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The Contribution of Biotechnology to Root-Knot Nematode Control in Tomato Plants

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ABSTRACT

Root-knot nematodes (*Meloidogyne* spp.) represent a particularly serious pest for tomato crops. These pathogens have evolved a sophisticated interrelationship with the roots of their host where they induce a specific type of nurse cell system, classified as multinucleate giant cells. The structural and physiological transformation of the initial cell to become the nematode feeding site is paralleled by modifications in plant gene expression. The recent characterisation of several parasitism genes specifically expressed within oesophageal gland cells of root-knot nematodes suggests that their products can influence the host cellular metabolism. In plants with genetic disease resistance, these secreted molecules might serve as virulence factors for successful parasitism. The *Mi* gene, which confers resistance to several species of root-knot nematodes, is present in many modern tomato cultivars. Resistance mediated by *Mi* is associated with localized necrosis of host tissue at the nematode feeding site and occurs very early after nematode infection. However, how *Mi* mediates recognition of and resistance to root-knot nematodes is largely unknown. In parallel with the use of such natural resistance, several biotechnological strategies have been experienced to improve tomato resistance. They are mainly based on the over-expression of anti-nematode and/or anti-giant cell genes placed under the control of specific promoters. Here, we review the recent progress in determining the role of signal transduction pathway(s) in tomato responses during both susceptible and resistant interactions, and how such knowledge should allow the development of alternative strategies for engineering durable resistance against root-knot nematodes in tomato.

Keywords: plant defence, avirulence genes, resistance genes, signal transduction, durable resistance
Abbreviations: **AA**, ascorbic acid; **AVR**, avirulence; **dsRNA**, double-stranded RNA; **ET**, ethylene; **HR**, hypersensitive response; **H_2O_2**, hydrogen peroxide; **HRGP**, hydroxyproline-rich glycoproteins; **IAA**, indole-3-acetic acid; **JA**, jasmonic acid; **J2**, second stage juvenile; **LOX**, lipoxygenase; **LRR**, leucine reach repeats; **NO**, nitric oxide; **NOS**, nitric oxide synthase; **O_2^-**, superoxide; **PAL**, phenilalanine ammonia lyase; **POX**, peroxidase; **R**, resistance; **RBO**, respiratory burst oxidase; **RKN**, root-knot nematode; **RNAi**, RNA interference; **ROS**, reactive oxygen species; **SA**, salicylic acid; **TAL**, tyrosine ammonia lyase

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Special Feature

INTRODUCTION

Plants defend themselves from pathogen using a variety of mechanisms including rapid induction of localized necrosis at the site of infection (the hypersensitive response, HR), increased expression of defence-related proteins, production of antimicrobial compounds, lignin formation, and the oxidative burst. These defence responses occur between some pathogens carrying a specific avirulence (*avr*) gene and plant hosts with a complementary resistance (*R*) gene. In this gene-for-gene interaction mutation of either the avirulence gene or the resistance gene leads to the failure by the plant to recognize the pathogen and this renders the pathogen to be successful in inducing host disease. In the last decades, several *R* genes conferring resistance to a wide spectrum of plant pathogens including virus, bacteria, fungi, and nematodes were successfully cloned (Hammond-Kosack and Jones 1997). These *R* genes were expected to encode components of signalling pathways which lead, at least, to defence response, and a number of cloned genes revealed that they encode members of protein family characterized by the presence of a putative nucleotide binding site and a region of leucine rich repeats (LRR) that are required for resistance against viruses, fungi, bacteria, and nematodes (Staskawicz 1995; Williamson 1998; reviewed in Teixeira da Silva 2006). Plant-parasitic nematodes and especially sedentary endoparasitic nematodes are a very economically important group of parasites for a large variety of crops throughout the world, including cultivated tomato. Their impact on yield losses has been estimated to billion euros annually. Current nematode control includes application of nematicides, crop rotation and the introduction into crops of natural resistance traits by conventional breeding. However, all nematicidal products are very toxic to the environment and crop rotation, although widely practised, rarely provides an adequate solution alone and is not practical for growers. Natural resistances have been found for a limited range of crops, exhibit often high pathogen specificity and, more, tend to break down due to the emergence of new virulent populations.

Sedentary endoparasites, cyst and root-knot nematodes, represent the most advanced and successful type of parasitism, as reviewed by Sijmons *et al*. (1994). The root-knot nematodes *Meloidogyne* spp. have evolved a very sophisticated mode of parasitism in that they are able to alter gene expression in specific host cells and to modify them into specialized feeding cells. Infective second stage juveniles (J2) migrate in the soil and are attracted to root tips where they penetrate and migrate towards a suitable site in the host root. The juveniles become sedentary and establish an intimate relationship with their host by induction and maintenance of specialized feeding cells from which they are completely dependent for their life cycle (Endo 1975). The most obvious morphological response of compatible plants to infection by *Meloidogyne* is the characteristic root galling, which alters the uptake of water and nutrients. Shunting of the latter from the plant to the nematode reduces plant growth and crop yield. In the incompatible interactions a hypersensitive response (HR), resembling those described for other pathogen resistance genes, is associated with root-knot nematodes infection (Williamson and Hussey 1996). Numerous genes that confer resistance to root-knot nematodes have been described. One of the best studied of these genes is *Mi-1*, which confers resistance against three species of root-knot nematodes in tomato (Williamson and Gleason 2003). A combination of cytological, biochemical, and molecular approaches has established that superimposed on the establishment of a compatible host response is the potential for the plant to detect the presence of the pathogen and rapidly activates a series of defence responses leading to incompatible reaction. The changes in gene expression identified so far are likely to be some steps downstream from the initial plant response to signals from the nematode. Specific compounds in nematode secretions could bind to plant cell receptors to elicit a signal transduction cascade to modulate gene expression (Williamson and Hussey 1996). The nature of the genes switched on/off during the nematode-host interaction is subject of great interest as some of the products of these genes are directly responsible for promoting nematode parasitism of plants or for fending off the nematode attack. Moreover, the timing in the surveillance system of receptor molecules that can recognize elicitor molecules of pathogen origin is fundamental for the host response and is functional against a wide spectrum of pathogens (Nürnberger *et al*. 2004). Following this initial recognition, multiple biochemical pathways are activated and lead, working synergistically, to a cascade of reactions, ultimately resulting in the formation and accumulation of secondary metabolites which help the plant to overcome the parasite. Understanding these events might lead to the development of resistant plants in which the resistance mechanisms act before the nematode has reached its sedentary stage. This review aims to highlight the new technologies applied to discover nematode parasitism genes and focuses on recently identified pathogen signals that are deciphered by the plant system of surveillance. Several realistic approaches to achieve durable disease control in tomato crop against root-knot nematodes, based on recent knowledge on the interaction, are discussed, including both natural and engineered resistance.

BIOLOGY AND GENETICS OF THE COMPATIBLE INTERACTION BETWEEN TOMATO AND ROOT-KNOT NEMATODES

Biology of the interaction

Life cycle

Like all other biotrophic plant pathogens, root-knot nematodes must have a plant host to feed upon to complete their life cycle. *Meloidogyne* spp. have a wide host range, causing problems in more than 2000 species of annual and perennial crops. Tomatoes are among the most seriously affected, with the nematodes causing problems in all growing areas. The most widely distributed species are *M. arenaria*, *M. incognita*, *M. javanica*, *M. hapla*, the latter being adapted to colder climates. The simple life cycle has four juvenile stages in addition to egg-laying adult female. Unlike other life cycle strategies, the J2 (**Fig. 1.1**) is the only infective stage and burrows into the root, usually near the root tip. The J2 enters the plant root behind the root cap, in the zone of elongation (**Fig. 1.2**) where it then migrates toward the root tip. Once it reaches the root meristem, it turns round and migrates back up into the differentiating vascular cylinder until it arrives at the zone where the protoxylem starts to develop. At this stage, specific xylem parenchyma cells are selected for the development of the feeding structure, a system of multinucleate giant cells (**Fig. 1.3**). Each cell is initiated by the injection of salivary secretions originating from the dorsal oesophageal gland, which are thought to be of different nature than those secreted during migration (Sijmons *et al*. 1994). These giant cells act as metabolic sinks that actively transfer nutrients from host plant to the developing nematode. Cells around the developing juvenile also become hypertrophic and more numerous, which ultimately results in the formation of the characteristic root galls, associated with *Meloidogyne* infection. Once feeding begins, the J2 losses its ability to move within the root. J2 causes little physical damage to the roots during the penetration process. Most damage to the host plant results from physiological and biochemical changes caused by nematode feeding. Giant cells, which are approximately 10 times larger than normal root cells, interfere with the development of the root because transport of water and nutrients from the soil are cut off. In addition, some of the sugars the plant produced by photosynthesis to support normal root growth are diverted to the giant cells to sustain the developing nematode. The juvenile transforms into a male or a female after several moults. Reproduction of *Meloidogyne* is by

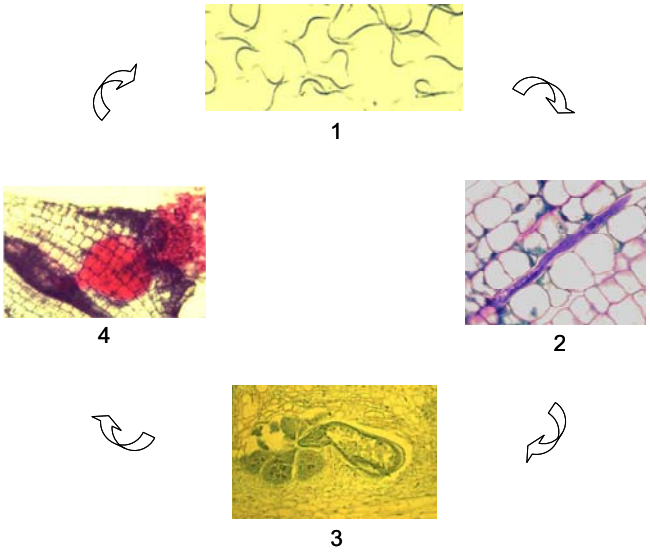

Fig. 1 The main steps of the biological cycle of *M. incognita*. (**1**) Infective second-stage juveniles (J2) (**2**) Intercellular migration of J2 in the root (**3**) Induction of giant cells by a young female (**4**) Egg-mass production by a mature female.

parthenogenesis, although males are commonly found. The process of sex differentiation depends on the stress and host response. The male is released outside the root without feeding, the immobile female remaining in the plant tissue. Approximately three weeks after root penetration, the female becomes swollen, pear-shaped, and lays eggs (**Fig. 1.4**). Egg production occurs without sexual cross-fertilization. Even though adult males may be present, they are not required for reproduction. The adult female deposits eggs in a gelatinous mass, and an egg mass contains from 500 to 2000 eggs. The egg masses project from the surface of young roots (**Fig. 1.4**). The female dies soon after laying eggs. Eggs in matrices often remain attached to root fragments in the soil after the female dies. Eggs may hatch immediately and juveniles may re-infect the root, or may over-winter and hatch next spring. The number of days required for the root-knot nematode to complete its life cycle depends on soil temperature. One life cycle, from egg to reproducing adult, will occur every 28 days when the soil temperature is 26-27°C. Several generations may succeed one another when conditions are favourable, infestations reaching considerable proportions. In case of heavy attacks on susceptible plants (e.g. tomato), galls can become very large, the root system being reduced to a swollen stump without hairs. The growth of the aerial part can be stunted and leaves can turn yellow. Infected plants are very susceptible to drought. Moreover, the presence of this nematode favours or worsens attacks by fungi and other phytoparasites.

Invasion strategies

Root-knot nematode parasitism of plants is a complex and dynamic interaction which involves hatching stimuli, attraction to the host, penetration of host tissues, recognition of tissues suitable for feeding site induction, modification of the host tissues, and an active response of the host. Detailed *in vitro* observations of *Meloidogyne* infection on *Arabidopsis* roots (Wyss *et al*. 1992) and tomato root explants (Guida *et al*. 1991) provided the most practical way to obtain step-by-step information on the development of host-nematode interaction. In general, the invasion of a root tip by one juvenile attracts others which enter the same site and they can all be successful in inducing pathogenesis (**Fig. 2A**). The migratory pathway of the juveniles is intercellular and they migrate between cortical cells until they reach the differentiating vascular tissue. Here they stop and select a parenchyma cell as their permanent feeding sites. The key to understanding the complex interrelationship between plant and parasite lies in the sensory system of the nematode. Nematodes have sense organs constituted by gland cells surrounded by nerve processes and consequently producing secretions. The secretions of the largest sense organs, the amphids, which are located in the anterior portion of the head of the nematode are suggested to be involved in chemoreception such as host attraction, identification of penetration sites, and initiation of feeding. Premachandran *et al*. (1988) demonstrated that the amphids of *M. incognita*, together with the other sensory organs (excretory pores and phasmids), are capable of producing a great amount of secretions, following chemical induction. Studies focused on the characterization of secretions identified a glycoprotein (gp32) specifically associated to the amphids of *M. incognita* when it locates tomato roots (Perry 1996). This glycoprotein appears to be very important as it seems to be involved directly or indirectly in the primary transduction of chemical stimuli. It is known that J2 are attracted to the host roots and that the juveniles inside the roots are highly selective in their choice of the host cell with which to establish a feeding site (Endo 1975). Assuming that the amphids serve a chemosensory role in *Meloidogyne*, the specific localization of gp32 in J2 may suggest its involvement in the location of host roots and in selecting the feeding site. Once the juvenile reaches its feeding site, electron microscopy observations revealed the presence of viscous materials, probably secreted from the amphidial pouches and filling the intercellular space between the nematode and host cells. The presence of this secretion may be related either to feeding site recognition during the moulting phase of the juveniles or with the production of protective and/or lubricating glycoproteins (Bleve-Zacheo and Melillo 1997). So far, the function of several proteins which have been identified as amphidial secretory products is still unknown. Among others, the MAP-1 putative avirulence protein, isolated after AFLP fingerprinting of near-isogenic lines of *M. incognita*, was suggested to be involved in the early recognition step between resistant plants and avirulent nematodes (Semblat *et al*. 2001). Intercellular migration of *Meloidogyne* does not seem to induce severe injuries in the root, as showed, for the first time, in an ultrastructural investigation of clover roots during early stages of infection by *M. incognita* (Endo 1975). The author concluded that the physical pressure exerted by the migrating juvenile on the cortical cells cause separation along their middle lamellae. Later, immunological studies demonstrated that the entering nematode may force the cells and, more, is able to separate the cells by means of digestion of the middle lamella. In addition, a surface coat was observed to labelling the cuticle of the juvenile and deposited on the root cell walls in contact with the nematode during its migration in *Arabidopsis* (Gravato-Nobre *et al*. 1999). More recently, characterization of specific molecules expressed at the surface cuticle of *Meloidogyne* spp. and released into plant tissue has been reported (Lima *et al*. 2005). The authors suggest that the cross-reactive immunodominant epitopes present in the amphids and cuticle of nematodes can be involved in the physiological mechanisms of the pathogenesis. Moreover, the cuticle of the nematode has been shown to regulate selectively the flow of fluids through the body wall and it is hypothesized that it could be a source of secreted compounds recognized as signal molecules by plants (Abad *et al*. 2003). An inhibitory effect of antibodies on nematode movement in plants has been previously demonstrated and this indicates that amphidial and cuticular antigens might represent a good target for devising novel nematode control strategies (Sharon *et al*. 2002). During migration through the plant tissue, the J2 use an arsenal of parasitism proteins which include amphidial and cuticle secretions combined with oesophageal gland secretions. Different strategies used in the last years provided great information concerning a large set of genes potentially involved in the early stage of nematode attack. Root-

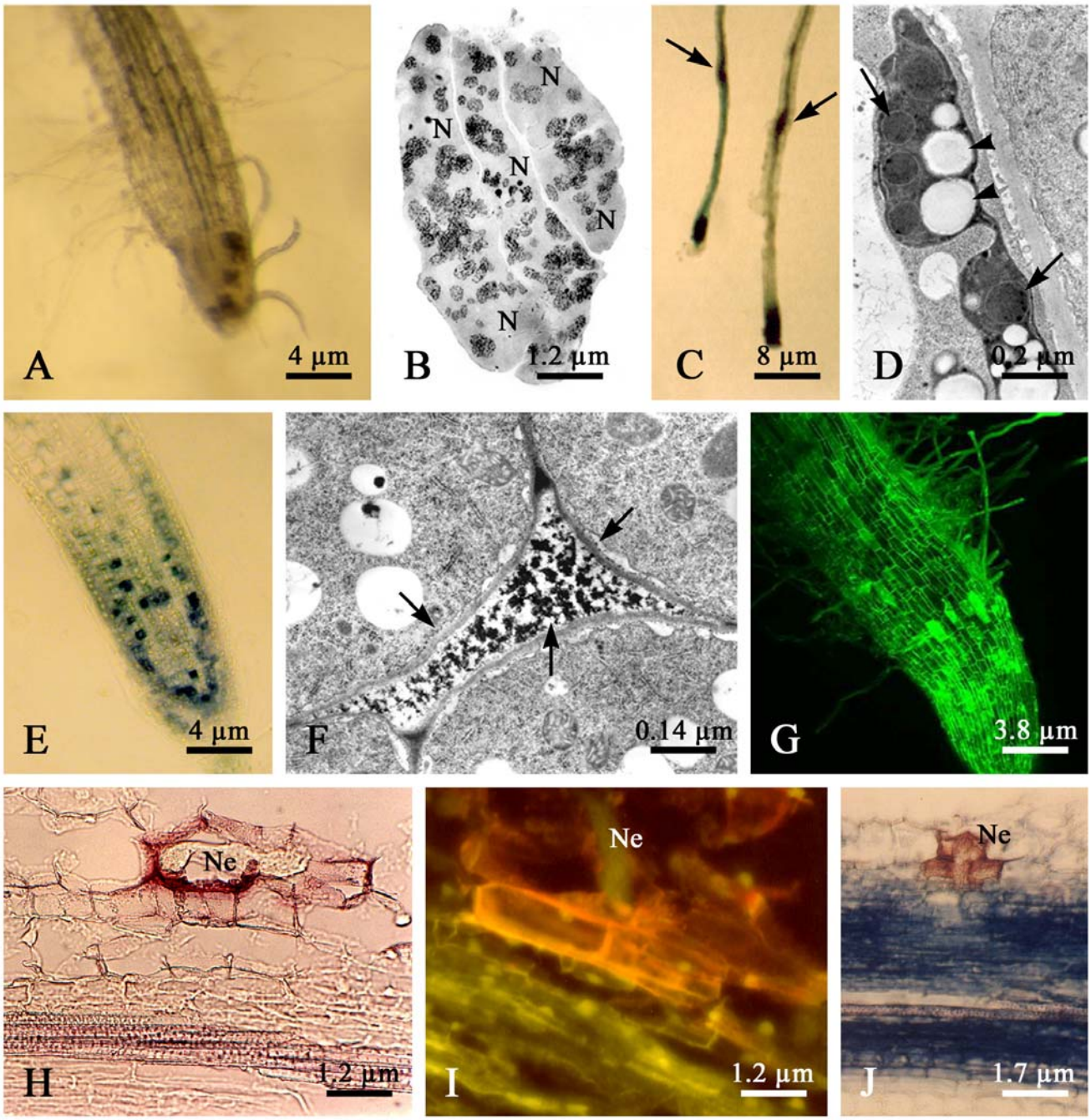

Fig. 2 Impact of *M. incognita* infection on tomato root cell metabolism. (**A**) Invasion of a resistant tomato root tip by three avirulent second-stage juveniles, showing HR reaction of cells at the point of nematode invasion. (**B**) A single, Feulgen-stained dissected giant cell induced by an avirulent pathotype in a susceptible tomato root with at least 90 mitotic nuclei (N) visible. (**C**) Injection of oesophageal *M. incognita* secretions in transgenic Arabidopsis induces re-differentiation, similar to that induced by virulent pathotype and indicated by slight swelling and positive GUS reaction, of injected parenchyma vessel cells (arrows). (**D**) Abnormal shape of plastids in a resistant root infected with avirulent pathotype. A number of globular crystalline bodies (proteins) (arrows) together with starch accumulation (arrow heads), as effect of pathogenesis in cell metabolism, can be seen. (**E**) NBT reaction, in 12 h inoculated root cells of resistant tomato infected with avirulent nematodes. Bluish formazan precipitates are formed by the reaction between NBT and superoxide. (**F**) Sub-cellular localisation of H_2O_2 production (arrows) in a resistant tomato root injured by avirulent nematodes, as shown by EM using the cerium perhydroxide technique. (**G**) Confocal laser scanning microscope detection of intracellular DAF-2DA fluorescence, indicative of NO production in a 12 h infected tomato root with avirulent population. (**H**) Syringaldazine oxidase activity in lignified red-stained cells close to avirulent penetrated nematode (Ne), and (**I**) Orange fluorescence-homovanillic oxidase and (**J**) Napthol AS-D esterase activity in 24 h tomato infected tissues with avirulent nematode, respectively.

knot nematodes have been shown to produce a panel of cell wall degrading enzymes such as pectate lyases and polygalacturonases that degrade the pectic polysaccharides, including the middle lamella (Jaubert *et al*. 2002a), and β1-4-endoglucanases, effective to degrade the cellulose (Abad *et al*. 2003). Characteristics of the efficient method used by the juveniles to silence the host response during its migration, may be suggested by the finding of ESTs homologous to detoxifying oxidoreductases such a superoxide dismutase in *Meloidogyne*.

Compatible interaction

Plants usually recognize the parasite and react by switching on a defence response. The more rapid the response, the sooner the blocking of invaders. Too weak or too late host response leads to successful nematode invasion and to the establishment of a compatible interaction. Expression of the compatible interaction is galling of invaded roots where several giant cells are induced by the nematodes. In response to repeated stimulation from the parasite, cells selec-

ted as feeding sites undergo additional nuclear division without cytokinesis, and assume a new differentiate state termed the giant cells. The giant cells act as sinks, diverting plant nutrients to provide metabolic energy for the nematode. Each nematode triggers the development of five to seven giant cells. Under optimal conditions, giant cell expansion can result in a final size of 600-800 µm long and 100-200 µm in diameter (Goverse *et al*. 2000), each containing as many as 100 enlarged nuclei (**Fig. 2B**). Concurrently, cortical cell division also occurs in the area around the nematode, forming the characteristic galls and the distorted root structure. This leads to a malfunctioning root system with disruption of continuity of vessel elements and alteration of all the vascular tissue. The magnitude of such dysfunction is often related to the number of nematodes that penetrated and became established within the root tissue of young plants. Severe nematode attack results in a dense, bushy root system where water and nutrients absorption and upward translocation is seriously affected. Giant cell development and gall formation are important parts of the sophisticated physiological interaction that occurs between the plant and root-knot nematodes as the parasitic relationship progresses. Disturbance caused by *Meloidogyne* in compatible hosts has been extensively reviewed (Bird 1974; Jones 1981).

Induction of giant cells

The first sign of giant-cell induction by root-knot nematode is the formation of a binucleate cell (Jones 1981; Melillo *et al*. 2006). Binucleate cells are found associated with juveniles 24 h after root infection, and become multinucleate within 48 h. Nuclei in the giant cells are enlarged and lobate, and may contain 14-16 times more DNA than do normal root tip nuclei (Goverse *et al*. 2000). The physiological function of the giant cell to supply nutrients to the nematode is underlined by additional cellular features namely small vacuoles, proliferation of endoplasmic reticulum, ribosomes, mitochondria, and plastids. Analogous to transfer cells, thickening of cell walls adjoining the xylem vessels increases the surface of plasma membrane by means of finger-like invaginations and favours the water transport from vessels to the giant cell (Jones 1981). Later, giant cells have also been demonstrated to have a proton-coupled transport system located between the plasma membrane at wall ingrowths and xylem vessels (Dorhout *et al*. 1992). Amino acids and sugar transport into the higher plant cells is generally thought to be mediated by a proton motive force and there is evidence in support of a chemiosmotic model of proton-amino acid symport (Bush 1993). It is now recognized that plant plasma membrane H^+-ATPases are encoded by a multigene family which are subjected to differential, temporal, and spatial gene regulation and encode isoprotein with different kinetic and regulatory properties (Palmgren and Christensen 1994). The large increase of the plasma membrane in giant cells suggests an increase in the proton pumping system and molecule carriers involved in the translocation of nutrients for the nematode into the giant cells. As a consequence, it seems likely that the genes encoding certain constitutive enzymes and structural proteins may be up-regulated or, at least, altered in their expression in order to support the increased cellular metabolic activity that is related to nematode feeding. More likely, the substantial alteration of cells of the root vascular system means that some plant genes have their normal expression patterns either quantitatively or qualitatively altered to meet the demands of the nematode (Opperman *et al*. 1994). Although not yet completely proven, there is growing evidence that the nematode induces these changes directly either modifying pre-existing plant transcription factors or by introducing proteins which can function as specific plant transcription factors (Gheysen and Fenoll 2002; Abad *et al*. 2003). Although the mechanism by which the nematodes alter plant gene expression is unknown, secretions from the oesophageal gland cells injected through the nematode stylet seem to be the principal components for plant parasitism. A specific structure "the feeding tube", strictly associated with the stylet, is produced by root-knot as other endoparasitic nematodes in the cytoplasm of nurse cells. The feeding tube, which is produced at the stylet orifice each time new secretions are injected before ingestion, is suggested to serve as a molecular sieve for host cell contents that are ingested by the nematode (Williamson and Hussey 1996). As the stylet does not pierce the plasma membrane of the nurse cells, the feeding tube might serve to collect nutrients from the distal part of fed cells and, more, to select molecules large enough to pass through the opening of the plasma membrane at the stylet orifice. The elaborate membrane system, involving both rough and smooth endoplasmic reticulum, arranged within the feeding tube, probably synthesizes and transports nutrients into the tube (Bleve-Zacheo and Melillo 1997). It is widely accepted that the early events for giant cell induction occur in the presence of nematode derived products. The extensive use of different molecular techniques and the efforts of a number of researchers have acquainted us with a catalogue of the so-called nematode-responsive plant genes (Gheysen and Fenoll 2002) and genes encoding secretions from the nematode and promoting parasitism of the host, termed "parasitism genes" (Davis *et al*. 2004). The authors suggest that particular signals from the nematode interfere with fundamental aspects of plant biology and differentiation. This means that the nematode-encoded factors used to initiate giant cell induction might closely resemble normal plant effectors, and might work in concert with endogenous host signals. Plant peptide signals are known to regulate the physiological processes leading to plant growth and development. Phytohormones (cytokinin and auxin) are proposed to be also implicated in successful nematode infection and a rapid activation of auxin-responsive promoter during gall formation in white clover by *Meloidogyne* has been demonstrated (Hutangura *et al*. 1999). In addition, a direct relation between auxin and nematode development has been found in an auxin-insensitive tomato mutant, where most of the penetrated juveniles failed to induce feeding sites. These observations suggest that auxin signalling is essential in giant cell formation and implies a change in the local auxin balance upon nematode infection (Goverse *et al*. 2000). These authors propose a range of options about the concentration of auxin at the feeding sites: i) auxin is originating from both the plant and the nematode, ii) nematode can manipulate auxin household by secreting substances, such as flavonoids (nematode secretions contain chorismate mutase, an enzyme involved in flavonoid synthesis), which inhibit auxin efflux, and iii) nematode locally increases sensitivity towards indole-3-acetic acid (IAA) and roots are very auxin-sensitive organs. The three options could be valid all-together in regulating the complex machinery of affected cells which leads to the phenotype of feeding sites.

Plant and nematode genes involved in the interaction

Putative gene parasitism in oesophageal glands

Among nematode secretions, those produced by two subventral and one dorsal oesophageal glands and secreted through the stylet in the plant tissue are thought to be the main factors involved in pathogenicity. Secretions seem to interact with receptors that trigger the signal transductions and also to provide specific transcription factors influencing the developmental program of the host. It is quite obvious that the substantial changes occurring in plant cell structure and metabolism require that plant gene expression must be reprogrammed in fed cells. As such, feeding sites must be seen as an extended phenotypic effect of nematode genes. It has been demonstrated that the maintenance of giant cells are an essential requisite for the nematode to complete its life cycle and requires a permanent stimulation

by the parasite (Bird 1974). Extensive efforts have been performed to characterize the molecular triggers of the nematode involved in this manipulation. However, little is known about the spectrum of genes expressed in giant cells and how nematodes manage to orchestrate the changes. Biochemical, cytochemical and molecular techniques evidenced different enzyme activities in the feeding sites but their correlation with nematode feeding action remains to be proven. Most studies for the identification of stylet secretions from root-knot nematode have been conducted at the gene and transcript level. Genes coding for such secretions are considered nematode parasitism genes and many of them have been found to be differently expressed in fed cells compared with normal cells (Gheysen and Fenoll 2002). However, tools for functional analysis of nematode genes involved in pathogenicity are still lacking and their molecular identification has been matter of the past few years (Davis *et al*. 2004). Direct purification of nematode secretions and analysis of their components have given limited results because of difficulty in collecting sufficient material for analyses. Recently, the technique was greatly improved by using chemical reagents which stimulate secretory production. Differential gene expression, cDNA library screening, direct analysis of secreted proteins, and whole nematode expressed tag (EST) analysis identified gland expressed genes (Doyle and Lambert 2002; Jaubert *et al*. 2002a). High sensitive analytical tools combined with a micro-sequencing system allowed the identification and a direct qualitative analysis of seven proteins secreted by *M. incognita* juveniles. Among these proteins, a calreticulin was found to be expressed in the subventral oesophageal glands and its role as a potential candidate acting in the differentiation of giant cells was hypothesized (Jaubert *et al*. 2002b). The direct approach of micro-aspirating the cytoplasm of oesophageal cells from parasitic nematodes to generate cDNA libraries of gland expressed genes provided a profile of 37 candidate parasitism genes and a gland-cell specific library of *M. incognita*. Moreover, EST analysis combined with *in situ* hybridization determined the level of specific parasitism genes in gland cells and their different expression pattern during the life stage of the nematode (Huang *et al*. 2003, 2005; Long *et al*. 2006). In the past few years, a consistent number of candidate parasitism genes were found to be expressed in the subventral gland cells of preparasitic and migratory parasitic J2. However, most of the parasitism genes identified had no similarity with any reported genes in database and very few homologs in *Coenorhabditis elegans* (Davis *et al*. 2004; Vanholme *et al*. 2004). This indicates that many of these genes are novel and/or must be considered as potential adaptations for parasitism. In spite of lack of information in databases, some evidence in the elucidation of gene function has been achieved by looking, for example, for specific protein domain architecture and for localisation of the secreted protein in plants at cellular and subcellular levels (Vanholme *et al*. 2004; Jaubert *et al*. 2005). Besides overexpression of a specific gene in the plant, a more comprehensive functional analysis is provided by RNA interference (RNAi). This technology is widely applied in plant and animal models and has been extensively used to characterise gene function. The availability of a protocol for RNAi in plant parasitic nematodes provided a powerful tool for investigating the knock-down of genes potentially involved in parasitism, such as calreticulin and polygalacturonase genes (Rosso *et al*. 2005) and peroxidase and NADPH oxidase (Bakhetia *et al*. 2005). Furthermore, knocking-out of the *Mi-cpl-1* gene expressed in the intestine led to the reduction of *M. incognita* feeding efficiency based on less egg-laying females and, more, a delay in egg-laying (Shingles *et al*. 2007). Recent evidence also suggests that nematodes may have evolved a mechanism to mimic plant signal peptides for parasitic modification of host cells. A nematode-secreted parasitism peptide, 16D10, has been demonstrated to function as a signalling molecule to induce root proliferation by specifically targeting the host plant SCARECROW-like transcription factors (Huang *et al*. 2006a, 2006b). A comprehensive review on root-knot and cyst nematode parasitism genes can be found in Baum *et al*. (2007).

Gene up-regulated in giant cells

The complex morphological and physiological changes that take place in the establishment and maintenance of giant cells rely on altered gene expression in the host cells. Many changes in plant gene expression at feeding sites (giant cells) have been excellently reviewed (Gheysen and Fenoll 2002). Most of these genes are induced in both compatible and incompatible interaction with some difference in levels and timing. This general response can be enhanced or depressed later on, depending on the signals between host and parasite at the establishment of their interaction. 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 (Abad *et al*. 2003). Molecular databases may give indication on the function of the products they encode. As examples, genes encoding peroxidase and lipoxygenase are induced very early during nematode infection and analysis of cellular expression together with biochemical investigation indicate that they are not only involved in the defence response but also include pathway resulting in secondary metabolite production (phytoalexins and physical barriers such as lignin) (Williamson and Hussey 1996). A tomato gene (*LeMir*), found to be rapidly induced after infection with *M. javanica* was related to defence response but the protein encoded by *LeMir* contains a putative signal peptide, indicating that it enters the secretory pathway and may have a role in plant maintenance and development (Brenner *et al*. 1998). The active metabolism of giant cells is maintained by a cohort of house-keeping or general metabolism genes that are up-regulated in these structures and can also be coupled in functional pathways. Among early induced genes there are several cell cycle genes, which are involved in the acytokinetic mitosis (repeated nuclear division without cell division) of giant cell formation. The massive water transport from the xylem through cell wall ingrowths of giant cells requires up-regulation of corresponding genes (*TobRB7*) for regulatory physiology and water status (Gheysen and Fenoll 2002). Screening of the genes up-regulated in infected roots demonstrated that these genes are regularly expressed in healthy plant and that their normal function is recruited by pathogen-induced plant promoters. This is the case of promoter activation and protein accumulation of HMGR, the key enzyme for phytosterol biosynthesis, in giant cells. Sterols are essential for hormone (auxin) signalling, contribute to membrane-associated metabolic processes, and are required for correct vesicle trafficking in the developmental control of the plants (Lindsey *et al*. 2003). Increasing of membranes for the extensive plasma membrane/wall biogenesis in giant cells and the finding that the HMGR enzyme, an endoplasmic reticulum-integral protein, colocalises with tubulin and kinesin (this means an active vesicle transport in giant cells) seem to support this role (Bleve-Zacheo and Melillo 1997). Nematode-responsive promoters have been shown to up-regulate specific genes involved in the biochemical make-up of giant cells (Gheysen and Fenoll 2002; Abad *et al*. 2003). Recently, a group of plant proteins, expansins, identified as wall loosening factors and as facilitators of cell expansion, have been identified and the induction of a tomato expansin expression in gall cells adjacent to feeding cells has been demonstrated (Gal *et al*. 2006). Recent studies have identified a system that regulates the flux of the growth factor auxin through the plant tissues via the subcellular asymmetric localization of specific transporters (Fleming 2006). Auxin stimulates the binding of Aux/IAA proteins to the receptor TIR1, a component of the ubiquitin–mediated protein degradation. Aux/IAA proteins influence gene transcription through interaction with members of auxin res-

ponse factors, which means that altering the Aux/IAA protein level mediates a transcriptional response to auxin. Aux/IAA proteins are encoded by a large gene family and different combinations of Aux/IAA genes might give specific but different read-out of the same input (Fleming 2006). Nematodes could locally manipulate the auxin levels by perturbing the polar auxin transport through the induction of the flavonoid pathway. Nematode secretions contain chorismate mutase, a precursor for the synthesis of aromatic amino acids and various secondary metabolites through the shikimate pathway of plant (Long *et al*. 2006) and of chorismate-derived compounds involved in the biosynthesis of the plant hormone indole-3-acetic acid (Goverse *et al*. 2000). The auxin-responsive promoter *GH3* has been also found to be rapidly and transiently activated during root gall initiation by *Meloidogyne* and associated with the activation of the flavonoid pathway (Hutangura *et al*. 1999). Plasma membrane ATPases are other genes that are up-regulated in giant cells. ATPases are activated by auxins to pump protons out of the cell, resulting in acidification of the cell wall. The so called acid-growth may be required for the rapid expansion of the giant cells (Gheysen and Fenoll 2002). Data available in literature seem to suggest that nematodes may use not all but only genes essential for feeding site induction from developmental pathways made available by the plant. Direct analyses of micro-aspired secretions of pre-parasitic and parasitic stage of nematodes gave further understanding of the parasitic process in the plant. Moreover, injection of *Meloidogyne* secretory components in *Arabidopsis* roots induced gall-like swellings (**Fig. 2C**), very similar to those observed in roots infected with root-knot nematodes, with hypertrophy of cortical cells and dense cytoplasm in injected parenchyma cells (Bleve-Zacheo, Rosso, Abad, unpublished results).

TOMATO RESISTANCE AGAINST ROOT-KNOT NEMATODES

The incompatible interaction induced by the *Mi-1* resistance gene

Nematode resistance genes have been defined as host genes that restrict or prevent nematode multiplication in a host species. In tomato, the single dominant gene *Mi-1* gene confers effective resistance against the three main root-knot nematode species, *M. incognita*, *M. arenaria* and *M. javanica*. All currently available root-knot resistant tomato cultivars are derived from this source. The phenotypic expression of incompatibility to *M. incognita* in tomato roots consists of an early hypersensitive reaction which involves localized host-cell necrosis, cellular disorganization and restricted nematode development at the infection site (Williamson and Hussey 1996).

The *Mi-1* gene was isolated by a positional cloning strategy and was shown to encode a protein sharing structural features with the nucleotide-binding site leucine-rich repeat (LRR)-containing class of plant resistance genes (Milligan *et al*. 1998; Vos *et al*. 1998). Although the role of the *Mi-1* gene-encoded protein has not yet been elucidated, data are accumulating that provide new insights into the signal transduction pathway leading to resistance. *In vitro* mutagenesis experiments showed that both the LRR region and the N terminus of the protein have a role in signalling and regulating the localized cell death, respectively (Hwang *et al*. 2000; Hwang and Williamson 2003). Moreover, the NBS domain of the protein was shown to bind and hydrolyze ATP, which may allow recruiting additional proteins (Tameling *et al*. 2002). Recent results also indicated that salicylic acid (SA) is an important component of the signalling that leads to nematode resistance and the associated hypersensitive response (Branch *et al*. 2004), as shown in the resistance response of many plants to various pathogens. In addition, the tomato *Rme-1* gene was demonstrated to be required for *Mi-1*-mediated resistance (de Ilarduya *et al*. 2001), probably *via* an indirect interaction involving some nematode product (i.e. the 'guard hypothesis'; Dangl and Jones 2001).

Variation in resistance of tomato genotypes carrying the *Mi-1* gene

Although the *Mi-1* gene should block nematode development at an early stage of the interaction, it does not confer total immunity, and occurrence of and variation in *Meloidogyne* spp. reproduction on resistant tomato genotypes has been documented (Tzortzakakis *et al*. 1999; Lopez-Perez *et al*. 2006). The reproduction of *M. javanica* isolates with partial virulence (i.e. with a low rate of reproduction on resistant cultivars) was much greater on tomato genotypes heterozygous for the *Mi-1* gene than on homozygous genotypes, suggesting a dosage effect of the *Mi* gene on partially virulent isolates only (Tzortzakakis *et al*. 1998). More recently, the influence of both the allelic state at the *Mi-1* locus (homozygous vs. heterozygous) and the tomato genetic background was demonstrated (Jacquet *et al*. 2005). Similar results were obtained with tomatoes carrying the *Mi-3* resistance gene (see below), with plants homozygous for *Mi-3* expressing more effective resistance than heterozygous ones (Yaghoobi *et al*. 2005).

Other resistance genes identified in wild tomato

The resistance conferred by *Mi-1* is no longer effective at soil temperatures above 28°C (Williamson and Hussey 1996), and can be overcome by virulent isolates of the nematode (see below). Due to the current reduction of chemical control strategies, new sources of resistance that could circumvent these drawbacks are urgently needed. For that purpose, the wild tomato relative *S. peruvianum* complex, from which the *Mi-1* gene was identified, has been extensively surveyed, and new resistance factors were found in different accessions. Indeed, new resistance genes (i.e. that segregate independently of *Mi-1*) were genetically characterized, that proved to be heat-stable at temperature above 28°C (i.e. *Mi-2*, *Mi-4*, *Mi-5*, *Mi-6* and *Mi-9*; Cap *et al*. 1993; Veremis and Roberts 1996a, 1996b; Veremis *et al*. 1999), or confer resistance to *Mi-1* virulent isolates (i.e. *Mi-7*, *Mi-8*; Veremis and Roberts 1996a), or share both properties (i.e. *Mi-3*; Yaghoobi *et al*. 1995). Among them, *Mi-3* and *Mi-9* have been studied more extensively, and their cloning is in progress. The *Mi-3* gene has been mapped to the short arm of tomato chromosome 12 (Yaghoobi *et al*. 1995), and fine mapping allowed the construction of a DNA contig which spans the *Mi-3* locus, with flanking markers within 0.25 cM of the gene (Yaghoobi *et al*. 2005). The *Mi-9* gene has been localized on the short arm of chromosome 6, in a similar genetic interval as *Mi-1* (Ammiraju *et al*. 2003). Very recently, a molecular genetic analysis of the *Mi-9* locus identified seven *Mi-1* homologues in the corresponding chromosomal region, and further experiments of virus-induced gene silencing showed that *Mi-9* is in fact a homologue of *Mi-1* (Jablonska *et al*. 2007).

Virulence in root-knot nematodes

The interaction between *M. incognita* and resistant tomatoes carrying the *Mi-1* gene probably constitutes the plant-nematode model system for which most research efforts have been devoted (Williamson 1998). Although it has been highly efficient in most agronomic situations, the intensive use of the *Mi-1* gene for more than sixty years, along with the pathogenic variability of root-knot nematodes, raises concern about the durability of the resistance in the next future. First, although the *Mi-1* gene should block nematode development at an early stage, occurrence of and variation in *Meloidogyne* spp. reproduction on *Mi-1*-resistant tomato genotypes has been documented. Second, *Meloidogyne* spp. biotypes virulent against the *Mi-1* gene (i.e. able to reproduce on *Mi-1*-resistant tomatoes) have

been reported from most of the tomato growing areas in the world (Castagnone-Sereno 2002). Moreover, previous experimental works showed that selection of *Mi-1*-virulent lines from the progeny of single *M. incognita* avirulent females was also possible under laboratory conditions (Jarquin-Barberena *et al*. 1991).

As with many other plant parasites, a gene-for-gene relationship has been postulated as a model for the interaction between *M. incognita* and tomato. However, in contrast to amphimixis, obligate parthenogenesis in the nematode precludes any possibility for male × female crossing, and thus Mendelian genetic approaches cannot be performed to study the mode of inheritance of (a)virulence in this species. To avoid such a drawback, isofemale line selection studies were designed that demonstrated the genetic determinism and inheritance of *M. incognita* virulence against the tomato *Mi-1* resistance gene (Castagnone-Sereno *et al*. 1994). Although the precise number of genes involved could not be inferred from these experiments, the occurrence of a polygenic system was nevertheless suggested as a consequence of the progressive increase observed in nematode reproduction on resistant plant over successive generations of selection (Castagnone-Sereno *et al*. 1994). No genetic mapping of the loci involved in (a)virulence can be performed, but the occurrence of molecular markers correlated with this character has been investigated, with contrasted results. Fingerprinting of avirulent and virulent *M. incognita*, *M. javanica* and *M. arenaria* isolates (the two latter being also controlled by the tomato *Mi-1* resistance gene) with 1550 AFLP markers did not allow to cluster them in correlation with their (a)virulence phenotype, which suggested that the virulent populations did not share a common origin and probably resulted of independent mutational events (Semblat *et al*. 2000). RAPD analyses identified a marker specific for some virulent isolates of the three same RKN species, but this *Mi*-virulence correlated marker proved to be significantly different between natural and selected virulent isolates, indicating that the genetic events leading to virulence against *Mi* may be different between the two types of isolates (Xu *et al*. 2001). Moreover, this result was obtained with virulent isolates originating from East Asia, and the applicability of the marker to virulent isolates from other geographic origins could not be demonstrated (Castagnone-Sereno, unpublished data). All together, these data tend to indicate that (a)virulence in RKN against the *Mi-1* resistance gene in tomato is not governed by one single gene, but rather involves a more complex genetic system.

At the molecular level, the determinants of the interaction between resistant tomato and (a)virulent root-knot nematodes have not yet been elucidated. The first candidate gene coding for a nematode avirulence (Avr) protein was isolated based on comparative AFLP fingerprinting of *M. incognita* virulent and avirulent near-isogenic lines selected on *Mi-1*-resistant and susceptible tomatoes, respectively, just differing in their ability to reproduce or not on resistant plants (Semblat *et al*. 2001). This gene, name *map-1*, encoded a putative protein containing a predictive N-terminal secretion signal peptide, but lacking any known homology with proteins in databases. Interestingly, antibodies raised against MAP-1 specifically labelled amphidial secretions from infective second-stage juveniles (Semblat *et al*. 2001). In that respect, MAP-1 may be involved in the early steps of recognition between resistant plants and avirulent nematodes. Further functional analyses, including the recently developed RNAi procedure (Bakhetia *et al*. 2005; Rosso *et al*. 2005), will no doubt help to reveal the function of this protein in the plant-nematode interaction.

Using the same model system, a cDNA-AFLP-based transcriptomic approach has been developed to monitor differences in gene expression between avirulent and virulent *M. incognita* isogenic lines, which resulted in the identification of 22 transcript-derived fragments (over more than 24,000 generated) present in avirulent lines and absent in virulent lines (Neveu *et al*. 2003b). Fourteen of the sequences did not show any significant similarity in databases, while 8 matched reported sequences from nematodes and other invertebrates. Analysis of the full-length cDNAs revealed a signal peptide for some of these candidates, and further *in situ* hybridization experiments showed specific expression in the intestinal or esophageal gland cells of infective J2 (Neveu *et al*. 2003b). Among them, a cysteine protease gene, *Mi-cpl-1*, was shown to be expressed only in the developmental stages which are in close interaction with the root tissues (i.e. juveniles and females), which suggested that the cysteine protease in *M. incognita* is related to the parasitic aspects of the plant-nematode relationship, e.g. pathogenicity and/or evasion of primary host plant defence systems (Neveu *et al*. 2003a). Very recently, the knock-out of the *Mi-cpl-1* gene in independent RNAi experiments indeed showed a significant reduction of the number of nematodes infecting tomato plants, which confirmed the role of this gene in the parasitic success of *M. incognita* (Shingles *et al*. 2007; Rosso *et al*., unpublished data). In the near future, a more profound knowledge of the genes/functions involved in the incompatible interaction between *Meloidogyne* spp. and tomato will provide new basic information for the development of durable resistance in this crop.

THE TOMATO DEFENCE RESPONSE

Involvement and interaction of various signalling compounds in the defence response

Recent research on molecular mechanisms underlying plant defence has revealed molecular organisation of plant systems for non-self recognition and anti-microbial defence. The disease resistance program in plants is initiated upon recognition of microbial virulence factors (Hammond-Kosack and Parker 2003). Signal perception is the first committed step of the elicitor signal transduction pathway and much effort has been put into isolation of effective signal molecules from fungal and other pathogens and plant extracts and identification of the corresponding receptors from the plasma membrane. Signal transduction cascades linked to recognition and defence response does no show significant differences in plants upon perception of different pathogens or general elicitors (Nürnberger *et al*. 2004). However, individual recognition appears to dictate specific signalling routes that employ a distinct set of secondary messengers and activate a specific portion of the defence machinery. Among the numerous genes whose expression changes in infected plants, the activity of some genes involved in secondary metabolism are affected upon pathogen infection. In particular, the phenylpropanoid pathway that is specific to plants and provides an array of molecules with important functions in plant-microbe interaction such as phytoalexins, monolignols, the signalling molecule SA, and the shikimate pathway, which provides chorismate that can be converted into SA. These changes are correlated with the co-ordinated regulation of plastid primary carbon metabolism and the shikimate pathway transcripts (**Fig. 2D**). Later, genetic analysis of global gene expression profiles demonstrated that plant defences against pathogens are regulated by cross-communicating signalling pathways in which SA, jasmonic acic (JA), and ethylene (ET) play key roles. Monitoring the dynamics of SA, JA and ET signalling in *Arabidopsis*, following the attack with different pathogens, showed that the signal signature characteristic of each *Arabidopsis*-attacker combination is orchestrated into a complex set of transcriptional alteration in which, in all cases, stress-related genes are overexpressed (de Vos *et al*. 2005). Increasing evidence indicates that JA-induced changes in secondary metabolites constitute a ubiquitous plant defence response. The widespread phenomenon of JA-and stress induced hydroxycinnamic acid amide production is consistent with a role of this compound in the defence of plants, including tomato (Chen *et al*. 2006). Jasmonic acid is a lipid-derived signal synthesized by the octadecanoid

pathway. The initial reaction comes from lipoxygenases (LOXs) and linoleic and linolenic acids, the most abundant fatty acids in the lipid mojety of plant membranes that represent their major substrates. The oxygenation step leads to a reaction cascade (called the LOX pathway), in which the hydroperoxides, produced by the LOX activity, are substrates for JA, methyl jasmonate, conjugated dienoic acids, and volatile aldehydes (Veronico *et al*. 2006). Recently, tomato plants were used as a model system since the peptide systemin and the lipid derived JA were recognised as essential signals in wound-induced gene expression (Wasternack *et al*. 2006). The occurrence of such hydroxyproline-rich glycosilated systemins in tomato seems to confirm biochemical data of hydroxyproline-rich glycoprotein (HRGP) increasing in hypersensitive response of tomato to *Meloidogyne* spp. infection (Zacheo and Bleve-Zacheo 1995). Furthermore, the transcript levels and enzymatic activity of a methyl jasmonate esterase, a member of the αβ-fold hydrolases, in tomato roots are in agreement with histochemical findings of an increased activity of carboxy and acetyl-esterases in roots of tomato resistant to *M. incognita* (Melillo *et al*. 1989). Application of exogenous methyl jasmonate to roots is known to induce nematode resistance in spinach and oats (Soriano *et al*. 2004). Mutagenized *Arabidopsis* lines that are deficient in jasmonate signalling have increased susceptibility to some soil fungi, and a tomato mutant deficient in JA synthesis is also highly susceptible to *Fusarium oxysporum* and *Verticillium dahliae* (Thaler *et al*. 2004). The systemic nature of the induced response to foliar JA treatment in tomato and grape also supports the hypothesis that plant defences can mediate indirect interactions between above-and below-ground pests (van Dam *et al*. 2003). A foliar application of JA to activate induced resistance to root-knot nematode in two near-isogenic lines of tomato with and without the *Mi-1.2* resistance gene showed that JA induces a systemic defence response that reduces avirulent nematode reproduction on susceptible tomato plants (Cooper *et al*. 2005).

The oxidative burst

Phytohormones and other endogenous signalling molecules such as SA and JA are known to regulate plant growth and development as well as to activate a defence response. However, biochemical and genetic studies have identified other classes of molecules that also have significant signalling properties in plants. Early signalling events between host and pathogen that occur in the apoplast and membrane surface in the host-pathogen interaction, where the pathogen does not cross the host plasma membrane (as endoparasitic nematodes seem to do), lead to the generation of hydrogen peroxide (H_2O_2) in the apoplast and to calcium influx in the symplast. Alkalinisation of the apoplast is an inevitable direct result of the chemistry of the oxidative burst on the outer surface of the plasma membrane. Moreover, it has been shown that apoplastic pH increases during the hypersensitive response and, more, apoplastic changes in pH are very important indicators of specific host defence response that are mediated by recessive or major resistant genes (Pignocchi and Foyer 2003). A stress-induced oxidative burst is commonly caused by many abiotic and biotic stresses and it is a signature of the HR to pathogen attack. The oxidative burst has several functions in addition to signal transduction and it is required to limit the spread of the pathogen, by favouring, for example, the cross-linking of the cell walls. Most importantly, the oxidative burst also sends signals to the attacked cell cytoplasm and to the other non-attacked inducing local cell death. This signalling is complex and involves both plant hormones and alteration in the concentration and redox status of antioxidants such as ascorbic acid (AA). Application of AA to susceptible tomato plants has been shown to inhibit *M. incognita* invasion and, conversely, a decrease in its endogenous content in resistant plant to increase nematode infection. Moreover, it has been demonstrated that not the endogenous level of AA differentiates susceptible and resistant plants, but the biosynthetic capability of the plant to synthesize a large quantity of AA following nematode attack (Arrigoni *et al*. 1979). AA might be utilised for the synthesis of mitochondrial hydroxyproline proteins which control the cyanide resistant respiration. Evidence for a potential activity of an alternative respiratory pathway in tomato roots infected with *M. incognita* has been reported by Zacheo and Bleve-Zacheo (1995). Enhanced cyanide resistant respiration in resistant tomato has been proposed to be linked to some terminal oxidases, including ascorbic acid peroxidases, and to be related to the HR. The activation of AA and the ascorbate pool would generate in the apoplast an inactive or oxidised state which activate or inactivate the defence processes. Pignocchi *et al*. (2006) showed that decreasing of AA in the apoplast alone, independent of the cytoplasmic AA pool, is able to mimic the effect of reactive oxygen species accumulation in the apoplast of tobacco cells infected with *Pseudomonas syringae* and that plant defence can be brought about by regulation of ascorbate oxidase activity. In addition, they suggest that regulation of the AA pool in the apoplast could be used to modulate cross-talk between different defence pathways in a similar manner to that already described for reactive oxygen species (Torres and Dangl 2005).

Reactive Oxygen Species

The production of reactive oxygen species (ROS) is among the earliest temporal events following pathogen recognition in plants. ROS are proposed to orchestrate the establishment of the plant defence response and HR. Key lines of evidence implicated an NADPH oxidase, analogous to that which generates superoxide during the respiratory burst in mammalian phagocytes, as the source of ROS detected in plants upon successful pathogen recognition. The mammalian NADPH oxidase, also known as the respiratory burst oxidase (RBO), consists of two plasma membrane proteins, $gp91^{phox}$ and $p22^{phox}$. Plant species contain *Respiratory burst oxidase homolog* (*Rboh*) genes that have homology to the neutrophil $gp91^{phox}$. The $gp91^{phox}$ homologs from *Arabidopsis*, *Nicotiana*, *Lycopersicon esculentum*, and other plant species have been shown to be required for ROS accumulation and as a signal transducer of stress and developmental responses (Torres and Dangl 2005). Plant Rboh is regarded as a quantitative player in dictating the cellular *milieu* of ROS flux, and their modulation would demand metabolic adjustment as suggested by compensatory fine-tuning in transcriptome profiles. *Rboh* genes are reported to be transcriptionally up-regulated by pathogens. Genetic proof of the function of *Rboh* in the pathogen-induced oxidative burst comes from analysis of *Rboh* mutants and antisense lines (Yoshioka *et al*. 2003). Plants possess a battery of scavenging systems, including ascorbate peroxidases, glutathione, superoxide dismutases and catalases, that maintain ROS homeostasis. When down-up regulation of scavenging systems in response to development, abiotic stress and pathogen infection may be made between multiple cellular responses, the strength, pulse length, and spatial context, as well as the interaction of ROS with other signals, are likely to play a role (Sagi and Fluhr 2006). Whereas much attention has focused on the role of pleiotropic effects of ROS overproduction, evaluation of ROS generation in different amount, or with different rates and times that might provoke different effects not only as dose-dependent result, but as a consequence of the activation of different metabolic pathways has been recently reported (de Pinto *et al*. 2006). A number of studies have also demonstrated that different ROS may trigger opposite effects in plants, depending on the intensity and time of their generation (Delledonne *et al*. 2001). Recently, it has been shown that tomato plants react to root-knot nematodes by mounting ROS production (**Fig. 2E**). The timing and extent of the ROS production differed between compatible and incompatible reaction, providing some clues as to which of these responses

may be effective in defence (Melillo *et al*. 2006). In the compatible interaction, ROS and H_2O_2 generation was seen at the time of nematode invasion (12 h after inoculation) and became cytologically undetectable at 48 h, concomitantly with giant cell induction. These changes in the oxidative response of the plant are reminiscent of other plant-pathogen interactions where different levels and kinetics of ROS production activate different responses. The noticeable differences in ROS production and the time course of ROS generation observed in the different tomato-nematode interactions indicate that superoxide induction is an early event during nematode infection. High concentration of H_2O_2, essential precursor of hydroxyl radicals, appears to play a key role in the oxidative events during early stages of infection, too. It is known that H_2O_2 acts as a signalling molecule that triggers gene activation, or as a cofactor in a process that requires new gene expression for both localised cell death and induction of defence genes in adjacent cells (Mellersh *et al*. 2002). Excess of H_2O_2, produced during the HR in incompatible interaction, suggests its direct role as an antimicrobial agent and as the cause of localised membrane damage at the site of nematode infection. The largely apoplastic location of cerium perhydroxide deposits in both internal cell wall regions and on the surface of the plasma membrane (**Fig. 2F**) indicate that either the plasma membrane or the cell wall is the primary site of the ROS/H_2O_2 generator. Furthermore, the cerium deposits on the plasma membrane have a distinct spatial pattern which suggests the presence of a single origin, presumably NADPH oxidase complex. Experiments with inhibitors of possible sources for H_2O_2 in the cell walls also provided a good system in defining the sub-cellular site for ROS production. The different pattern of ROS and H_2O_2 in compatible and incompatible interaction, between 12 and 48 h after nematode inoculation, may suggest that the first 24 h are critical for determining the plant response to invading nematodes (Melillo *et al*. 2006). According to the literature, during the incompatible reaction, when the nematode is detected and defence response including cell death is induced, the initial and rapid accumulation of H_2O_2 is followed by a prolonged burst of H_2O_2 production. In the compatible interaction, only the first peak of H_2O_2 occurs, confirming that hydrogen peroxide not only acts as causal trigger for HR but also activates genes encoding enzymes that prevent cells from oxidative damage (Apel and Hirt 2004). This suggests that if general oxidation of the root tissues, attributable to H_2O_2 generation, had occurred, it was transient and reversed by 24 h after inoculation. ROS are known to be produced in different sub-cellular compartments and to influence the expression of a large number of genes in plants. During the incompatible-pathogen interaction, superoxides (O_2^-) are produced enzymatically outside the cell and are rapidly converted to hydrogen peroxide that can cross the plasma membrane. Extracellular peroxidases are considered to catalyse H_2O_2-dependent mechanism of ROS generation. It is also well recognized that plants possess a plasma membrane (PM)–NADPH oxidase as a source of ROS production, and this suggests that both POXs and PM-NADPH oxidase are the major sources for ROS production as defence mechanisms during biotic stresses (Yoshioka *et al*. 2003). Alternatively, different sub-cellular antioxidants might contribute to local redox changes as visualized by distinct NBT staining patterns and DCFH reaction in the compatible interaction. Overall, the spatio-temporal differences in the production and accumulation of ROS and H_2O_2 generation in the nematode-host interaction might form a discrete unit for defence response (Melillo *et al*. 2006). However, no single defence mechanism has been unequivocally proven to operate in plant cells.

Nitric oxide

Several lines of evidence suggest that nitric oxide (NO) is an important signal in plant-pathogen interactions and the best characterised relationship between NO and ROS refers to its role in plant defence, in particular in the establishment of HR (Wendehenne *et al*. 2004; Zaninotto *et al*. 2006). NO is endogenously produced from L-arginine, NADPH, and molecular oxygen, by constitutive and inducible form of nitric oxide synthase (NOS). Although it is widely presumed that NO production is catalysed by NOS, neither a gene nor a protein homologous to mammalian NOS has been isolated from plants to date. However, Guo *et al*. (2003) identified a NO-generating enzyme, *AtNOS1*, within the *Arabidopsis* genome. *AtNOS1* is smaller than mammalian NOS and exhibits no sequence homology to NOS and seems to produce NO by oxidising arginine. In contrast, there is a clear evidence that plants can produce NO from nitrite via NADPH-dependent nitrate reductase. In mammals, the cytotoxic effects of NO derive from the diffusion-limited reaction of NO and O_2^- to form the potent oxidant, peroxynitrite. Peroxynitrite is a lipid permeable molecule, with a wider range of chemical targets than NO, which can oxidise proteins, lipids, RNA, and DNA, and lead to cell injury and cell death. In plants, peroxynitrite does not appear to be an essential mediator of NO-ROS-induced cell death, which seems to be activated by the relative level of NO and H_2O_2 that is formed by dismutation of O_2^- (Delledonne *et al*. 2001). Furthemore, by abrogating O_2^--mediated cytotoxic effects through the conversion of O_2^- into peroxynitrite and by increasing the level of cyto-protective proteins such as catalase, superoxide dismutase, glutathione, and alternative oxidases, NO may protect cells against oxidative stress (Delledonne *et al*. 2001; Wendehenne *et al*. 2004). During the hypersensitive response, death of certain plant cells is beneficial for the plant. However, plants may also have developed mechanisms against high intercellular NO concentrations. The presence of various molecules acting as NO scavengers could help to prevent unwanted NO reactions. NO affects the mitochondrial cytochrome oxidase, but not the alternative oxidase, whose transcription and activity can be stimulated by NO. Studies on the NO induction of gene expression in plants revealed that NO can modulate the expression of transcription factors, receptors, and several pathogen-induced genes in addition to genes known to respond to oxidative stress such as several POXs and key enzymes of JA biosynthesis. Moreover, analysis of gene expression during NO-ROS-mediated cell death identified genes specifically induced by NO or by H_2O_2, as well as the set of genes regulated by both. This indicates the complex inter-connection between the two molecules in the cell death signalling (Zaninotto *et al*. 2006). In that respect, the presence of an NO generation system (NO and NOS) and its relation to the oxidative burst in the HR of tomato roots to *M. incognita* were investigated (Leonetti P, Melillo MT, Leone A, Bleve-Zacheo T, submitted). Confocal laser microscope (**Fig. 2G**) and cytofluorimetric analyses indicated NO production at a very early stage of nematode infection. In addition, occurrence of NOS activity, increasing in parallel with NO production, and accumulation of H_2O_2 was detected in cells undergoing HR. Hypersensitive cell death, occurring in the incompatible interaction 24h after nematode inoculation and its increasing in intensity in a time dependent manner, clearly induced DNA laddering, obviously not observed in the compatible interaction (Leonetti P, Melillo MT, Leone A, Bleve-Zacheo T, submitted). Jointly, these data support the existence of an interplay between signalling networks in both pathogen and hosts that has evolved to allow successful infection by pathogens and host resistance to disease.

Secondary metabolite production

The general cellular process and regulatory principle for activation of plant secondary metabolite biosynthesis is that an extracellular or intracellular signal is perceived by a receptor on the surface of the plasma membrane or endomembrane. The elicitors induce rearrangement of metabolic fluxes between a constitutively expressed pathway and an

elicitor-inducible pathway, which regulate the expression of biosynthetic genes involved in the plant secondary metabolism. These differential regulations of branch compound biosynthesis reflect a feature of elicitor induction of plant secondary metabolites (Zhao *et al*. 2005). It is well known that attack by incompatible pathogens causes an array of defence reactions, including a range of plant defensive secondary metabolites such as phytoalexin-like compounds that act as antimicrobial or repellent agents to kill microbes. Moreover, almost all pathogen elicitors stimulate the phenylpropanoid pathway that leads to biosynthesis of flavonoids as well as lignin and of phenolic compounds. The enzymes chalcone synthase, phenylalanine ammonia lyase (PAL) and 4-coumarate-CoA ligase are rate-limiting enzymes in this pathway to various products. PAL and tyrosine ammonia lyase (TAL) are the key enzymes in the synthesis of lignin. In association with phenol biosynthesis, the activity of PAL and TAL is enhanced in nematode-infected plants. Both enzymes are involved in the synthesis of cynnamic and *p*-coumaric acid and, by ferulic and synapic acid production and their subsequent oxidative polymerisation, lignin is formed (Zacheo and Bleve-Zacheo 1995). Because H_2O_2 is required for cross-linking of cell wall lignin, the oxidative burst is also involved in secondary metabolite production (Zhao *et al*. 2005). Increasing of H_2O_2 and peroxidase (POX) activity during pathogenesis has been demonstrated and accumulation of POXs has also been correlated with nematode infection (Zacheo *et al*. 1997). A group of fast migrating POXs has been purified from resistant isolines of tomato following *M. incognita* infection, and identified as anionic POXs (Zacheo *et al*. 1997). The major changes in peroxidase isozymes, induced by nematode infection, were related to the group of POXs having a particular affinity for syringaldazide, a specific electron donor for lignifying POXs, and indicating a correlation between the level of anionic POXs and lignification (**Fig. 2H**). Electron-microscopy cytochemical analyses using 3-3'-diaminobenzidine/H_2O_2 technique, at acidic pH, showed that POX activity was mainly detectable along the plasma membrane, at basic pH being only located in the vacuoles. These findings clearly indicate a specific compartmentalisation of POXs related to differential and specific metabolic functions. In addition, resistant infected tissues incubated in homovanillic acid, a substrate closely related to lignin monomers, strongly reacted at the level of cell wall and intercellular spaces (**Fig. 2I**), this reaction being very weak in susceptible tissues. This suggests the existence of topologically distinct active sites of POX molecules. Acidic POXs are reported to be located in the cell walls and intercellular spaces and, because of their reactivity towards cynnamil alcohols and isoflavonoid phytoalexins, to be involved in cell wall strengthening (Zacheo *et al*. 1997). Moreover, increase of POX isoenzymes with high affinity for paraphenylenediamine-pyrochatecol, that are reported to be involved in the polymerisation of some phenolic monomers to generate the aromatic matrix of suberin, was also detected following nematode infection. Suberin, an aromatic, aliphatic polymer is a normal component of the Casparian strips of the root endodermis, and modulates the flow of water to the stele. It is also known that cells in which secondary cell wall changes are occurring or will occur, contain high levels of carboxylesterase activity. A correlation between increased carboxylesterase activity and HR has been demonstrated in root tips of resistant tomato infected with *M. incognita* (Melillo *et al*. 1989). The precocious increasing of esterase activity has been suggested to be an early event in the resistant response and the initiation of the program for lignification (**Fig. 2J**). Upon microbial infection, some structural glycoproteins play a major role in the reinforcement of the plant cell wall architecture. Of these structural glycoproteins, HRGP have been fond to increase following infection with different pathogens, including nematodes. This increase, which is correlated with resistance, is mediated by ethylene. Increase of HRGP production in mitochondria of tomato roots hypersensitively reacting to *M. incognita* infection has been attributed to the ability of the host to develop cyanide-resistant respiration, not detected in the compatible interaction. HRGP are one of the mediators for phytoalexin production and are shown to be greatly synthesised by the increased amount of AA in the infected resistant plant (Zacheo and Bleve-Zacheo 1995). Signal transduction leading to biosynthesis of plant secondary metabolites is a complicated network that is closely related to the regulatory machinery of plant defence response. A synergistic effect of multiple signalling molecules has been implicated in the production of secondary metabolites. Despite the evident overlap in signalling that is triggered upon pathogen attack, the plant response is highly dependent on the plant-attacker combination. Little is known about how plants coordinate attacker-induced signal into specific defence response. A well-accepted hypothesis is that modulation of the different defence signalling pathways involved plays an important role in this process. However, information is often highly specific for a given plant-pathogen interaction and, more, different studies are characterised by unique experimental conditions. The large variation in experimental conditions and plant–microbe combinations makes it difficult to integrate these results and draw overall conclusion. It is of paramount importance to understand interplay and modulation of the different signalling pathways and to elucidate whether such responses are triggered by similar initial stages of pathogen recognition. Development of functional genomics, proteomics and metabolomics represents a powerful tool to improve understanding of plant secondary metabolite production and to provide information on the time dependent profiling of metabolites.

BIOTECHNOLOGICAL APPROACHES TO ACHIEVE ROOT-KNOT NEMATODE CONTROL IN TOMATO

Natural resistance and marker-assisted selection in breeding programs

The selection of nematode-resistant cultivars is a major goal in tomato-breeding programs, and the successful introduction of the *Mi-1* gene from *S. peruvianum* into high-yielding varieties is a good example of this strategy. Indeed, the *Mi-1* gene was transferred from *S. peruvianum* PI128657 into *S. esculentum* using embryo rescue. From the initial interspecific cross, one single F_1 plant was used for further breeding by repeated backcrossing, and all the modern fresh-market and processing resistant tomato cultivars are derived from this single F_1 plant (Williamson 1998). Clearly, a conventional breeding program involves successive crossing and selection steps based on careful phenotypic analyses, which make this procedure a laborious and time consuming task. This is particularly true in the case of root pathogens such as plant-parasitic nematodes, for which reliable inoculation and screening of the plants is labour intensive. In this context, it is obvious that marker-assisted selection holds great promise in plant breeding. In fact, the use of DNA technology in plant breeding has opened a new realm in agriculture called 'molecular breeding' (Rafalski and Tingey 1993), based on the use of several kinds of DNA markers (for the description of the main molecular markers and their use, see the review by Kumar 1999).

The intensive labour devoted to the mapping and cloning of the *Mi-1* resistance gene has generated a huge number of molecular markers linked to the gene. For example, RFLP markers have been developed that exhibit a recombination rate with the *Mi-1* locus likely to be less than 1%, which is sufficient for high fidelity testing (Klein-Lankhorst *et al*. 1991; Messeguer *et al*. 1991). After cleavage with the restriction enzyme *Taq*I, the PCR-based REX-1 codominant marker can distinguish homozygous versus heterozygous individuals, and the procedure can be used for rapid, routine screening (Williamson *et al*. 1994). Newly developed AFLP and RFLP markers allowed the physical

localization of *Mi-1* to a region of the genome spanning less than 65 kb (Kaloshian *et al*. 1998), and its subsequent cloning (Milligan *et al*. 1998). Based on the sequence of the *Mi-1* gene, PCR markers located within the gene have recently been developed in order to avoid any segregation between the gene and the marker (El Mehrach *et al*. 2005). Since the gene has been cloned, this procedure represents the ultimate strategy to identify individuals carrying *Mi-1* in segregating progenies, and should be very useful to introduce it into cultivated varieties. Molecular markers of potential interest for marker-assisted selection have also been developed for the *Mi-3* resistance gene. The NR14 marker allowed mapping of the gene on the short arm of chromosome 12, although its linkage with the gene was not so tight (Yaghoobi *et al*. 1995). Recently, a marker that completely cosegregates with *Mi-3*, as well as flanking markers within 0.25 cM of the gene were characterized (Yaghoobi *et al*. 2005). These new markers should be used to aid in introduction of *Mi-3* into cultivated tomato.

However, marker-assisted selection is in some occurrences not sufficiently robust and should be used with caution. This is typically the case when linkage of the marker to the gene of interest is not tight enough. Another problem may occur when inter-specific crosses are needed, e.g. to transfer the resistance trait from a wild species into a cultivated related species, which is often the case for pathogen resistance characters. For example, the NR14 PCR marker was developed for the *Mi-3* gene in *S. peruvianum* (Yaghoobi *et al*. 1995), but works poorly when the gene is transferred into a *S. esculentum* genetic background (C. Caranta, pers. comm.). This is clearly a problem since several generations of back-crossing with the recurrent cultivated parent species are needed to restrict the introgression to its minimal size (i.e. the resistance gene itself), and thus to eliminate most of the wild genetic background. In this situation, efforts must be devoted to the development of new markers.

Transgenic plants harbouring natural resistance genes

Genetic engineering now offers a new alternative for breeding, i.e. directly transferring a previously isolated gene into a genotype of interest. For example, several genes conferring resistance to various plant pathogens have successfully been transferred in tomato, and the transgenic plants exhibited reduced disease (Tai *et al*. 1999; van der Vossen *et al*. 2003). Because it has been cloned, the *Mi-1* resistance gene constitutes a good candidate for such a strategy, and intraspecific transformation of susceptible tomato with *Mi-1* has been successful (Milligan *et al*. 1998). However, the agronomical use of such transgenic plants is hampered by the instability of the nematode resistance in subsequent T_2 and T_3 generations, probably due to some epigenetic effects (Goggin *et al*. 2004). Another practical goal for breeders is to transfer a resistance gene from one species into another one where natural resistance is not currently available, which implies that the gene must function in a heterologous genetic background. Phylogenetic relatedness among plants may explain the success or failure of such a resistance transfer, a phenomenon referred to as restricted taxonomic functionality (Tai *et al*. 1999). In fact, transformation of other Solanaceous crops with *Mi-1* led to contrasted results. In tobacco, none of 19 independent transformant lines exhibited nematode resistance (Williamson 1998), while heterologous expression of the gene in eggplant conferred resistance (Goggin *et al*. 2006). Moreover, *Mi-1* did not confer resistance in plants belonging to more distant families, i.e. *Arabidopsis* (Brassicaceae) and lettuce (Asteraceae) (Goggin *et al*. 2006). Clearly, further studies are still needed to understand the recognition events and signalling pathways underlying *Mi-1*-mediated resistance before transgenic plants harbouring this gene can routinely be used in the field.

Engineering root-knot nematode artificial resistance in tomato

The persistent research efforts pursued in many laboratories worldwide in the past decade have led to a better understanding of the plant response when challenged with root-knot nematodes. Along with the progress in the field of biotechnological applications, such knowledge should allow the identification of new targets and strategies for the implementation of engineered nematode resistance in crop species. There are several approaches for developing transgenic plants with improved nematode resistance. The objective of the following paragraphs is not to present an exhaustive list of all the possible strategies and targets experimented so far to engineer plants with resistance to nematodes, but rather to focus on some promising leads that could help reaching this goal, with examples based on the tomato-*Meloidogyne* interaction, when available.

Anti-nematode-based resistance

Anti-nematode effectors

Besides their effects on the parasite, the effective use of anti-nematode genes to generate artificial resistance relies on their lack of toxicity to the host. Because they are produced by the plant itself, one of the most promising example of this strategy is the use of proteinase inhibitors that block digestive processes upon uptake by the nematode. For example, constitutive expression of a modified rice cystanin gene (a cysteine proteinase inhibitor) in transgenic *Arabidopsis* plants resulted in reduced size and fecundity of *M. incognita* females (Urwin *et al*. 1997), which can be considered as partial resistance. When cystatin was directed with a promoter preferentially active in the roots, which limited transgene expression, a comparable level of resistance to root-knot nematodes was achieved in transgenic potato plants (Lilley *et al*. 2004). Different classes of proteinase genes have been characterized in *M. incognita*, e.g. the cathepsin L-like cysteine proteinase *Mi-cpl1* (Neveu *et al*. 2003a) and the serine proteinase *Mi-ser1* (da Rocha Fragoso *et al*. 2005). In conjunction with the specificity of action of proteinase inhibitors, this diversity may explain the only partial resistance achieved, and probably constitutes an objective limitation to this approach. Moreover, concerns about durability of such a transgenic resistance and its toxicological and environmental effects have still to be addressed.

Inactivation of parasitism genes

Parasitism genes expressed in the oesophageal gland cells of root-knot nematodes encode proteins that are secreted into host root cells to induce the formation of the nematode feeding site and its maintenance during the whole parasitic process (Davis *et al*. 2004). Therefore, such proteins have been considered as putative targets for the construction of artificial defence systems. Because its efficacy had previously been shown for plant viruses (Tavladoraki *et al*. 1995), one of the first approach that was experienced was the *in planta* expression of recombinant antibodies ('plantibodies') that bind to esophageal secretions of the nematode and therefore should interfere with its infection cycle. Indeed, plantibodies that specifically bind to *M. incognita* stylet secretions were produced and characterized (Baum *et al*. 1996; Rosso *et al*. 1996). However, although they were expressed in leaves, stems, roots, and galls, plantibodies had no influence on root-knot nematode parasitism of transgenic plants, probably because of unappropriate cellular compartment targeting (Baum *et al*. 1996). Plantibodies possibly accumulated apoplastically whereas nematode stylet secretions might be injected into the cytoplasm of the parasitized cell, precluding plantibody interference with the secretion function in parasitism. In spite of subsequent efforts to improve plantibody expression levels in the plant

cytosol (Schouten *et al*. 1997), optimization is still needed to remove the remaining obstacles to the widespread use of plantibodies against root-knot nematodes.

More recently, research has focused on the inactivation of *Meloidogyne* parasitism genes *via* RNAi. RNAi was performed *in vitro* by soaking, i.e. incubation of juveniles into double-stranded RNA (dsRNA) derived from the target genes. Silencing of genes expressed in the oesophageal glands of the nematode was achieved, that led to up to 92% depletion of the targeted transcripts in the case of *Mi-crt*, a gene coding for *M. incognita* calreticuline (Rosso *et al*. 2005). Moreover, *in planta* infection with juveniles silenced for a dual oxidase gene resulted in a reduction in nematode egg numbers at 35 days up to 70% (Bakhetia *et al*. 2005). Such *in vitro* effects provided the opportunity to explore further the potential development of new transgenic RNAi-based resistance strategies. In root-knot nematodes, the parasitism gene 16D10 has been shown to encode a secretory peptide that stimulates root growth and functions as a ligand for a putative plant transcription factor (Huang *et al*. 2006b). *In vivo* constitutive expression of 16D10 dsRNA in transgenic *Arabidopsis* induced RNAi of the gene in nematodes feeding on these plants, and a subsequent 69-93% reduction of the parasite reproduction (Huang *et al*. 2006a). Interestingly, the transgenic plants were resistant to the four major *Meloidogyne* species, i.e. *M. arenaria*, *M. hapla*, *M. incognita* and *M. javanica* (Huang *et al*. 2006a), a range larger than that conferred by the natural *Mi-1* resistance gene. Although it has still to be validated on crop plants, including tomato, *in planta* RNAi silencing of nematode genes involved in parasitism thus appears as a very promising approach for developing novel transgenic resistance against *Meloidogyne* species. More generally, targeting any nematode gene essential for its survival/development during interaction with the host using *in planta* RNAi-mediated silencing could represent a very flexible strategy for the control of root-knot nematodes. Among others, it has recently been suggested that genes involved in the neuromuscular function of plant-parasitic nematodes would represent excellent candidates for engineering such artificial host resistance (Kimber and Fleming 2005).

Anti-feeding site-based resistance

Since giant cells forming the feeding site are essential for the development and reproduction of root-knot nematodes, another strategy for engineering resistance against these parasites is thus to promote the selective disruption of these specialized cells. To achieve this goal, two complementary approaches may be developed, i.e. disrupting feeding sites with toxic proteins or preventing feeding site differentiation by blocking plant genes essential for its formation. In order to avoid any effect outside of the giant cells, specific promoters should be used to very precisely direct the transgene expression. Early works characterized targeted promoters that were either down-regulated (Goddijn *et al*. 1993) or up-regulated (Opperman *et al*. 1994) in feeding sites after infection by *M. incognita*, which gave rise to enthusiactic research on their use to drive the expression of a phytotoxic protein (e.g. the barnase RNase) in transgenic plants to achieve nematode resistance. However, there was limited success in applying this approach (Ohl *et al*. 1997), probably because of the side effects of transgene expression in undesired host tissues. Using a variety of molecular approaches, further studies have explored changes in gene expression in the feeding sites, and identified genes and metabolic pathways specifically regulated upon root-knot nematode infection (Gheysen and Fenoll 2002). For example, the first demonstration of the effect of inactivating a gene function essential for giant cell formation was provided by knockout of the *rpe* gene encoding a key enzyme in the pentose phosphate pathway (Favery *et al*. 1998). More recently, high-throughput strategies such as microarray technology have been applied to plant-nematode interactions, and have provided large-scale information about patterns of gene expression in giant cells induced by root-knot nematodes in *Arabidopsis* (Hammes *et al*. 2005; Jammes *et al*. 2005) and in tomato (Bar-Or *et al*. 2005). In the latter case, microarray experiments demonstrated significant changes in the steady-state levels of transcripts of several functional categories, at 5 and 10 days after inoculation with *M. javanica*, including pathogenesis-related genes, hormone-associated genes and development-associated transcription factors (Bar-Or *et al*. 2005). These recent studies support accumulating evidence that some plant genes are manipulated by pathogens, including nematodes, to allow the establishment of compatible interactions. Therefore, it is anticipated that the disruption of such genes should prevent nematode development. Among them, because they seem to be essential for feeding site maintenance and successful nematode feeding (de Almeida Engler *et al*. 2004), plant genes involved in cytoskeleton rearrangements in the giant cells during *Meloidogyne* infection have been identified as promising candidates for the creation of durable resistance against these pests (de Almeida Engler *et al*. 2005).

CONCLUDING REMARKS

What has been known for several decades is the extraordinary variety of chemical compound the plants are capable to synthesise, and many of these products are implicated in defence responses. The information we have at hand implies that there are many biochemical events and many pathways which are responsible for disease resistance, and there are also influences by different types of stimuli. All these factors play a role in gene regulation and trigger activation of the pathways leading to *in situ* production of a number of secondary metabolites, which in turn offer protection to the plant. Recent studies, for example, support the notion that lipid-derived jasmonic acid represents an essential signal in wound-induced expression, orchestrates systemic defence responses to herbivores and controls RKN infection in tomato plants used as a model system. The rapid production of ROS in the apoplast in response to pathogens has been proposed to orchestrate the establishment of different defensive barriers against pathogens as well as in tomato-RKN interaction. We are currently at an exciting time, when most of the technologies required to answer these questions are in place. Thus, a comprehensive analysis of gene expression using microarrays and chips, coupled with proteomics and metabolomics to follow different antioxidants and related compounds during oxidative stress, should answer many of these questions. A better knowledge of the molecular mechanism(s) of cellular components with important roles in defence response signalling, including certain transcription factors, will be instrumental in improving the plant ability to perceive stress stimuli more rapidly, thereby coping with different form of stress more efficiently and in a natural way. The emerging link between the enhanced activation of defence response in challenged cells during abiotic and biotic stresses points to the possibility of improving the plant natural defence potential against multiple forms of stress simultaneously. Through such a holistic approach in which many parameters are measured and in which biostatistic tools are used for identifying correlations, similarities and differences related to different stresses, should provide new insights in the complex and dynamic processes of defence responses.

Because of the recent progress in both knowledge about the interaction between plants and RKN and plant biotechnology, it is expected that genetic engineering will gain more and more importance in plant breeding, including tomato, with the major challenge of developing durable resistance. While classical breeding strategies can only result in the introgression of one single resistance gene into a new cultivar, the simultaneous combination of classical and transgenic approaches will allow to produce plants with more than one resistance mechanism, and thus provide the

possibility of breeding new cultivars with a more stable resistance. In addition, and although environmental concerns still exist with the generic use of GMOs in agriculture, the deployement of transgenic resistant crops may be justified by the urgent need of an efficient alternative to the extremely toxic chemical nematicides currently used, some of which being already prohibited.

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