23.5.1 Synthetic Biology

Very recently, the first microorganism was grown in which all the metabolic processes were controlled from an artificially constructed genome (Gibson et al. 2010). This arguably heralds the beginning of the epoch of synthetic biology, but the vision of designer organisms was first articulated in the 1970s when it was stated (Szybalski 1974):

> But the real challenge will start when we enter the synthetic biology phase of research in our field. We will then devise new control elements and add these new modules to the existing genomes or build up wholly new genomes.

This naturally begs the question as to whether the development of designer biological control agents is a future possibility. To date, the isolation, mass production and re-introduction of potential biological control agents has only met with limited success, but the idea of construction *de novo* of microorganisms with exactly the functionality required offers a multitude of possibilities. Although such approaches are in the medium to long term future, the development of this type of technology will not be beyond criticism, and many of the societal issues that confront the development of genetically modified crops will also need to be addressed regarding designer biological control agents (Davies and Spiegel 2010).

23.5.2 Commercial Development and Future Directions

In the relatively short term biological control will continue to offer products that will be of value to niche markets (Whipps and Davies 2000; Gowen et al. 2007; Hallman et al. 2009). However, the commercial market for the control of plant-parasitic nematodes in arable crops is immense and with the continued withdrawal of nematicides from the market due to legislation, the challenge to produce alternatives will remain. The continued study of biological control is important, not only for developing robust agents that can be applied and be expected to work with confidence, but also in the long term, in the epoch of synthetic biology, new combinations of functional traits can be assembled in novel systems that so far are unimaginable.

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Chapter 24
Nematode Resistant GM Crops in Industrialised and Developing Countries

Catherine J. Lilley, Tina Kyndt and Godelieve Gheysen

24.1 Introduction

Plant parasitic nematodes rarely inflict fatal damage on their host plants; nevertheless they are one of the most destructive uncontrollable biotic stresses on crops. The general symptoms of stunted growth, chlorosis and wilting that result from the disruption of water and nutrient transport have a considerable impact on agricultural crop yields (Bird and Kaloshian 2003). In addition, secondary pathogens can cause significant necrosis of infected root tissues in plants already weakened by nematode attack. The lack of clear, specific disease symptoms can lead some growers to underestimate nematode-related yield loss or to wrongly attribute it to the more obvious secondary diseases. Despite the likely under-reporting of crop losses and the lack of recent, comprehensive field data it is clear that plant-nematode parasitism is a severe constraint to productivity across temperate, tropical and subtropical crops (Chitwood 2003; Koenning et al. 1999; Sasser and Freckman 1987). A seminal survey in 1987 canvassed the opinions of nematologists around the world to reach an estimate of US $ 70 billion annual losses across 40 crops (Sasser and Freckman 1987). Adjusting for inflation, this figure was revised to US $ 125 billion in 2003 (Chitwood 2003) and a more detailed study relating the original loss estimates to crop value data for 2001 extrapolated the value to be US $ 118 billion per year (McCarter 2009). Yield losses in individual susceptible crops can reach 20% whilst those sustained by staple food crops of subsistence farmers in the developing world can be devastating (Atkinson et al. 1995; Koenning et al. 1999). Nematodes tend to have a greater impact in tropical and subtropical regions where there is wider species diversity, a lack of resources for control and management practices and conditions that are conducive to nematode population growth (De Waele and Elsen 2007).

Nematodes of the order Tylenchida are responsible for the majority of crop damage. Agronomically important species include migratory endoparasites such as *Radopholus* spp. and *Pratylenchus* spp. and the more specialised sedentary

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endoparasites, the root-knot nematodes (*Meloidogyne* spp.) and cyst nematodes (*Heterodera* and *Globodera* spp.). Root-knot nematodes are the major contributors to yield losses (Koenning et al. 1999; Sasser and Freckman 1987) and probably pose the greatest single biotic threat to resource-poor farmers in Africa and other developing world regions (Coyne et al. 2009). The high impact of this group of species can be attributed to its global distribution and combined ability to infect almost every cultivated crop. Cyst nematode species typically have a much narrower host range and those that attack potato (*Globodera rostochiensis* and *G. pallida*), soybean (*Heterodera glycines*) and sugar beet (*H. schachtii*) are of particular economic importance. The cereal cyst nematode complex of species are widespread pests of temperate cereal crops such as wheat, barley and oats (Subbotin et al. 2003). They are severe endemic pathogens of wheat and barley in India as well as in large areas of southern Australia and elsewhere (Rivoal and Cook 1993).

Management and control of plant parasitic nematodes is therefore essential to maintain economically viable production of crops in intensive agricultural systems and to ensure food security for resource-poor subsistence farmers. Integrated use of chemicals, resistant crop varieties and cultural and biological practices provides the most successful management strategy. Conventional intensive farming methods rely largely on the use of nematicides, but the range of compounds available continues to diminish as environmental and health concerns prompt legislation for their withdrawal in many countries. For example, 1,2-Dibromo-3-chloropropane (DBCP) was de-registered in the 1970s following the discovery that it caused male sterility, and methyl bromide, which causes ozone depletion, is undergoing a phased withdrawal under the United Nations' Montreal Protocol (http://ozone.unep.org/). In the European Union two effective nematicides, aldicarb and 1,3-dichloropropene, have already been withdrawn and the currently available compounds (fosthiazate, ethoprophos and oxamyl) are at risk from amendment to EU Directive 91/414/EEC. This directive may be centred on a hazard based approach that could phase these compounds from use over ten years or possibly more abruptly (Clayton et al. 2008). Chemical control imposes an additional financial burden on growers (Sorribas et al. 2005) and is not an option for many subsistence farmers. In some situations crop rotation can be an effective strategy to limit nematode infestation, particularly those species with a narrow host range such as many of the cyst nematodes. It has limited utility, however, against a species such as *M. incognita* that has a wide host range and the ability to parasitise up to 3,000 plant species (Abad et al. 2003). Even where the strategy has potential, the long rotation periods required for efficacy may be economically unviable for specialist growers.

24.2 Manipulation of Plant Resistance

The most cost effective and sustainable strategy for limiting crop damage by plant parasitic nematodes is the use of resistant plants. Naturally occurring resistance has been exploited successfully in a number of crop species following transfer of

resistance genes from wild relatives or breeding lines through conventional breeding programmes. Particular economic benefits have been achieved with soybean and potato cultivars resistant to the cyst nematodes *H. glycines* and *G. rostochiensis* respectively and with tomato cultivars harbouring the *Mi-1.2* gene that confers resistance to three *Meloidogyne* species (Starr et al. 2002). Despite these successes, there are many more crops where commercially attractive resistance is not available. The number of published reports of natural resistance far exceeds the number of cases where resistant cultivars have been successfully deployed in agricultural systems. In some cases an identified resistance trait may be linked to other undesirable characteristics with associated yield penalties as occurred upon the transfer of *H. schachtii* resistance from *Beta procumbens* to cultivated sugar beet (Panella and Lewellen 2007). Where resistance is conferred by a single dominant resistance gene (R-gene) this drawback could potentially be overcome by using molecular techniques to directly introduce the isolated R-gene to a favoured crop variety. Transfer of R-genes in this manner is more likely to be successful between different cultivars of the same species, or between closely related plant species. For example *Rpi-vnt1.1*, a late blight R-gene from the wild species *Solanum venturii* is able to confer resistance to *Phytophthora infestans* in transgenic potato and tomato (Foster et al. 2009). Importantly, no reduction in tuber size or yield was reported for *P. infestans* resistant transgenic potato crops expressing the *RB* gene from *S. bulbocastanum* (Halterman et al. 2008). Reports of functional R-gene expression in heterologous species are less common, although a gene from pepper confers resistance to bacterial spot in tomato (Tai et al. 1999) and expression of the rice *Xa21* gene in sweet orange cultivars reduces their susceptibility to citrus canker (Mendes et al. 2010).

Attempts to introduce nematode R-genes directly into crops have met with variable success. As with other pathogens, transgenic resistance is much more likely to result following intraspecific transfers than interspecific ones. Transfer of *Hero A* from *Lycopersicon pimpinellifolium* into a susceptible tomato cultivar conferred comparable levels of resistance to *Globodera* species as that seen in an introgressed *Hero A* line. No significant resistance was achieved, however, in transgenic potato expressing the same construct (Sobczak et al. 2005). Similarly, although *Mi-1.2* confers resistance when transgenically expressed in a susceptible tomato cultivar (Milligan et al. 1998), attempts to transfer *Mi-1.2*-mediated root-knot nematode resistance from tomato to tobacco have been unsuccessful (Williamson 1998). Transgenic expression of *Mi-1.2* in aubergine (*S. melongena*), taxonomically related to tomato, yielded more promising results. The transgenic lines supported significantly lower levels of *Meloidogyne* reproduction with fewer egg masses (Goggin et al. 2006). A putative homologue of *Mi-1.2* was isolated from the hot pepper *Capsicum annuum* using a PCR-based cloning approach with degenerate primers designed to conserved regions of known R-genes. Expression of this gene, *CaMi*, in susceptible tomato cultivars conferred resistance to *M. incognita* (Chen et al. 2007). Most instances of successful interspecies transfer of R-genes have, like those just described, involved transformation of a species that is closely related to the parent plant. Recently, however, the tomato *Mi1* gene has been introduced into a root-knot nematode susceptible cultivar of lettuce (*Lactuca sativa*) resulting in transgenic

lines resistant to *M. incognita* (Zhang et al. 2010). If such heterologous transfer proves possible for other plant species then the utility of the currently small number of cloned nematode R-genes will be widened considerably. Downstream components of the response cascade must be present in the susceptible recipient plant in order for transferred R-genes to confer the desired resistance, and it remains to be determined whether or not this limitation can be overcome for most crops.

Not all naturally occurring nematode resistance is conferred by a single, dominant R-gene that can be manipulated with relative ease once identified. Some sources of nematode resistance are complex traits inherited in a polygenic manner (Tomczak et al. 2009) and the genes involved in these resistance mechanisms have not been identified. Such quantitative resistance is not readily amenable to transgenic manipulation at the current time. An additional drawback to the heterologous expression of R-genes lies with their specificity. Nematode R-genes are typically effective against one or a limited range of nematode species—many are functional only against specific pathotypes—whilst crops are often exposed to a range of these parasites (Roberts 1992). Such specificity also contributes to a lack of durability if plants containing a single resistance gene are grown repeatedly. If variability exists within a field population then virulent species or pathotypes will be selected and the resistance will appear to break down. Such a situation occurred in the UK where the widespread use of potato cultivars carrying the *H1* resistance gene successfully controlled *G. rostochiensis* but led to an increase in the prevalence of *G. pallida*.

There is thus the potential for alternative biotechnological approaches to produce benefits for nematode control including the possibility of achieving durable, broad spectrum nematode resistance (Atkinson et al. 2003).

24.3 Development of Biotechnological Solutions to Nematode Control

Substantial progress has been made over the last decade in understanding the often complex interactions that take place between parasitic nematodes and their host plants, as described in other Chapters of this book. The ultimate goal of much of this fundamental work is to apply the enhanced knowledge to the development of new control strategies. The potential impact of our increased understanding is starting to be realised as approaches aimed at expressing proteins to reduce root invasion or disrupt growth and development of the nematode are trialed. Promising results have also been achieved by delivering dsRNA from a host plant to bring about RNAi silencing of genes in the feeding nematode.

24.3.1 Proof-of-principle Using Model Systems

Whilst new nematode control options are urgently required in a range of crop species, genetic manipulation of many crop plants can be a slow and technically

challenging process. Novel nematode resistance strategies are therefore frequently tested in model systems and their practical utility remains to be demonstrated in a field situation. One test system that allows rapid evaluation of transgenic nematode resistance is the hairy roots that arise from transformation with *Agrobacterium rhizogenes* (Guillon et al. 2006; Shanks and Morgan 1999). Wounded plant tissue is inoculated with *A. rhizogenes* that transfers T-DNA segments from the Ri-plasmid into the plant genome. The hairy roots that arise are characterized by a high growth rate with extensive branching that is independent of added hormones (Guillon et al. 2006). The hairy root cultures can be additionally transformed to express a gene of interest. Plant parasitic nematodes develop successfully on hairy roots of a range of species that can be readily maintained *in vitro* (e.g. Alpizar et al. 2006; Cho et al. 2000; Elsen et al. 2000; Kifle et al. 1999; Kumar and Forrest 1990; Wubben et al. 2009). Consequently they have been used to investigate the efficacy of a number of new technologies (e.g. Cai et al. 2003; Li et al. 2007, 2008; Marra et al. 2009; Urwin et al. 1995) although care is required to ensure that adequate controls are in place to overcome an inherent variability of nematode infection (Plovie et al. 2003). Moreover, it is possible to regenerate complete composite plants from transformed hairy roots, widening the scope of this system (Collier et al. 2005). Soybean plants with transgenic root systems produced by this method are being used to evaluate RNAi-mediated effects on *H. glycines* (Klink et al. 2009; Li et al. 2010).

Arabidopsis thaliana has been used as a model host in numerous studies of plant-nematode interactions largely due its ease of transformation, its fully sequenced genome and the vast array of molecular resources available (see also Chap. 8). As a host to representatives of the economically important cyst and root-knot nematodes (Sijmons et al. 1991) it has also been used in preliminary demonstrations of potential resistance strategies (Huang et al. 2006; Sindhu et al. 2009; Urwin et al. 1997a, 1998, 2000).

24.3.2 Proteinase Inhibitors

Transgenic expression of proteinase inhibitors (PIs) in plant roots is the most widely explored approach for engineered resistance to plant parasitic nematodes. A range of different inhibitors, most of them naturally occurring plant proteins, have been shown to be detrimental to feeding nematodes, reducing their growth and fecundity. Inhibitors of all four main classes of proteinases (serine, cysteine, aspartic and metallo-) occur in plants and are often induced in response to wounding or herbivory (Koiwa et al. 1997). Correspondingly, proteinase genes and activity have been identified in plant parasitic nematodes (Fragoso et al. 2005, 2009; Lilley et al. 1996, 1997; Neveu et al. 2003; Shingles et al. 2007; Urwin et al. 1997b). A digestive role has been proposed for these enzymes and corroborated for some by expression in the intestine. With digestion of protein being a common requirement of nematodes, PI-based control could have efficacy against a wide range of species, irrespective of their parasitic strategy. This would have particular utility in those field situations where a number of different nematode pests occur concurrently.

Cysteine proteinase inhibitors, termed cystatins, have received the most attention. Initial experiments utilized the rice cystatin Oc-I, modifying its coding region to remove an amino acid and improve its inhibitory activity 13-fold over the native protein. Expression of this engineered variant (Oc-IΔD86) in tomato hairy roots using the cauliflower mosaic virus (CaMV35S) promoter resulted in significantly smaller female *G. pallida* after 6 weeks when compared to control roots (Urwin et al. 1995). Expression of Oc-IΔD86 in a second model system *Arabidopsis*, using the same promoter, allowed the cystatin to be tested against additional nematode species. The size of female *H. schachtii* and *M. incognita* was considerably reduced relative to controls, with growth arrested prior to egg-laying. This effect was correlated with detection of the cystatin in the feeding nematodes and reduced cysteine proteinase activity in the intestine of female *H. schachtii* recovered from plants (Urwin et al. 1997a). The same *Arabidopsis* plants also suppressed growth and egg production of the reniform nematode, *Rotylenchulus reniformis*, with cystatin expression level influencing reproductive success (Urwin et al. 2000). Although *Arabidopsis* is not a favoured host for this nematode the study is an example of a model system providing preliminary data to support later cystatin expression in crops of interest such as pineapple (Wang et al. 2009) where transformation is limited by a slow rate of regeneration. An alternative model host plant, alfalfa, was used to demonstrate that the rice cystatins Oc-I and Oc-II expressed at a low level in alfalfa under the control of a wound-inducible promoter conferred some resistance to the root-lesion nematode *Pratylenchus penetrans* (Samac and Smigocki 2003).

A rather different approach was taken to inhibit cysteine proteinases of *H. glycines* parasitizing transgenic soybean hairy roots (Marra et al. 2009). The propeptides that are cleaved from cysteine proteinase precursors can often act as inhibitors of their cognate enzymes (e.g. Roche et al. 1999; Silva et al. 2004). The propeptide region of the *H. glycines* HGCP-I cathepsin L enzyme was expressed in roots and caused a reduction in the number and fecundity of female nematodes (Marra et al. 2009). The prodomain inhibitor displays greater specificity for target enzymes than do typical plant PIs (Silva et al. 2004) and, whilst this may limit the utility of the approach to control a wide range of nematode species, it could have biosafety advantages for non-target organisms.

Although less widely studied, serine proteinase inhibitors also have demonstrated potential for nematode control. In another model system study, transgenic expression of the sweet potato serine PI, sporamin, inhibited growth and development of female *H. schachtii* parasitising sugar-beet hairy roots (Cai et al. 2003). In this case, the severity of the effect was clearly correlated to the level of trypsin-inhibitory activity detected in the transformed root lines.

24.3.3 Cry Toxins of Bacillus Thuringiensis

The δ–endotoxins, or Cry proteins produced in the sporulation phase of the soil bacterium *Bacillus thuringiensis* (Bt) are the basis of both biocontrol and trans-

genic approaches to insect control (Sanchis and Bourguet 2009). The toxicity of Bt to nematodes was first demonstrated for the free-living bacteriovore *Caenorhabditis elegans* (Borgonie et al. 1996) and a range of different Cry proteins (Cry5B, Cry6A, Cry14A and Cry21A) were subsequently shown to have efficacy against this and other bacterial-feeding nematodes (Marroquin et al. 2000; Wei et al. 2003). The Cry5B toxin has been studied the most extensively. It targets the intestine of *C. elegans* in a similar manner to that in which Cry proteins exert their effects on insects (Marroquin et al. 2000). Cry5B is a three domain protein with a predicted structure analogous to that of the insecticidal proteins and it binds to an invertebrate-specific glycolipid in the nematode intestine (Barrows et al. 2007; Griffitts et al. 2005). *C. elegans* mutants resistant to Cry5B remain susceptible to the unrelated Cry6A, suggesting that although this toxin also damages the intestine it has a dissimilar mode of toxicity, possibly binding to a different receptor (Marroquin et al. 2000). Both Cry5B (Li et al. 2008) and Cry6A (Li et al. 2007) confer resistance to *M. incognita* when expressed in hairy roots of tomato with the greatest effect observed on egg production, which is reduced by up to 64 and 76% respectively. These studies also substantiated those carried out with *C. elegans* that suggest dissimilar modes of action for the two toxins. Cry6A-expressing root lines showed only a slight reduction in gall number but a substantial reduction in both number of egg masses and total numbers of eggs relative to controls (Li et al. 2007). Cry5B expressing roots, on the other hand, supported significantly reduced numbers of galls. This was reflected in a reduced total egg production but there were no significant differences in the number of eggs per egg mass between transgenic and control lines (Li et al. 2008). Therefore Cry5B appears to exert its strongest effect on larval stages whilst reproduction is most sensitive to Cry6A. A combination of the two toxins expressed in the same plant may therefore afford a higher level of protection and also reduce the risk of resistant nematodes developing. The size of Bt toxins may be a constraint to their efficacy against all economic plant parasitic nematodes. The full-length Cry6A protein is 54 kDa and a truncated 43 kDa version has reduced toxicity (Wei et al. 2003), decreasing *M. incognita* egg production by 56% when expressed in hairy roots (Li et al. 2007). The 54 kDa form of Cry6A was detected within feeding females of *M. incognita* and although similar studies were not carried out for the truncated version of Cry5B that was used in the hairy root assays (79 kDa; Li et al. 2008) its efficacy strongly suggests that it too is ingested. This provides valuable insights into the constraints to uptake imposed by the feeding tube of *M. incognita*, a self-assembling, blind-ended structure most likely produced from pharyngeal gland secretions that forms at the stylet orifice and extends into the cytoplasm of the feeding cell. The feeding tube probably acts as a molecular sieve, permitting the uptake of certain molecules whilst excluding others and its structure differs between root-knot (Hussey and Mims 1991) and cyst nematodes (Sobczak et al. 1999; C.J. Lilley, unpublished observations). *H. schachtii* excludes dextrans of 40 kDa but not 20 kDa (Böckenhoff and Grundler 1994) and proteins of 23 kDa and 28 kDa but not 11 kDa (Urwin et al. 1997c, 1998) so this and similar species may not ingest Cry proteins.

24.3.4 Peptide Repellents

Location and invasion of host roots by plant parasitic nematodes requires them to sense and respond appropriately to distinct chemical, thermal and physical cues, a process that is mediated by sensory neurons. Disruption of this process is a proven target as the nematostat aldicarb impairs the response to known stimuli without inducing either paralysis or death of plant parasitic nematodes at low concentrations (Perry 1996). J2 stages of sedentary endoparasitic nematodes are vulnerable to sensory intervention prior to feeding cell initiation, whilst migratory ecto- and endo-parasites remain motile and could therefore be affected throughout their lifecycle. A key feature of chemical nematicides is that they are able to affect nematodes in soil before root invasion thus reducing the root damage that can affect crop yield and leave the plant vulnerable to secondary infection. A transgenic resistance strategy has been developed that also targets nematode chemoreception in a non-lethal manner (Lilley et al. 2011; Liu et al. 2005). Biopanning of a phage display library led to the isolation of two different peptides that either bind and inhibit acetylcholinesterase or bind to nematode nicotinic acetylcholine receptors, two targets in the cholinergic nervous system of nematodes. Both peptides inhibited cyst nematode chemoreception, blocking their response to an attractant, at concentrations from 1 nM (Winter et al. 2002). Fluorescently tagged peptide has been used to demonstrate that it functions only after retrograde transport along certain chemoreceptive neurons to their cell bodies and subsequent interneurons. Disruption of chemoreception of *G. pallida* was correlated with transport of the peptide to the nerve ring but not merely with its uptake into the amphids (D. Wang 2009, personal communication). The strategic development of this approach has provided prototype *Arabidopsis* and potato plants expressing secreted forms of the two peptides (Liu et al. 2005; Lilley et al. 2011; D. Wang 2009, personal communication). In *Arabidopsis* plants that secrete the acetylcholinesterase inhibiting peptide under control of the constitutive CaMV35S promoter, the number of established *H. schachtii* was suppressed by more than 80% (Lilley et al. 2010). The same plants also supported lower numbers of the migratory endoparasite *Pratylenchus penetrans* (J. Green 2008, personal communication). Similar constitutive expression in potato roots caused a 52% reduction in the number of female *G. pallida* that developed in the roots (Liu et al. 2005). The peptide that binds to nematode nicotinic acetylcholine receptors also suppressed *G. pallida* infection in potato hairy roots (Liu et al. 2005). The two peptide repellents have been placed under control of the *Arabidopsis mdk4-20* promoter that directs restricted expression at the root tips of both *Arabidopsis* and potato, resulting in equivalent or higher levels of resistance to cyst and root-knot nematodes (Lilley et al. 2011; D. Wang 2009, personal communication). The levels of secreted repellent detectable in hydroponic solution or soil water are low (<1 µg/ml) and they are not stable enough to persist in soil water. Therefore, the effect is probably due to suppressing invasion at the rhizoplane or at least very close to the root surface (Atkinson et al. 2009).

24.3.5 Silencing of Nematode Genes via Host-Delivered RNAi

RNA interference (RNAi), the process in which dsRNA can trigger the silencing of particular target genes through mRNA degradation, has been proposed as one of the most promising techniques for functional genetic studies in nematodes. The phenomenon was first described for *C. elegans* (Fire et al. 1998) and a number of studies have now reported its application for functional analysis of genes from a range of plant parasitic nematode species (see Chap. 10). These studies have relied on soaking infective, non-feeding nematodes in dsRNA to achieve ingestion. Many factors appear to influence the degree of gene silencing observed including incubation time, size of the dsRNA, the nematode species, and the target gene/domain (Lilley et al. 2007). As with animal parasitic nematodes (Knox et al. 2007), inconsistent outcomes of RNAi soaking experiments may prohibit the routine application of this technique as a screening tool for potential control targets. Host-generated RNAi has been suggested as an efficient strategy to continuously introduce dsRNA (or siRNAs) into the feeding plant parasitic nematode, to silence essential genes and hence to control the infection (Auer and Frederick 2009; Fire et al. 1998; Gheysen and Vanholme 2007; Lilley et al. 2007; Reimann-Philipp and Beachy 1993; Urwin et al. 2002). This would prolong the effective exposure to dsRNA/siRNA and should maximise the RNAi effect. Essentially, both sense and anti-sense cDNA sequences of the target gene, separated by a spacer region or intron, are expressed under the control of a strong plant promoter. The transcribed RNA then forms into a self-complementary hairpin structure with either the spacer region forming a loop or, more commonly, the intron sequence being removed by splicing. The resulting dsRNA is processed by the plant RNAi machinery resulting in siRNAs that may become incorporated into the RISC (RNA-induced silencing complex). The half-life of transgenically expressed dsRNA and the rate of accumulation of siRNAs are unknown. However, both molecules have been detected in plants (Fairbairn et al. 2007; Huang et al. 2006; Sindhu et al. 2009; Steeves et al. 2006) although not always in the same study. Similarly, it is not clear if the target transcript suppression observed arises from ingestion of plant derived siRNAs or dsRNA that is subsequently processed by the nematode.

Some proof-of-concept studies have shown extremely promising results: almost complete resistance against root knot nematode infection was observed in tobacco plants expressing hairpin RNAs corresponding to a nematode-specific collagen gene (patent application by Michaeli et al. 2005), splicing factor or integrase (Yadav et al. 2006). Both studies targeted genes presumed to be essential for nematode development based on the lethal RNAi phenotypes of their *C. elegans* orthologues. Surveys of genome sequence (Abad et al. 2008) and EST collections (Alkharouf et al. 2007) have identified further such orthologues in *M. incognita* and *H. glycines* that may prove to be equally effective targets. A high level of resistance to root-knot nematodes was also achieved by targeting a parasitism gene expressed in the subventral gland cells of *M. incognita* (Huang et al. 2006). In this case dsRNA complementary to the *16D10* gene was expressed in transgenic *Arabidopsis* plants

and the resulting lines displayed a significant reduction (63–90%) in the number of galls as well as an overall decrease in gall size and corresponding reduction in total egg production, compared to control vector-transformed lines. Importantly, the high level of homology between the *16D10* sequences of different *Meloidogyne* species led to broad-range resistance against *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*.

Not all host-delivered RNAi targeting *Meloidogyne* genes results in a resistance phenotype. Partial silencing of a putative transcription factor of *M. javanica* (*MjTis11*) did not significantly affect either nematode development or fecundity (Fairbairn et al. 2007). Possibly the degree of silencing was insufficient or the gene product does not have an essential, non-redundant role.

Cyst nematodes can also be targeted by expression of dsRNA molecules in plant roots although in this case it appears that the technology is less robust and requires optimization to achieve consistent suppression of nematode infection. In one report, nematodes infecting transgenic soybean plants expressing a hairpin construct targeting the major sperm protein (MSP) gene of the soybean cyst nematode *H. glycines* displayed an overall 68% reduction in egg production (Steeves et al. 2006) however the experimental set-up precluded clear interpretation of these results (Gheysen and Vanholme 2007). A number of studies have now demonstrated that hairpin RNAs expressed in the model plant *Arabidopsis* can at least partially reduce transcript abundance of targeted parasitism genes of *H. schachtii* (Patel et al. 2008, 2010; Sindhu et al. 2009). None of the range of genes delivered strong suppression of nematode development, but significant reduction in female numbers of between 23–64% was detected. A common observation seems to be a large but currently inexplicable variation in the infection results between and even within transgenic lines (Patel et al. 2008; Sindhu et al. 2009; Kyndt et al. unpublished data). Silencing of four essential cyst nematode genes with roles in mRNA metabolism (two ribosomal proteins and a spliceosomal protein) or neuronal transmission (synaptobrevin) achieved a more promising result (Klink et al. 2009). Tandem inverted repeat constructs of the *H. glycines* genes expressed in soybean plants resulted in 81–93% fewer females developing on the transgenic roots.

From the still relatively small number of studies carried out to date, host-delivered RNAi appears to achieve more effective resistance when it targets nematode genes involved in essential cellular processes. The only parasitism gene to offer a similarly high level of control is the *16D10* peptide of *M. incognita*. Possibly the majority of parasitism genes have combinatorial roles in the parasitic interaction or have redundant functions as members of gene families. Nevertheless, targeting parasitism genes by RNAi may offer advantages for developing transgenic nematode resistant crops. It guarantees that the dsRNA/siRNA uptake coincides with maximum expression of the target gene, since parasitism genes by definition are expressed during nematode feeding. This would need to be confirmed for genes not specifically linked to parasitism (Klink et al. 2009). Targeting parasitism genes should also minimise the possibility of the siRNAs inducing “off-target” gene silencing effects (Jackson et al. 2003). Many parasitism genes are nematode-specific and even genus-specific, which should facilitate regulatory approval of the trans-

genic plants. Housekeeping genes selected for their homology with *C. elegans* are more likely also to have similarity with genes found in non-target organisms.

24.4 Progress Towards Transgenic Resistance in Crop Plants

To date, biotechnological strategies for nematode resistance have largely been reported as proof-of-principle demonstrations in model systems and their utility to protect crop plants either in containment trials or in a field situation remains to be tested. As most of the molecular nematologists developing these approaches are based in Europe and North America their focus has been on the crop and nematode species of particular economic importance in these regions e.g. soybean, sugar beet, potato, tomato and their associated root-knot and cyst nematode pests (McCarter 2009). However, some of the technologies described above are being progressed to staple crops and vegetables in developing world countries where nematode control is also urgently required. Developments in three selected crops are discussed in more detail below. Potato is a crop where field testing of transgenic, nematode-resistant plants has been undertaken; banana and rice represent two staple crops of the developing world in which biotechnology could help to limit nematode-induced losses.

24.4.1 Potato

With the exception of maize, potato (*Solanum tuberosum*) is the most widely grown major food crop and the only significant tuber crop produced in developed countries. As potato production expands in subtropical and tropical countries away from its traditionally cooler environments, the impact of many pests and pathogens, including nematodes, is likely to increase (Scurrah et al. 2005). The economic importance of particular nematode species varies according to location and climate. In temperate regions of the world, together with cooler areas of subtropical and tropical regions, potato cyst nematodes (PCN, *Globodera* spp.) are the most important nematode pests and are often subject to strict quarantine regulations. Yield losses can be as high as 80% in countries such as Bolivia where continuous potato cultivation occurs (Franco et al. 1998). As mentioned in Sect. 2 above, the *H1* resistance gene has been successfully used against *G. rostochiensis* infection but it does not stop *G. pallida* from reproducing on potato. Other significant nematode pests of potato include the false root-knot nematode (*Nacobbus aberrans*) that is prevalent in parts of South America, *Meloidogyne* spp. and *Pratylenchus* spp. (particularly *P. penetrans*).

Engineered resistance based on PIs has been extensively tested in potato, primarily against *G. pallida*. The potential of plant PIs as anti-nematode effectors was first explored using the serine PI cowpea trypsin inhibitor (CpTI). CpTI expressed in transgenic potato influenced the sexual fate of newly established *G. pallida* (Hepher and Atkinson 1992) and as a result the population was biased toward

a predominance of the much smaller and less damaging males. Subsequent work focused on cystatins and culminated in successful field trials of transgenic potatoes. The best transgenic line of the fully susceptible potato cv Desiree, expressing chicken egg white cystatin from the constitutive CaMV35S promoter, displayed 70% resistance to PCN in the field (Urwin et al. 2001). When the same construct was used to transform two potato cultivars (Sante and Maria Huanca) that each display natural partial resistance to *G. pallida*, the best transgenic lines of each were enhanced to full resistance (Urwin et al. 2003). Subsequent field trials demonstrated that both the modified rice cystatin (OcIΔD86) and a sunflower cystatin expressed in cv. Desiree afforded similar levels of protection to chicken egg white cystatin (Urwin et al. 2003). Potato plants in which expression of the OcIΔD86 cystatin was limited mainly to the roots and, in particular, to the syncytia and giant cells induced by *G. pallida* and *M. incognita*, were shown to have similar resistance levels to those achieved with constitutive expression for both nematodes (Lilley et al. 2004). The conductance of field trials allowed environmental biosafety aspects of the technology to be investigated (Cowgill et al. 2002a, b, 2004; Cowgill and Atkinson 2003).

The potential of the peptide repellent technology has also been assessed in transgenic potato plants. In a containment trial, the best line constitutively expressing the acetylcholinesterase-inhibiting peptide supported 52% fewer female *G. pallida* than the control (Liu et al. 2005). Localised expression of the same peptide using a root tip specific promoter enhanced the resistance of the best line to 95% (Lilley et al. 2011). Containment and field trials have been carried out for plants secreting the peptide that binds nicotinic acetylcholine receptors with resistance demonstrated to both *M. incognita* and *G. pallida* (HJ Atkinson 2010, personal communication).

24.4.2 Banana

Although bananas and plantains (*Musa* spp.) are critical for food security in many (sub)tropical countries, they suffer severe yield losses due to nematode infestation when grown commercially or for local consumption. The most widespread and damaging nematodes to *Musa* spp. are of the migratory endoparasitic type: the burrowing nematode *Radopholus similis* and the root-lesion nematodes *Pratylenchus coffeae* and *Pratylenchus goodeyi* (Sarah et al. 1996; Bridge et al. 1997). They cause losses of 20 to 40% of yield. In regions where these migratory species are less abundant, root-knot nematodes *M. incognita* and *M. javanica* are also important pests on *Musa* spp. (De Waele and Davide 1998).

Nematodes in established banana plantations are mainly managed by means of nematicides (Gowen and Quénéhervé 1990), however chemical control is not only costly, it is toxic and largely inaccessible to most farmers in the developing world. The most sustainable method of nematode control requiring no changes to existing cultural practices is the use of resistant plants that suppress nematode reproduction (Starr et al. 2002). Sources of host resistance against migratory endoparasitic

nematodes are scarce, but have been found in citrus, groundnut, potato, alfalfa and lima bean (De Waele 1996). In the genus *Musa*, some existing cultivars, new hybrid crosses and wild relatives have been shown to be (at least partially) resistant towards *R. similis* and in some cases also towards the root-lesion nematodes (Wehunt et al. 1978; Price 1994; Fogain and Gowen 1998; Stanton 1999; Stoffelen et al. 1999; Elsen et al. 2002; Moens et al. 2005; Dochez et al. 2006; Quénéhervé et al. 2009a, b). From a biochemical point of view, increased amounts of condensed tannins and flavan-3,4-diols (Collingborn et al. 2000) and higher levels of vascular lignin and cell-wall bound ferulic acid esters in the cortex (Wuyts et al. 2007) have been observed in resistant cultivars. Dochez et al. (2009) provide some information on the genetic basis of banana resistance by evaluating the susceptibility of a diploid banana hybrid population against a population of *R. similis* from Uganda. They found 37 out of 81 hybrids to be resistant and the results indicated that resistance to *R. similis* is controlled by two dominant genes, with additive and interactive effects. However, neither these nor other nematode resistance genes have yet been isolated in *Musa* spp. and, hence, introduction of foreign genes is currently the most efficient route to nematode control.

The triploid nature of banana makes this crop particularly interesting for genetic transformation because the sterility of the cultivars hampers improvement by classical breeding. The sterility of the plants is in this case an advantage as the risk of gene flow to other related plants is minimized. Relative success in genetic engineering of bananas and plantains has been achieved recently. A range of transformation protocols are available based on electroporation of protoplasts derived from embryogenic cell suspensions, particle bombardment and *Agrobacterium*-mediated transformation (reviewed in Arvanitoyannis et al. 2008). Currently, a confined field trial with GM-bananas containing a resistance gene for the fungus Black Sigatoka is being undertaken in Uganda and Egypt is performing contained trials with genetically engineered virus-resistant banana (Karembu et al. 2009). In Australia, genetically modified strains of Cavendish bananas are being tested in the field. These plants were engineered to contain genes that are expected to increase nutrient content (source: http://www.gmo-compass.org/eng/news/339.docu.html).

A number of the nematode resistance strategies described above are being tested in banana and plantain. Cavendish dessert bananas that express the OcIΔD86 engineered variant of rice cystatin under the control of the maize ubiquitin promoter displayed 70 ± 10% resistance to *R. similis* in a glasshouse trial (Atkinson et al. 2004). Plants expressing the same cystatin under the control of a root-specific promoter that is upregulated in giant cells (Green et al. 2002; Lilley et al. 2004) were resistant (83 ± 4%) to *M. incognita* (HJ Atkinson 2007, personal communication). The approach is now progressing to cooking varieties of *Musa.* East African Highland banana plants constitutively expressing a maize cystatin support reduced multiplication of *R. similis* (CJ Lilley, unpublished data) and the plantain cv. Gonja has been transformed (Dr L Tripathi; IITA, Uganda) to express both a cystatin and a repellent peptide (HJ Atkinson 2010, personal communication). Similar additive cystatin plus repellent constructs have been introduced into East African Highland banana varieties (NARO, Uganda). There could be an additional advantage to cystatin-

mediated nematode resistance in banana as cystatin impairs feeding and development of banana weevils (Kiggundu et al. 2010).

Limited research has been carried out to determine the efficacy of RNAi in migratory endoparasitic nematodes such as *R. similis* or *Pratylenchus* spp. that are the key pests of banana. *R. similis* readily ingests molecules from soaking solution and uptake of dsRNA leads to efficient transcript suppression (CJ Lilley, unpublished data) although the extent of silencing can vary according to the region of the nematode gene targeted and the experimental occasion (Haegeman et al. 2009). Soaking *R. similis* in dsRNA homologous to a gland cell xylanase gene resulted in up to 60% reduction in subsequent infection of *Medicago truncatula* (Haegeman et al. 2009). It remains to be seen if host-generated RNAi will work efficiently against migratory parasitic nematodes. Currently, transgenic banana lines containing hairpin constructs targeting important genes of *R. similis* have been generated in the research group of Prof. R. Swennen (KULeuven, Belgium) and are under evaluation in collaboration with the group of Prof. G. Gheysen (UGent, Belgium) (unpublished data).

24.4.3 Rice

Rice (*Oryza sativa* L.) is a staple food crop for more than half of the world's population. It is the second most important cereal crop after maize (http://faostat.fao.org/). Estimated yield losses due to nematodes in rice are 10–25%, with an annual monetary value of about US $ 16 billion (Fortuner and Merny 1979). The most common plant-parasitic nematodes attacking rice are the root-knot nematode *Meloidogyne graminicola*, the cyst nematode *Heterodera oryzae*, the stem nematode *Ditylenchus angustus*, the root rot nematode *Hirschmanniella oryzae* and the white tip nematode *Aphelenchoides besseyi* (Bridge et al. 2005). *A. besseyi* and *D. angustus* are foliar parasites whilst the others are root parasites. Although *A. besseyi* is widely distributed and can cause damage in all rice cultivation systems (irrigated lowland, rainfed lowland, deepwater and upland) its economic importance varies between regions, countries and localities (Bridge et al. 2005). *D. angustus* has a restricted distribution, but when it occurs it is one of the most devastating of all diseases affecting rice. For instance, in the Mekong Delta (Vietnam) it has been a serious problem in the past, causing 50–100% loss of deepwater rice. Cyst nematodes on rice (mainly *H. oryzae*) have a restricted distribution and hence are only of local economic importance (Bridge et al. 2005).

About 75% of total rice production comes from irrigated lowland and, since *Hirschmanniella* spp. are well-adapted to flooded conditions and efficiently water-dispersed, these are the most important nematode pests for irrigated lowland rice worldwide (Islam et al. 2004). Approximately 60% of the world's rice fields are infested by *Hirschmanniella* spp., among which the most commonly recorded species is *H. oryzae* (Bridge et al. 2005). In the absence of hosts the nematodes can persist in the soil, and low soil moisture induces quiescence that extends longevity for up

to a year. Even at low population levels, *H. oryzae* has a high damage potential as affected rice plants show reduced resistance to other pathogens and a substantial decrease in sugars, amino acids and phenolic compounds. The majority of rice cultivars are good hosts of *Hirschmanniella* spp. but some tolerant or relatively resistant cultivars have been reported (Bridge et al. 2005).

M. graminicola has a very wide distribution and is a damaging parasite on upland, lowland and deepwater rice. As water scarcity is and will continue to be a major issue in the future, new ways of growing rice with less water are being explored. A promising new approach is upland or so-called 'aerobic' rice production, allowing a 50% reduction of water use (Bouman et al. 2002). However, under continuous cultivation, a gradual yield decline is reported. Nematode diversity is very high in upland rice ecosystems and the two genera with the highest potential to cause economic damage are *Pratylenchus* and *Meloidogyne* (*M. graminicola, M. incognita, M. javanica* and *M. arenaria*). There is evidence that root knot nematodes and, more specifically *M. graminicola*, contribute significantly to the yield reduction in aerobic rice (Nie et al. 2007). Under simulated upland conditions, yield losses caused by *M. graminicola* ranged from 20 to 80% and under intermittently flooded conditions, from 11 to 73% (De Waele and Elsen 2007). Several sources of resistance against *M. graminicola* have been found in *O. longistaminata* and *O. glaberrima* and crosses have been initiated to transfer these resistances into *O. sativa* cultivars (Soriano et al. 1999).

As a model monocot with a relatively small genome size of about 389 Mb, used as an important research tool to understand the biology of cereal crops, rice has great potential for future development of transgenic nematode resistant varieties. The available genetic maps, genome sequence and efficient transformation protocols are fundamental resources to work towards this goal. Although a GM rice cultivar resistant to glufosinate herbicides has been approved in the US, no large scale production of genetically modified rice is currently taking place. In 2004, the high vitamin A content Golden Rice underwent its first field tests and is planned to become available from 2011. Golden Rice will be provided free of cost to small-scale farmers in developing countries. China leads the pack in rice breeding research. In November 2009, the Chinese ministry issued biosafety certificates to two strains of genetically modified pest-resistant rice (developed by Huazhong Agricultural University) confirming it safe to produce and consume, taking a major step toward endorsing the use of biotechnology in the staple food crop of billions of people in Asia. The cultivation of GM rice in China, India, Indonesia and the Philippines is expected in the near future (James 2009).

To date, the only nematode resistance technology introduced into rice is the cystatin-based defence. Transgenic plants of four elite African rice varieties constitutively expressing the modified rice cystatin OcIΔD86 displayed 55% resistance to *M. incognita* (Vain et al. 1998). Only a low level of cystatin expression was observed, possibly due to a suboptimal CaMV35S promoter or homology-dependent silencing of the transgene in combination with the endogenous *OcI* gene. In subsequent work, a maize cystatin has been expressed in the rice variety Nipponbare under the control of a root promoter from *Arabidopsis* (*TUB-1*)

that is known to be up-regulated in the feeding cells of *M. incognita* parasitizing rice (Green et al. 2002). The construct also included an intron sequence from the maize ubiquitin gene to enhance expression levels and the best transgenic lines displayed 91 ± 7% resistance to *M. incognita* (HJ Atkinson 2008, personal communication).

24.4.4 Other Crops

In addition to the examples discussed above, biotechnological approaches for nematode resistance have been demonstrated in a number of other crop plants. Inhibitory activity of a potato serine proteinase inhibitor (PIN2) expressed in transgenic wheat showed a positive correlation with plant growth and yield following infestation with the cereal cyst nematode *Heterodera avenae* (Vishnudasan et al. 2005). A protective effect on the plant against nematode infection was inferred; however the effect of the PI on nematode development was not investigated. In a further demonstration of the potential of PIs, a cystatin from the tropical root crop taro (*Colocasia esculenta*) was expressed constitutively in a root-knot nematode-susceptible tomato cultivar. There was a 50% reduction in the number of galls formed by *M. incognita* on the transgenic plants compared to wild-type plants and a larger reduction in the number of egg masses produced per plant (Chan et al. 2010).

24.5 Future Developments

A number of biotechnological strategies have been described that disrupt the nematode/plant interaction and deliver a level of nematode control. Each has particular merits. The efficacy of proteinase inhibitors has been established against a range of plant parasitic nematodes. This is an important feature, as crops may suffer concomitant infection with more than one nematode species. Control of just the most prevalent may lead to an increase in importance of other, previously minor pests. Technology that is effective against a single nematode species may also be insufficiently attractive to justify the investment levels needed to bring the novel resistance to the market. The biosafety implications of transgenic plants expressing cystatins have been explored and the evidence to date suggests that they pose no threat to the environment, non-target organisms or human health (Atkinson et al. 2009). The emerging technology of RNAi holds potential, but there will need to be careful selection of gene targets and optimisation of construct design to ensure high efficacy. As the technology is developed further we will discover how generically it can be delivered to target different nematode species. Currently, host-delivered RNAi has only been reported against root-knot and cyst nematodes and the potential of the approach to control other economically important genera is unexplored. RNAi may prove to be very biosafe; its sequence specificity should limit deleteri-

ous effects on non-target organisms and no novel transgenic protein production is required, alleviating food safety concerns surrounding possible toxicity and allergenicity. A strategy that interferes with host plant location and invasion offers the additional benefit of potentially preventing the damage inflicted on roots during the invasion and migration process. To achieve this, optimum temporal and spatial expression in the roots is important to ensure effective levels of repellent are delivered to infective nematodes. The precedent set for commercialisation of insect-resistant crops expressing Bt Cry proteins may ease the regulatory approval of similar crops developed for nematode control.

To date, none of these demonstrations provide complete resistance to any nematode species and only a small number of studies have reported resistance greater than 90%. This sets the challenge to increase efficacy in order to achieve the level of resistance required for commercialisation. A general means of improving resistance could be to increase the level of effector protein or dsRNA/siRNA in the plant. A number of studies have shown a correlation between the level of PI expression or activity and the degree of nematode resistance obtained (Cai et al. 2003; Urwin et al. 2000). Alternative promoters, use of enhancer elements and optimisation of codon usage tailored to particular crops may lead to increased levels of resistance. There is evidence to suggest that a high level of hairpin RNA expression in feeding cells is required to produce gene silencing in feeding nematodes (Fairbairn et al. 2007). In *C. elegans* and plants, primary siRNAs derived from the Dicer complex-mediated cleavage of dsRNA are able to initiate the *de novo* synthesis of secondary siRNAs from the target mRNA. These molecules are more abundant than the primary siRNAs and highly effective in gene silencing (Rosso et al. 2009). For host-derived RNAi, the target transcript is not present in the plant cell and this amplification step cannot occur. Simultaneous expression of the nematode gene and its corresponding hairpin construct in the same transgenic plant should facilitate this process and may lead to a higher level of nematode resistance. When an *Arabidopsis* line expressing the *Meloidogyne 16D10* transgene was crossed with a line expressing the *16D10* dsRNA, the F1 plants contained higher levels of *16D10* siRNAs (Huang et al. 2006).

Efficacy can also be improved by stacking defences together in the same plant. This could be achieved by combining two or more aspects of the same technology as demonstrated for a cysteine and serine PI (Urwin et al. 1998). The possibility of simultaneously targeting two nematode genes by host-delivered RNAi is also being explored and shows promise (Charlton et al. 2010) although no additive effects were observed when *H. glycines* was soaked in dsRNAs targeting two pharyngeal gland genes (Bakhetia et al. 2008). The different technologies need not be exclusive and two or more could be combined in the same plant as techniques for introducing multiple genes into transgenic plants improve (Naqvi et al. 2010). Two approaches that target distinct aspects of nematode biology may offer greater durability. Intriguingly a recent study reported the synergistic effects of a Bt Cry protein and a nicotinic acetylcholine receptor agonist against *C. elegans* (Hu et al. 2010), suggesting that combining very different nematode control strategies may have high utility.

24.6 Prospects for Implementation of Biotechnological Control

It is clear that plant parasitic nematodes are a major constraint to global agriculture. Current chemical control, although successful where used, imposes too high a financial burden for subsistence growers and changes in legislation are likely to limit or ban the use of many more compounds. Despite this worsening situation, no crops with transgenic nematode resistance have reached commercialisation. This is in contrast to the successful adoption of insect-resistant biotech crops that cover an increased acreage year on year, with 25 countries planting biotech crops in 2009 (James 2009). To gain acceptance, new biotechnologies for nematode control must be sensitive to the needs of growers and consumers, safeguard the environment and demonstrate a level of resistance sufficient to warrant commercial investment. It is likely that biotech approaches will form part of a long-term strategy to control nematode-associated yield loss, in concert with improvements in traditional breeding processes, nematicide development and management practices (McCarter 2009).

If attitudes to biotech crops that are common in Europe prevail elsewhere then commercialization may be very slow, particularly for crops such as potato and sugar beet. One consequence of the limited uptake of transgenic plants in Europe is that some of its plant biotechnology businesses have relocated to the USA so reducing European influence on the directions of the industry (Atkinson et al. 2009). There is a pro-poor need for the deployment of nematode-resistant transgenic plants and the EU's unwillingness to accept transgenic crops may be having an adverse effect on development in Africa (Bodulovic 2005) and presumably elsewhere. Addition of extra traits to an existing transgenic crop is less controversial than a completely new introduction (McCarter 2009). Crops such as soybean and cotton, where a large percentage of the acreage is already planted with transgenic varieties may therefore be the first to benefit from commercialised nematode resistance technology.

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