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## Pathogen profile

**Root-knot nematode parasitism and host response: molecular basis of a sophisticated interaction**

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**SUMMARY**

**Taxonomy:** Eukaryota; Metazoa; Nematoda; Chromadorea; order Tylenchida; Tylenchoidea; Heteroderidae; genus *Meloidogyne*.

**Physical properties:** Microscopic-non-segmented worms. *Meloidogyne* species can reproduce by apomixis, facultative meiotic parthenogenesis or obligate mitotic parthenogenesis. Obligate biotrophic parasites inducing the re-differentiation of plant cells into specialized feeding cells.

**Hosts:** *Meloidogyne* spp. can infest more than 3000 plant species including vegetables, fruit trees, cereals and ornamental flowers.

**Symptoms:** Root swellings called galls. Alteration of the root vascular system.

**Disease control:** Cultural control, chemical control, resistant cultivars.

**Agronomic importance:** Major threat to agriculture in temperate and tropical regions.

**INTRODUCTION**

Root-knot nematodes (RKN), *Meloidogyne* spp., are obligate, sedentary endoparasites of many plant species. Their potential host range encompasses more than 3000 plant species. Among the many genera of nematodes having some economic impact, *Meloidogyne* spp. are responsible for a large part of the annual 100 billion € losses attributed to nematode damage (Sasser *et al.*, 1987). The most economically important species are the apomictic species *Meloidogyne incognita* and *M. arenaria*. *M. incognita* is found in every temperate and tropical country, and it is possibly the single most damaging crop pathogen in the world (as reviewed in Trudgill and Blok, 2001). The control of nematodes is often realized by the combination of several pest management strategies. Cultural control is widely practised, but rotation is of

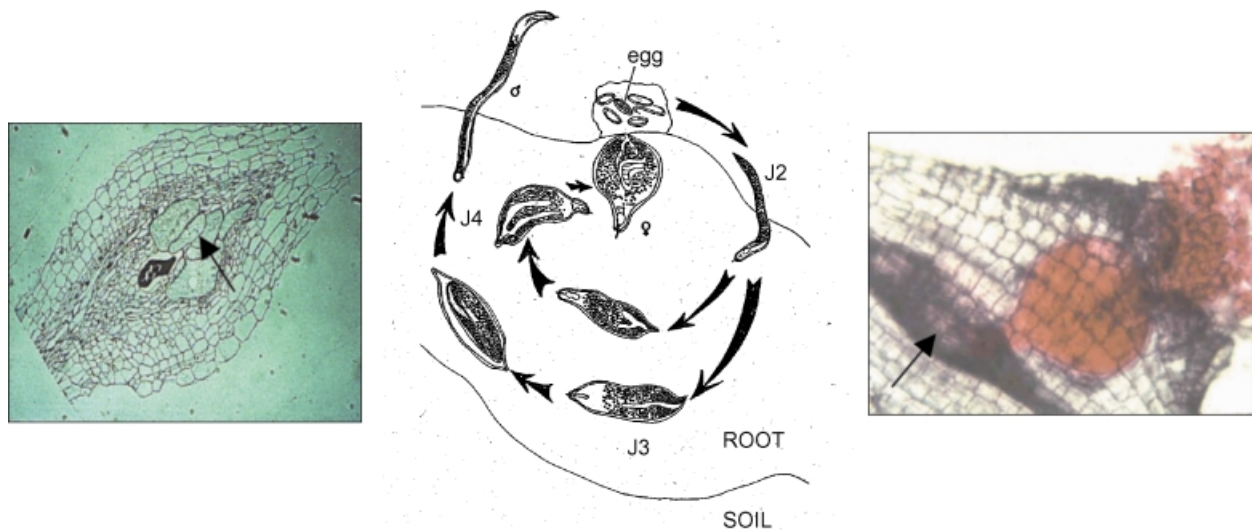
limited value for nematodes with a host range as wide as that of *Meloidogyne* spp. (Trudgill, 1997). The withdrawal from agriculture of many nematicides and soil fumigants further limits this control option, and emphasizes the need for alternative strategies. Resistant cultivars have proven commercially successful, for instance in the control of the most damaging species of *Meloidogyne* on tomato. However virulent biotypes of nematodes occur that challenge these resistant cultivars (Castagnone-Sereno, 2002).

The primary symptom of RKN infection is the formation of typical root galls on the root of susceptible host plants (Fig. 1). Nutrient and water uptake are substantially reduced because of the damaged root system, resulting in weak and poor-yielding plants. During parasitism, RKN establish and maintain an intimate relationship with their host. The worms hatch as second-stage juveniles and invade the root in the zone of elongation. They migrate intercellularly, first to the root apex and then to the vascular cylinder, where permanent feeding sites are established. The second-stage juveniles then undergo three moults to develop into adults. The saccate females remain sedentary, producing large egg masses. Males migrate out of the plant (Fig. 2).



**Fig. 1** Symptoms of *Meloidogyne incognita* attack on *Ficus* roots.

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**Fig. 2** Life cycle of *Meloidogyne* spp. Second-stage juveniles (J2) hatch from eggs in the soil and invade the root tissues towards the vascular cylinder. After three further molts, females become pear-shaped and sedentary and induce the formation of five to seven giant, multinucleate cells upon which they feed (left panel). Males remain vermiform and leave the root. After maturation, females lay eggs within a gelatinous matrix outside the root (right panel). The feeding site is indicated by arrows.

Nematode growth and reproduction depend on the establishment of specialized feeding sites within the root. Consequently, these nematodes do not kill the host cells from which they feed. Instead, they induce a redifferentiation process that leads to the formation of multinucleated feeding cells, named giant cells. Giant cell formation is the result of repeated nuclear divisions of the initial feeding cell without cytokinesis. Each RKN triggers the development of five to seven giant cells, each containing as many as 100 nuclei, which have undergone vast endoreduplication. These cells appear to be metabolically active, with a very dense cytoplasm containing numerous mitochondria, plastids, ribosomes, well-developed Golgi apparatus and smooth endoplasmic reticulum, generally organized in swirls. The central vacuole disappears and gives rise to many small vacuoles. In addition, cell wall ingrowths typical of transfer cells develop. These cell wall ingrowths are thought to enhance solute uptake from the vascular system. Concomitant with giant cell formation, hyperplasia and hypertrophy of the surrounding cortical cells lead to the formation of the typical root symptoms (reviewed by Williamson and Hussey, 1996).

Natural host resistance against *Meloidogyne* spp. has been found in several wild plant species and shown to reduce or suppress nematode development and reproduction (reviewed in Roberts, 1995). Some dominant resistance genes have been identified and mapped on plant chromosomes, e.g. the tomato *Mi*, *Mi-3* and *Mi-9* genes (Ammiraju *et al.*, 2003; Kaloshian *et al.*, 1998; Yaghoobi *et al.*, 1995), the pepper *Me3* gene (Djian-Caporalino *et al.*, 2001) or the peanut *Mae* and *Mag* genes (Garcia *et al.*, 1996). One of the best characterized nematode resistance

genes is *Mi*, originally found in the wild species *Lycopersicon peruvianum*, which confers resistance to several RKN species in tomato. The *Mi* gene has been cloned, and shown to belong to the NBS-LRR class of genes which also includes genes conferring resistance to viruses, bacteria and fungi (Hwang *et al.*, 2000; Milligan *et al.*, 1998). Moreover, the first element of the *Mi* resistance signalling pathway has been identified (Martinez de Ilarduya *et al.*, 2001). All the commercially available tomato cultivars resistant to RKN carry the *Mi* gene, and the emergence of nematode biotypes 'breaking' this gene is a serious threat to their future use.

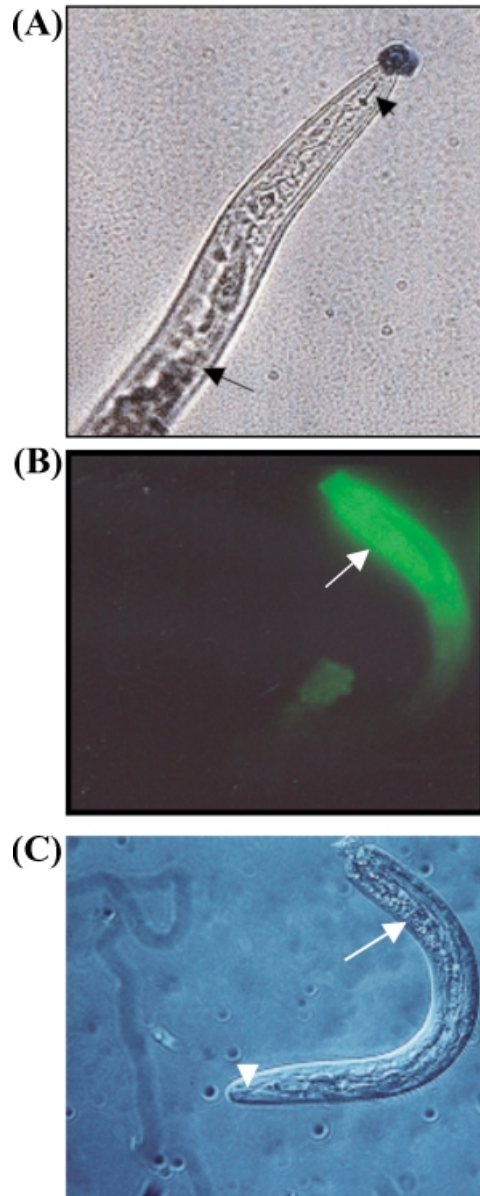
Recent investigations on the molecular basis of the plant–RKN interaction have focused on the parthenogenetic species *M. incognita*, *M. arenaria* and *M. javanica*. The characterization of *Meloidogyne* secretions, (a)virulence factors and nematode responsive plant genes will lead to a better understanding of the molecular events and regulatory mechanisms involved in plant parasitism by RKN and should allow the development of target-specific strategies to limit crop damage by these pathogens.

### Pathogenicity factors

Pathogenicity factors secreted by the nematode are assumed to play key roles during parasitism. Such secreted products could be involved in the invasion of the root tissues or the differentiation of the root cells into specialized feeding cells (Grundler and Wyss, 1994; Hussey, 1989). Such signals may be produced at distance from the differentiating feeding site or after injection of secretory

compounds in the plant cell. Electron microscopy observations showed that for feeding, the nematode perforates the cell wall with its stylet. The stylet reaches the plasma membrane of the cells and several feeding tubes are formed, probably issued from the assembly of nematode secreted proteins. The feeding tubes, closely associated with endoplasmic reticulum, may allow the puncturing of nutrients from distal parts of the feeding cell or act as molecular sieves to prevent the obstruction of the stylet (Hussey and Mims, 1991). Several structures of the nematode can be a source of secreted molecules involved in the plant–nematode interaction. The main secretory organs are the amphids, cephalic sensilla and phasmids, which are secretory chemoreceptors, the esophageal glands, the excretory system and the rectum, which produces the gelatinous matrix protecting the eggs from predators and dehydration (Eisenback, 1985). Moreover, the cuticle of the nematode selectively regulates the flow of fluids through the body wall and could be a source of secreted compounds recognized as signal molecules by the plant. Most efforts in analysing nematode secretions have focused on proteins secreted from amphids and oesophageal glands. Amphids are two sensory organs located bilaterally on the cephalic region of the nematode. They are composed of several cells including nerve cells and secretory cells. Amphids are in close contact with the plant cells that are differentiating in feeding cells. Several glycoproteins secreted by the amphids have been isolated, some of them being involved in the perception of signals from the environment (Stewart *et al.*, 1993). The oesophageal glands are specialized cells able to export secretions through the stylet. The activity of the oesophageal glands has been shown to vary during parasitism (Hussey and Mims, 1990). Whereas the subventral glands are large and filled with vesicles during the migratory phase of parasitism, the dorsal gland only starts the active production of secretions after sedentarization. While the nematode starts feeding, the subventral glands become less active. Therefore, subventral and dorsal glands seem to have different roles during parasitism.

The small size of infective juveniles (400  $\mu\text{m}$  long and 15  $\mu\text{m}$  wide) makes the purification of secretions challenging. However, a procedure was set up in order to induce *in vitro* the stylet secretion of *M. incognita* juveniles in quasi-sterile conditions. The harvested secreted proteins were analysed by 2D-electrophoresis and the most abundant proteins identified by microsequencing (Jaubert *et al.*, 2002a). Among the most abundant proteins, a 14-3-3 protein and a calreticulin, known to have multiple functions including the regulation of signalling and metabolic pathways and the regulation of the cell cycle and cell growth and differentiation were identified. The specific expression of the corresponding genes in the oesophageal glands of second stage juveniles has been demonstrated by *in situ* hybridization (Fig. 3). The 14-3-3 proteins have been shown to be secreted by animal parasitic platyhelminthes, and calreticulin is secreted by animal parasitic nematodes and trematodes. Although their role in animal



**Fig. 3** Accumulation of stylet secretions of *M. incognita* second stage juveniles visualized after Coomassie Blue staining (A). The calreticulin protein was detected by immunofluorescence on juvenile sections. The J2 sections were observed under fluorescent (B) or bright (C) light (from Jaubert *et al.*, 2002a). The oesophageal gland region is indicated by arrows, the stylet is indicated by arrowheads.

parasites is not clear, their implication in many host–parasite interactions supports the hypothesis of a role of these proteins in the plant–nematode interaction.

A second approach for the identification of secreted proteins is a candidate gene strategy, based on the knowledge of plant parasitism by bacteria, fungi and nematodes (Robertson *et al.*, 2000; Smant *et al.*, 1998). Major candidate genes are genes

encoding pectino-cellulolytic enzymes known to be involved in the softening of the plant cell walls during invasion by the parasite and genes encoding detoxification enzymes protecting the parasite from the oxidative defence response of the plant. Such genes can be isolated by PCR amplification using degenerated oligonucleotides designed from conserved motives of the proteins. Candidate genes can also be identified from expressed sequence tags (ESTs). Large sets of RKN ESTs have been produced in several laboratories, and about 36 000 ESTs from *Meloidogyne* are now available in public databases (Dautova *et al.*, 2001; McCarter *et al.*, 2000). The candidate strategy allowed the isolation of genes encoding cellulases ( $\beta$ -1,4-endoglucanases) and pectinases (pectate lyases and a polygalacturonase) from *M. incognita* (Jaubert *et al.*, 2002b; Rosso *et al.*, 1999). The specific expression of these genes in the oesophageal glands was demonstrated by *in situ* hybridization. The actual secretion *in planta* of a  $\beta$ -1,4-endoglucanase was demonstrated in the closely related cyst nematode *Heterodera glycines* (Wang *et al.*, 1999). ESTs homologous to detoxifying oxidoreductases such as superoxide dismutase are present in *Meloidogyne* EST databases. The involvement of the corresponding genes in the plant–nematode interaction is to be determined.

However, a candidate gene strategy is not relevant when looking for genes involved in the induction and maintenance of the feeding sites, since the formation of such structures is specific to the plant–nematode interaction. Therefore, differential analyses of expressed genes in parasitic stages were developed. RNA fingerprinting was carried out to seek for genes up-regulated in *M. incognita* parasitic juveniles as compared to preparasitic juveniles. Genes encoding a cellulose binding protein and a putative secretory venom allergen AG5-like protein, both transcribed in the subventral oesophageal glands were isolated with this strategy (Ding *et al.*, 1998a, 2000). A further differential analysis was conducted that aimed at isolating genes specifically expressed in the oesophageal glands of *M. javanica* juveniles extracted from roots. cDNAs obtained from the oesophageal gland region of the nematode were differentially screened against cDNAs from the posterior part. Two genes expressed in the glands were identified, one coding for a pectate lyase and one coding for a chorismate mutase (Doyle and Lambert, 2002; Lambert *et al.*, 1999). Interestingly, chorismate is a precursor for the synthesis of aromatic amino acids and of chorismate-derived compounds involved in cell wall formation and synthesis of plant hormone and defence compounds. A refined strategy for the construction of gland-specific cDNA libraries was developed with the microaspiration of the content of dorsal oesophageal gland cells (Ding *et al.*, 1998b).

Taken together, the different strategies all provide a large set of genes expressed in the oesophageal glands of the nematode and potentially involved in the plant–nematode interaction. It appears that RKN produces a panel of cell wall degrading

enzymes which are potentially involved in the invasion of the root tissues. The isolation of several isoforms of  $\beta$ -1,4-endoglucanases and a cellulose binding protein devoid of enzymatic activity indicates that nematode cellulases could act synergistically or be associated with multienzymatic complexes to produce an efficient degradation of cellulose. Moreover, a panel of proteins potentially involved in feeding site induction is now available. Understanding the function of these proteins in the interaction is hindered by the multiplicity of functions described in other eukaryotes, the absence of homologous proteins in databases, or the lack of known function for homologous proteins. All attempts in plant parasitic nematode transformation thus far failed. However, developing tools for the functional analysis of these genes by reverse genetics is now crucial and the recent report of gene silencing by RNA interference in cyst nematodes (Urwin *et al.*, 2002) should open new perspectives for the study of nematode pathogenicity factors.

#### Towards understanding (a)virulence in root-knot nematodes

The specificity of plant–pathogen relationships is often governed by the direct or indirect interaction between a single resistance gene in the host plant and a corresponding avirulence gene in the pathogen. Following recognition of the (avirulent) pathogen by the plant carrying a monogenic resistance gene, elicitation of host defences is activated and the so-called hypersensitive response leads to localized cell necrosis at the infection site (De Wit, 1997). For example, such a plant response has been well documented for tomatoes bearing the *Mi* RKN resistance gene (Williamson, 1999). Although *M. incognita*, *M. arenaria* and *M. javanica* are clonal organisms that reproduce asexually by mitotic parthenogenesis (Triantaphyllou, 1985), these species exhibit a considerable level of variability in terms of host range (Roberts, 1995; Trudgill and Blok, 2001). In particular, virulent populations have been reported that are able to reproduce on plants carrying a resistance gene, e.g. the *Mi* gene from tomato, the *Rk* gene from cowpea, or the *Me3* gene from pepper (Castagnone-Sereno, 2002). To date, the most studied interaction involves *M. incognita* and the tomato *Mi* resistance gene. Because of parthenogenesis, the genetic basis of (a)virulence cannot be tested in RKN. However, artificial selection experiments indicated that virulence is genetically inherited, and suggested that a polygenic system may be involved in that character (Bost and Triantaphyllou, 1982; Castagnone-Sereno *et al.*, 1994). Since plant resistance is actually the most effective and environmentally safe method of controlling RKN, understanding the mechanisms involved in *Meloidogyne* virulence is of great importance for the durable management of these pathogens.

In order to investigate the genetic relationships among avirulent and *Mi*-virulent RKN, the molecular characterization of



natural populations of various origins has been performed, using either RAPD or AFLP markers (Semblat *et al.*, 2000; Tzortzakakis *et al.*, 1999; Xu *et al.*, 2001). However, concerning the identification of DNA polymorphisms correlated with nematode (a)virulence, the results of these analysis appeared somewhat contradictory. Two independent studies showed a lack of correlation between the genetic diversity of the nematodes and their (a)virulence against the *Mi* gene (Semblat *et al.*, 2000; Tzortzakakis *et al.*, 1999), which demonstrated that virulent populations might not share a common origin. In addition, these results were obtained in populations from two very contrasting sampling scales, i.e. an isolated and restricted geographical area (the Crete Island, Tzortzakakis *et al.*, 1999) vs. a world-wide collection (Semblat *et al.*, 2000). Conversely, Xu *et al.* (2001) reported the development of a SCAR marker specific for some virulent *Meloidogyne* spp. populations from China and Japan, but this marker was not always discriminant, according to the origin of the virulence of the populations (natural vs. selected). Overall, these results indicate that virulence is probably not the result of a single mutational event, and suggest that the genetic determinants for virulence may be of different origin between different virulent populations.

In order to identify genes involved in the (a)virulence of the nematodes, differential strategies have been developed, based on the comparative analysis of near-isogenic lines which differ in their ability to reproduce or not on *Mi*-resistant tomatoes. Because of their parthenogenetic mode of reproduction, it has been hypothesized that any difference between these clonal lines should be the molecular determinants of the character of interest. AFLP fingerprinting of such biological material confirmed that the lines were nearly identical at the genomic DNA level, and allowed the identification of a few fragments reproducibly differential between avirulent and virulent lines (Semblat *et al.*, 2001). One of these amplified fragments, only present in avirulent lines, and used as a probe to screen a cDNA library from *M. incognita* second-stage juveniles, led to the cloning of a full-length sequence, named *map-1*, encoding a putative proline-rich protein of 458 amino acids. No significant homology was found in the databases for the MAP-1 protein, which contained a predicted N-terminal secretion signal peptide and several extremely conserved repetitive motives of 13 and 58 amino acids in its internal part. The differential expression of *map-1* between avirulent and virulent lines was confirmed in RT-PCR experiments, and sequences homologous to *map-1* were found in populations of *M. arenaria* and *M. javanica* which are, together with *M. incognita*, the three species controlled by the *Mi* resistance gene (Semblat *et al.*, 2001). In order to obtain insight into the putative function of MAP-1, antibodies were raised against the protein which strongly and specifically labelled the amphidial secretions of *M. incognita* second-stage juveniles in immunofluorescence microscopy experiments. Since amphids are the most important

chemosensory organs of nematodes, it has been hypothesized that MAP-1 could be involved in the recognition events occurring in the plant–nematode interaction. At the transcriptome level, cDNA-AFLP analyses of near-isogenic lines have been developed to monitor the differential gene expression between avirulent and virulent nematodes. Using this approach, differentially expressed candidate genes have recently been identified and are currently being investigated in either *M. javanica* (V.M. Williamson, personal communication) or *M. incognita* near-isogenic lines (Neveu *et al.*, 2002). However, at this stage, a current challenge is the functional validation of the direct implication of *map-1* and further candidates in the (a)virulence phenotype of the nematodes. As mentioned above, no transformation system is available yet for plant-parasitic nematodes, but the application of RNA interference to RKN will be of particular interest for the understanding of functions and genes involved in the interactions between RKN and their hosts. Undoubtedly, such knowledge is a key point for the future development and implementation of durable strategies for the control of these pests.

### Plant genes involved in giant cell formation

The identification of nematode responsive plant genes represents a major challenge in the understanding of how nematodes dramatically alter root development to produce and maintain giant cells. Little is known about the plant genetic requirements involved in the establishment and maintenance of these specialized cells. The mechanisms by which nematodes influence plant cell metabolism should share regulatory features in different plant species, since *Meloidogyne* is able to develop giant cells in several thousand host plants. A better knowledge of the plant response during the compatible interaction would allow an identification of targets for the development of novel approaches to engineer plant resistance.

The complex morphological and physiological changes that take place during the establishment of giant cells are reflected by altered gene expression in affected root cells (Gheysen and Fenoll, 2002; Williamson and Hussey, 1996). Approaches based on protein analysis and differential gene expression between healthy and infected roots have allowed the identification of up-regulated, and also down-regulated genes in galls. Computer searching of molecular databases may indicate a putative function for the products they encode. As examples, cDNAs homologous to a Myb-type transcription factor (Bird and Wilson, 1994), late embryogenesis-abundant protein (Van der Eycken *et al.*, 1996) and a 20S proteasome  $\alpha$ -subunit (Vercauteren *et al.*, 2001) have been shown to be up-regulated. However, this approach must be coupled with precise cellular expression pattern analysis, knockout mutant characterization and biochemical investigations for their actual function to emerge. The recent development of gene silencing technology in various plants should help an

analysis of the role of nematode responsive genes in feeding cell development.

To monitor gene expression changes in giant cells, the use of promoter-GUS fusion constructs represents a sensitive approach allowing spatial and temporal expression analysis. Several plant genes that could be expected to play a role in the establishment or maintenance of the feeding sites have been studied. In this way, the expression of genes encoding proteins involved in the regulation of the cell cycle, cell wall reorganization, metabolic pathways, osmoregulation and hormone responses has been analysed during giant cell formation. It has thereby been shown that the root-specific gene *TobRB7*, which encodes a presumed water channel expressed in root meristematic and immature vascular cylinder regions, is reactivated in tobacco giant cells induced by *M. incognita* (Opperman *et al.*, 1994). Similarly, the transcriptional activation of cell cycle markers such as the cyclin-dependent kinase *CDC2a* and the mitotic cyclin *CYC1At* is observed during the early stages of feeding cell formation (de Almeida Engler *et al.*, 1999; Niebel *et al.*, 1996). Moreover, other genes are down-regulated in nematode feeding sites. For example, the promoter of the plant phenylalanine ammonia-lyase I gene, which is highly active in non-infected roots, is silenced within a few days following nematode infection (Goddijn *et al.*, 1993).

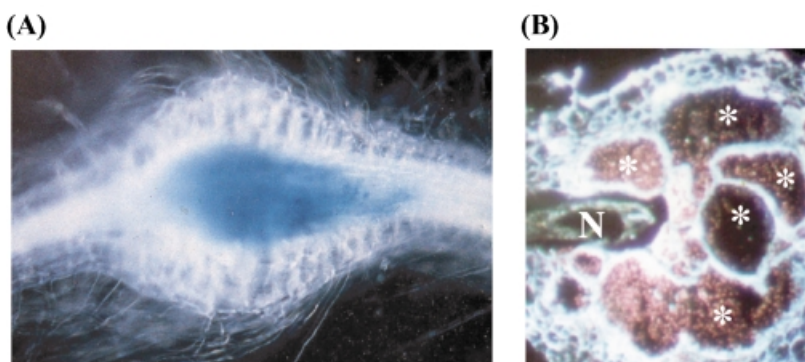
To identify new genes and to obtain a more comprehensive view of the molecular mechanisms underlying the induction and maintenance of giant cells, a promoter trapping strategy was developed with a promoter-less GUS gene construct being introduced randomly into the *Arabidopsis* genome via *Agrobacterium* transformation (Barthels *et al.*, 1997; Favery *et al.*, 1998). Screening for GUS expression after *M. incognita* infection showed that the genes up-regulated in giant cells are also expressed in the healthy plant in different cell types or at different developmental times (Favery *et al.*, 1998). These results from *Arabidopsis* support the hypothesis that 'normal' biochemical functions have been recruited to play key roles in allowing pathogen growth and confirm the complex morphological and physiological changes that occur in cells during their modification into

nematode feeding sites. This promoter trap strategy was validated by the molecular characterization of a strongly up-regulated plant gene, *RPE*, involved in the early steps of giant cell formation (Fig. 4, Favery *et al.*, 1998). This gene is likely to be directly involved in the biochemical make-up of giant cells. *RPE* encodes a D-ribulose 5-phosphate 3-epimerase, a key enzyme in the pentose phosphate pathway. This metabolic pathway plays a crucial role in actively growing cells by producing the NADPH required in numerous biosynthetic reactions (for example, fatty acids and isoprenoid compounds such as sterols) and by generating carbohydrate intermediates for the synthesis of nucleotides and cell wall polymers. Analysis of *rpe* homozygous mutants showed that *RPE* is essential for the early steps of giant cell formation. Finally, the root expression of *RPE* in the proliferating cells of root tips and in the small subset of cells involved in the initiation of lateral roots indicates that the genetic control of nematode feeding sites and root formation share common steps.

In the near future, the use of microarray technology will give large-scale information about patterns of gene expression during the plant–nematode interaction and particularly about the genes which are down-regulated during giant cell establishment. Few of them have been identified thus far. Finally, determining how a nematode selects particular root cells and modifies them to serve as a feeding site will enhance our knowledge of normal cell development and may serve to identify genes that regulate aspects of cell division and differentiation. Therefore, RKN represents an efficient tool for the understanding of plant development.

## CONCLUSIONS AND FUTURE PROSPECTS

RKN are biotrophic parasites that have evolved subtle strategies to successfully infest a diversity of plant species. Invasion of the root tissues by RKN involves mechanisms common with plant pathogenic bacteria and fungi, i.e. the production of cell wall degrading enzymes. However, the molecular signals inducing the differentiation of plant cells into specialized feeding cells are still to be determined. The success of RKN in colonizing various



**Fig. 4** GUS expression in galls of *Arabidopsis* RPE-tagged line induced by *Meloidogyne incognita*. (A) Localized GUS expression (blue) in a root gall 7 days after infection. (B) Cross-section of a gall 10 days after infection. Galls were excised from histochemical  $\beta$ -glucuronidase (GUS) stained plants, fixed, and sectioned. Sections, examined under dark field light, clearly showed GUS expression (pink precipitate) limited to giant cells. The head of the feeding nematode (N) can be seen at the edge of the giant cells (\*).

environments is illustrated by their ability to adapt to selection pressure and to develop on resistant cultivars. Genes potentially involved in feeding cell induction and in (a)virulence are emerging. Understanding the function of these genes greatly depends on the development of reverse genetic tools applied to plant parasitic nematodes.

In parallel with this, elucidating the early response of the plant to the pathogen will allow an understanding of the key steps involved in the intimate interaction leading to the formation of the feeding sites. Because plants have engaged associations with a wide range of mutualistic and parasitic biotrophic organisms, it is quite conceivable that these biotrophic interactions might have evolved certain common core components affecting cellular processes. Recent data suggest that certain cellular processes dealing with endoreduplication, cell cycle regulation, cell to cell communication and water transport are shared by the complex developmental processes of nodule organogenesis and gall formation (Favery *et al.*, 2002; Koltai *et al.*, 2001). A refined analysis of the affected signal cascades and their comparison with different plant/microorganism interactions such as symbionts and mycorrhizae should highlight the common reactions of the plant and the specific responses which lead to the formation of the feeding sites.

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