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RESEARCH PAPER

The plant apoplasm is an important recipient compartment for nematode secreted proteins

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Abstract

Similarly to microbial pathogens, plant-parasitic nematodes secrete into their host plants proteins that are essential to establish a functional interaction. Identifying the destination of nematode secreted proteins within plant cell compartment(s) will provide compelling clues on their molecular functions. Here the fine localization of five nematode secreted proteins was analysed throughout parasitism in *Arabidopsis thaliana*. An immunocytochemical method was developed that preserves both the host and the pathogen tissues, allowing the localization of nematode secreted proteins within both organisms. One secreted protein from the amphids and three secreted proteins from the subventral oesophageal glands involved in protein degradation and cell wall modification were secreted in the apoplasm during intercellular migration and to a lower extent by early sedentary stages during giant cell formation. Conversely, another protein produced by both subventral and dorsal oesophageal glands in parasitic stages accumulated profusely at the cell wall of young and mature giant cells. In addition, secretion of cell wall-modifying proteins by the vulva of adult females suggested a role in egg laying. The study shows that the plant apoplasm acts as an important destination compartment for proteins secreted during migration and during sedentary stages of the nematode.

Key words: Apoplasm, effector, giant cell, immunocytochemistry, intercellular migration, *Meloidogyne incognita*, plant-parasitic nematode.

Introduction

Pathogen effectors are secreted compounds able to modify the physiology of infected cells or to modulate the host defence response to assist the infection process. Identifying the final destination of effectors in plant cells is essential to decipher their precise role in the success or failure of the disease (reviewed by Ellis *et al.*, 2009; Hogenhout *et al.*, 2009; Mosquera *et al.*, 2009; Khang *et al.* 2010; Rafiqi *et al.*, 2010).

Similar to microbe pathogens, plant-parasitic nematodes (PPNs) secrete a repertoire of proteins with diverse functions. Root-knot nematodes (RKNs) and cyst nematodes (CNs) are the most successful PPNs and are major threats to agriculture worldwide (Bird and Bird, 2001). These obligate endoparasites display a highly specialized and complex feeding relationship with their hosts. The RKN life cycle consists of five stages punctuated by four moults. After the first moult within the egg, RKN second-stage juveniles (J2s) hatch into the soil and are attracted to plant roots by root exudates. J2s invade the root elongation zone

and migrate intercellularly towards the root tip and then upwards into the vascular cylinder where they select 6–8 parenchyma cells in which they insert their stylet and inject secretions (Wyss and Grundler, 1992). These secretions induce the differentiation of root cells into 'giant cells' that form the permanent feeding site and are the unique source of nutrients needed for nematode development and reproduction. The hypertrophied and polynucleated giant cells result from repeated mitosis without cytokinesis (de Almeida Engler *et al.*, 2005). Once the nematode has established the feeding site, it remains sedentary and the interaction with the host is maintained for several weeks. During this period, the nematode overcomes plant defences and maintains the feeding cells (Jammes *et al.*, 2005; Barcala *et al.*, 2010). The nematodes further develop into adult females which lay eggs in a gelatinous matrix on the root surface.

More than 100 RKN proteins have been identified to date that are potentially secreted by the amphids or through the stylet. Amphids are bilateral chemosensory organs located on the nematode head and open to the exterior via a prominent pore. Amphids participate in the perception of chemotactic environment stimuli and secrete proteins and carbohydrates that may act as signalling molecules and elicit defence responses from the plant during the interaction (Perry, 1996). Among amphidial secretions, the protein MAP-1 was identified as a putative avirulence (Avr) factor in *Meloidogyne incognita*, and its secretion by pre-parasitic J2s suggested that it might be involved in the early recognition steps between the plant and the nematode (Semblat *et al.*, 2001). On the other hand, the proteins secreted through the stylet are synthesized in three unicellular oesophageal glands and transported via secretory vesicles along the gland cell extension towards the secretory ampulla where they are released into the oesophagus. The secretions are then injected into the host tissues through the stylet, a hollow protrusible structure that acts as a syringe for injection of secretions and for nutrient uptake from the giant cell cytoplasm (Hussey, 1989; Sobczak *et al.*, 1999). The plant cell wall acts as a physical barrier to pathogen invasion, and one important function of some of the proteins secreted through the stylet is cell wall softening during migration. In the genome of the RKN *M. incognita*, >60 genes encode cell wall-degrading or -modifying enzymes including cellulases (glycoside hydrolase family GH5) and pectate lyases (PL3) (Abad *et al.*, 2008). A role in root invasion has been shown for cellulases and pectate lyases in CN juveniles that migrate intracellularly and destroy the walls of the cells they go through (Wang *et al.*, 1999; Chen *et al.*, 2005; Bakhetia *et al.*, 2007). In contrast, RKNs only destroy the epidermal cells of the root apex during penetration and then migrate intercellularly without damaging plant cells (Wyss and Grundler, 1992). Although a role in plant cell wall softening can reasonably be proposed for RKN cellulases and pectate lyases, cellulases are also expressed in RKN sedentary adult females and their function at this stage has not been explored (Rosso *et al.*, 1999). Interestingly, recent studies suggested that a cellulose-binding protein (CBP) from the CN *Heterodera schachtii* might participate in the formation of the nematode feeding site (Hewezi *et al.*, 2008). The feeding site induced by CNs is a syncytium formed by cell wall dissolution of adjacent plant cells. Conversely, induction of hypertrophied giant cells by RKNs requires cell wall expansion and thickening (reviewed in Caillaud *et al.*, 2008). Whether RKN secreted proteins are involved in these cell wall modifications has not been clearly determined.

In addition, several nematode proteins produced in the oesophageal glands have been characterized, although with no predicted or known function to date. For example, the *M. incognita* 6D4 protein is a glycoprotein of high molecular weight for which the amino acid sequence could not yet be determined. Immunolocalization studies on juveniles showed that 6D4 is transported by the secretory vesicles of the subventral and dorsal oesophageal glands towards the gland cell ampulla for secretion through the stylet. 6D4 is also produced in the dorsal gland of females extracted from infected roots, suggesting that it may have a role *in planta* throughout infection (Davis *et al.*, 1992).

To date, only a few nematode proteins have actually been shown to be secreted within the host plant during parasitism (Curtis, 1996; Lopez de Mendoza *et al.*, 2002; Doyle and Lambert, 2003; Zhang *et al.*, 2006) and little is known about the destination compartments of the secreted proteins in infected roots. Cellulases and pectate lyases have been localized by immunocytology in front of the nematode's head during migration and along the migratory path of *Heterodera glycines* and *Meloidogyne javanica* juveniles, respectively (Wang *et al.*, 1999; Doyle and Lambert, 2002). However, immunolocalization studies were largely focused on the migratory stage of the nematode, and one single study provided a clear demonstration of secretion of a nematode protein (the calreticulin Mi-CRT) by the sedentary nematodes in the feeding site and showed abundant accumulation along the cell wall of giant cells in the vicinity of the stylet tip (Jaubert *et al.*, 2005).

Here the fine localization throughout parasitism of a panel of RKN candidate effectors produced from the amphids or the oesophageal glands and secreted *in planta* during root infection and parasitism is presented. For that purpose, an immunocytochemical procedure was adapted that can be applied to localize proteins secreted by parasitic nematodes during all phases of infection. The method described here has a great advantage since it preserves tissues from both the plant host and the pathogen. An additional plus is that it can be useful to localize secreted proteins of other pathogens during parasitism within their plant hosts. Showing the secretion of MAP-1 from the amphidial glands during infection, the present experiments reinforce the assumption that it is a candidate Avr protein from *M. incognita*. In order to better understand their respective roles throughout parasitism, the secretion of CBM2-bearing proteins and pectate lyases during migration within the root, giant cell formation, and female development was compared. The spatio-temporal secretion *in planta* of a new aspartyl protease-like protein (Mi-ASP2) produced in the

subventral oesophageal glands was also analysed. Finally, the 6D4 protein which is constantly present within the dorsal glands during infection was localized within all parasitism phases of plant roots. Overall, the study showed the apoplasm as an important destination compartment for nematode secreted proteins during migration and feeding cell formation in the host plant.

Materials and methods

Antibodies and serum production

Sera directed against Mi-ASP2, CBM2, and Mi-PEL3 were raised by immunization of rabbits with synthetic peptides (Eurogentec Polyclonal Antibody Production, France). In order to avoid background, the peptides were selected based on the absence of similarity to other nematode proteins or to plant proteins. As additional criteria, the selected peptides located on hydrophilic regions of the proteins with good accessibility to solvents as predicted by the Porter and PaleAle servers (http://distill.ucd.ie/paleale/; Pollastri *et al.*, 2007). For Mi-ASP2, rabbits were immunized with two synthetic peptides: STYKVDGRPFSDNNT and RVKRGRKNSRAGRLA. For CBM2 and Mi-PEL3, rabbits were immunized with the peptides NGPATPPQIKVLGDGKC and WKYSYKPGEAGTSDKC, respectively. The anti-MAP-1 serum was obtained from a synthetic peptide as described by Semblat *et al.* (2001). Hybridoma supernatants specific to effector 6D4 were obtained from a mouse immunized with protein homogenate from *M. incognita* females and selected based on specific reaction on the oesophageal glands of the nematode (Davis *et al.*, 1992).

Immunolocalization on pre-parasitic J2s

For whole-mount immunolocalization, pre-parasitic freshly hatched J2s were fixed for 18 h at 4 °C in phosphate-buffered saline (PBS) containing 2% paraformaldehyde and subsequently for 4 h at room temperature. Immunolocalizations were performed essentially as described by Semblat *et al.* (2001). Primary antibodies (anti-Mi-ASP2, anti-CBM2, and anti-Mi-PEL3) were diluted 1:50 in PBS (containing 1 mg ml^{-1} of horse serum–phenylmethylsulphonyl fluoride), and secondary antibody (Alexa 488 goat anti-rabbit IgG, Molecular Probes) was diluted 1:200 in PBSTB buffer (PBS containing 0.1% Tween-20 and 0.1% bovine serum albumin). Control samples were incubated with pre-immune serum in the absence of primary antibody. Antibody- and pre-immune serum-treated whole-mount J2s (cut in two parts) were transferred to multitest polylysine-coated slides and observed with a microscope equipped for epifluorescence microscopy and differential interference contrast optics (Axioplan 2, Zeiss). Images were captured with a digital AxioCam camera (Zeiss).

Seed sterilization and plant growth

Surface-sterilized seeds of *Arabidopsis thaliana* (L.) Heyhn var. Columbia were grown on Gamborg B5 medium (Sigma, St Louis, MO, USA) containing 1% sucrose, 0.8% agar (plant cell culture tested; Sigma) and kept under a growth chamber with a light regime of 16 h overhead illumination and 8 h darkness at 21 °C. Seven-day-old seedlings were then transferred to Knop medium (Sijmons *et al.*, 1991) and kept inclined so that roots could grow on the surface of the medium, facilitating nematode infection.

Nematode infection and fixation

Roots of *A. thaliana* cv. Columbia grown *in vitro* were inoculated with the surface-sterilized *M. incognita* J2 population Calissane. Galls were dissected from infected *Arabidopsis* roots at various time points after nematode inoculation [7, 14, 21, 30, and 55 days after inoculation (DAI)] and fixed in 4% formaldehyde in 50 mM PIPES buffer (pH 6.9) for 2–10 d (depending on the size of the gall) at 4 °C.

Dehydration and embedding of nematodes in methacrylate

After fixation, dissected galls were dehydrated in an ethanol series (1 h each: 15, 30, 50% v/v), with gentle shaking, at 4 °C or in ice, and incubated overnight at 4 °C in 70% ethanol. Samples were then further dehydrated in 85% ethanol and three times in 100% ethanol (1 h each) on ice. After dehydration, the ethanol was replaced by 50% ethanol-butyl-methylmethacrylate [BM, 4:1 containing 0.1 mM dithiothreitol (DDT)] at 4 °C overnight (Kronenberger *et al.*, 1993). The following day, the solution was replaced by 100% methacrylate mixture containing 0.1 mM DTT, and kept overnight at 4 °C (and up to 1 week depending on the size of the gall). The solution was replaced by fresh BM embedding medium containing 0.5% benzoin ethyl ether (100% BM and BEE) for 1 h up to overnight (or longer). Finally, samples were placed in plastic capsules containing 100% BM, 0.5% BEE, and 0.1 mM DDT, and polymerized at 4 °C for 6 h under UV light. After polymerization, samples can be kept for an undetermined time.

Sectioning methacrylate-embedded nematode material

Polymerized samples were removed from the capsule and sectioned to 5 µm (greater thickness may cause loss of sections from slides). Sections were floated on drops of sterile water on polylysine-coated glass slides, dried on a hot plate at 60 °C, and incubated overnight at 42 °C, so that sections will adhere to the slides. Slides were microscopically screened for sections containing complete longitudinal sections of migrating nematodes within the roots and for longitudinal sections of parasitic nematodes containing their heads. The slides were then kept in dry slides boxes at room temperature until used for immunolocalization.

Immunocytochemical analysis of secreted nematode proteins

Slides were treated for 30 min with 100% acetone to remove embedding medium and to improve antibody access to epitopes in infected roots. Sections were checked under the microscope to monitor if embedding medium was well dissolved, dipped in 100% ethanol, rehydrated progressively in an ethanol series (85, 70, 50, 30, 15%), and finally placed in 50 mM PIPES buffer (pH 6.9). On each slide ~200 µl of blocking solution (1% bovine serum albumin in 50 mM PIPES, pH 6.9) was added and covered with a coverslip to allow incubation for 30 min (or longer) at room temperature in a humid box. Primary (AB1) and secondary antibodies (AB2) were diluted 50- and 300-fold, respectively, in blocking solution containing 0.2% dimethylsulphoxide (DMSO). Antibodies were incubated at 37 °C for 30 min and centrifuged for 5 min. The coverslip was then carefully removed and BS was replaced by ~150 µl of each AB1 (anti-Mi-ASP2, anti-CBM2, anti-PEL3, anti-MAP-1, or anti-6D4) or pre-immune serum of each antibody (PIS) as a control. The humid box containing the slides was incubated overnight at 4 °C and then placed at 37 °C for 90 min. Subsequently, the coverslips were carefully removed, and slides were placed in slide racks and washed for 30 min in 50 mM PIPES buffer (pH 6.9) with gentle stirring. Excess PIPES was removed and replaced immediately by ~150 µl of AB2 (Alexa 488 goat anti-rabbit IgG, Molecular Probes), covered with a coverslip, and incubated for 3 h at room temperature or for 1 h at 37 °C. A set of slides was incubated only with AB2 to control potential unspecific binding of the secondary antibody. The slides were then washed again for 30 min with PIPES protected from the light. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; 1 µg ml^{-1} in water or PBS) for 5 min at room temperature and rinsed briefly in deionized water to remove salts. Finally, slides were mounted with ProLong antifade medium (ProLong antifade kit; Invitrogen

Molecular Probes), and observed with a microscope (Axioplan 2; Zeiss) equipped for epifluorescence and differential interference contrast optics. Representative cytological images of migratory and parasitic stages of nematodes within the plant root for each protein analysed were collected using a digital camera (AxioCam; Zeiss). Images of immunolabelled sections, differential interference contrast transmission, and DAPI-stained DNA (nuclei) were overlaid. Slides can be kept for a long period (up to 2 months) in dark dry boxes for later observation.

Results

The amphidial MAP-1 protein is secreted in the apoplasm during root invasion and early sedentary stages

The MAP-1 protein was previously shown to be secreted as two close spots on the surface of the head of pre-parasitic *M. incognita* J2s, suggesting the secretion of this protein by the amphids (Semblat *et al.*, 2001). In order to find out if MAP-1 was secreted *in planta* during parasitism, immunocytochemical analysis was performed on root tissue sections of *A. thaliana* during nematode infection at 7, 14, and 21 DAI. The localization of this protein by the anti-MAP-1 antibody was consistent with the results observed on the pre-parasitic stage, showing antibody label in both amphids within the nematode head. The localization of the protein (7 DAI) was constantly observed in the amphids of the migrating J2s (Fig. 1A, B) and in some sections also observed along the nematode body (Fig. 1C, D), suggesting a secretion of the protein during the migratory phase through root tissues. Surprisingly, MAP-1 was also detected within the amphids of sedentary nematodes in mature root galls (21 DAI), and in the exterior of the nematode head along the cell wall of the adjacent giant cells within the plant apoplasm (Fig. 1E, F). Control experiments using the pre-immune serum instead of anti-MAP-1 on infected root sections and at different time points after nematode inoculation, showed no background fluorescence (see Supplementary Fig. S1A–D available at *JXB* online).

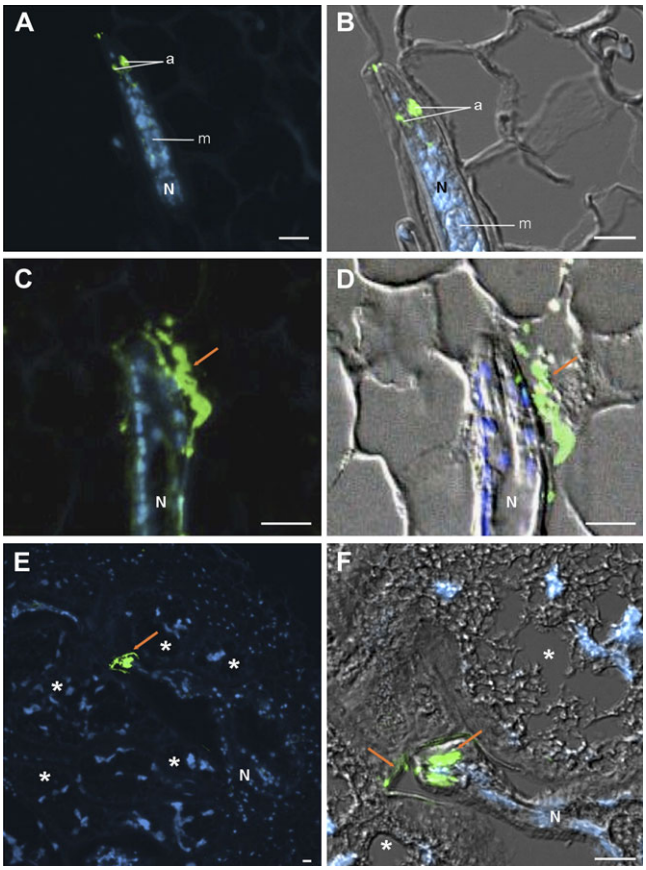

Fig. 1. Immunodetection of the MAP-1 protein (green) during *Meloidogyne incognita* parasitism in roots of *Arabidopsis thaliana*. (A, B) Localization of MAP-1 in the amphids of a migrating juvenile within the roots (7 DAI); (C, D) MAP-1 secretion alongside the nematode surface (arrow) during migration within the host root (7 DAI); (E, F) gall containing a sedentary nematode, showing the significant accumulation of MAP-1 in the amphids of the nematode and along the giant cell wall (arrow) within the apoplasm (21 DAI). Micrographs A, C, and E are overlay images of MAP-1 (green) and DAPI-stained nuclei (blue). Micrographs B, D, and F are overlays of images of MAP-1 (green), DAPI-stained nuclei (blue), and differential interference contrast (grey) to better visualize plant and nematode tissues. a, amphid; m, metacorpus; N, nematode. *, giant cells. Bars=10 μm.

The aspartyl protease-like Mi-ASP2 is secreted from the subventral oesophageal glands into the apoplasm during migration and early sedentary stages

Mi-asp2 was identified as a new nematode candidate effector by a PCR-based strategy (Neveu *et al.*, 2003). The *Mi-asp2* full-length cDNA (accession number FN179274) encodes a predicted 403 amino acid protein and has a predicted N-terminal putative signal peptide of 17 amino acids with congruent prediction with neural network and HMM methods (Emanuelsson *et al.*, 2007). An Interproscan search for conserved domains (Zdobnov and Apweiler, 2001) revealed the presence of two possible overlapping Interpro family domains (IPR001461 and IPR009007), both referring to aspartic proteases. A CD search (Marchler-Bauer and Bryant, 2004) confirmed that the Mi-ASP2 protein sequence contained a eukaryotic aspartyl protease domain and the two conserved asparate residues of the catalytic centre (James *et al.*, 2004) were found (see Supplementary Fig. S2 at *JXB* online). Pairwise alignment of Mi-ASP2 with Mi-ASP1, a cathepsin D-like aspartic protease recently identified in *M. incognita* (Fragoso *et al.*, 2009), returned only 26.30% identity at the amino acid level (see Supplementary Fig. S3 at *JXB* online), indicating that the two proteins are encoded by clearly different genes. A similarity search using BlastP against the NCBI non-redundant protein database (nr) revealed a large set of significant hits matching on the whole length of the predicted aspartic protease domain, the 26 best hits all belonging to the Nematoda phylum (see Supplementary Table S1 at *JXB* online). Six predicted proteins annotated as aspartic proteases, with e-values <e-15 and length >300 amino acids, were retrieved from the *M. incognita* genome

and their percentage identity to Mi-ASP2 ranged from 26% to 28% (see Supplementary Table S2 at *JXB* online). A phylogenetic analysis including the 100 best nr Blast hits and the matching *M. incognita* predicted proteins showed that Mi-ASP2 formed a highly supported cluster together with Minc02830 and Minc04744, clearly distinct from all other eukaryotes, including other nematodes (see Supplementary Fig. S4 at *JXB* online).

To find out if *Mi-asp2* transcripts are present in nematode secretory organs, tissue localization of mRNA was examined in pre-parasitic *M. incognita* J2s by *in situ* hybridization. A specific hybridization signal was observed in the subventral oesophageal glands (see Supplementary Fig. S5 at *JXB* online). Aiming to find out if this protein was secreted *in planta* during the host infection process, a polyclonal antiserum was raised against the Mi-ASP2 protein. Western blots of total *M. incognita* J2 homogenates have shown that the antibody specifically binds to an individual protein band of ~50 kDa (see Supplementary Fig. S6A at *JXB* online), suggesting some possible post-translational modifications of the protein (the theoretical expected molecular weight of Mi-ASP2 is 43.9 kDa). Whole-mount immunolocalization assays of Mi-ASP2 on freshly hatched pre-parasitic J2s showed that the antibody strongly labelled the content of the subventral oesophageal gland cells, their cytoplasmic extension, and ampullae located immediately behind the pump chamber in the medium bulb (Fig. 2A, B). To analyse whether *M. incognita* secretes this protein during parasitism, a similar approach was taken to that described above for MAP-1 using infected root sections. During migration of *M. incognita* J2s within the host root (7 DAI), Mi-ASP2 was localized in the subventral gland ampullae behind the pump chamber, inside the lumen of the oesophagus, and along the nematode head. A large number of sections showed a similar labelling pattern, suggesting that Mi-ASP2 is continuously secreted in the plant apoplasm by the migrating nematode (Fig. 2C, D). During the nematode sedentary stages (14 and 21 DAI), Mi-ASP2 was consistently observed accumulated on the head of the nematode, at the tip of the stylet, and within the plant apoplasm flanking the giant cells. However, the secretion here was less abundant than during the migratory phase (Fig. 2E–H). Control experiments using the pre-immune serum instead of anti-ASP2 revealed no background fluorescence (see Supplementary Figs S1E–H and S8A, B at *JXB* online).

CBM2-bearing proteins and pectate lyases are secreted in the plant apoplasm during root invasion but only CBM2-bearing proteins have a role in egg laying

The genome of *M. incognita* contains 12 cellulase (β-1,4-endoglucanase) and three expansin-like genes that have a cellulose-binding module from family 2 (CBM2) separated from the catalytic domain by a flexible linker (Abad *et al.*, 2008). In addition in *M. incognita*, four CBPs have been identified containing a signal peptide, a linker, and CBM2, but no catalytic domain (Ding *et al.*, 1998; Adam *et al.*, 2009). In order to assess their roles throughout parasitism, RKN CBM2-bearing proteins were localized by using a polyclonal serum directed against CBM2. A 17 amino acid peptide was selected from the C-terminal part of the GH5 cellulase Mi-ENG1 to raise the serum. An exhaustive search for CBM2 in the genome and expressed sequence tag (EST) data of *M. incognita* showed that the peptide sequence is highly conserved in the CBM2 of six GH5 cellulases. In addition, the peptide sequence shared up to 13 amino acids with other GH5 cellulases and had lower similarity to CBM2 from expansins and CBP (seven and nine conserved amino acids, respectively; see Supplementary Fig. S9 at *JXB* online). Western blot analysis suggested that the serum reacted with cellulases (50 kDa and 76 kDa) and with expansins (35–41 kDa), but not with CBP (see Supplementary Fig. S6B at *JXB* online).

Proteins recognized by the anti-CBM2 serum were localized in whole mounts of pre-parasitic J2s within the subventral oesophageal glands, along their cytoplasmic extensions, and in the ampullae located in the posterior part of the metacorpus (Fig. 3A, B). During migration within the root tissue, CBM2-bearing proteins were localized within the nematode in the same structures. In addition, the proteins were localized along the nematode head and in the apoplasm of root cells (7 DAI), indicating their secretion during migration of the juvenile nematodes (Fig. 3C, D). In some infected root sections (7 DAI), the proteins were also localized along the nematode surface, potentially due to the deposition of proteins along the cuticle as the nematode migrated within the root (see Supplementary Fig. S10A, B at *JXB* online). During the sedentary phase of the nematode in roots (14 DAI), CBM2-bearing proteins could still be detected throughout the stylet. However, this secretion was much less abundant than during migration (Fig. 3E, F). In some root sections, a strong labelling was observed inside the male still coiled within the cuticle of the fourth stage juvenile (30 DAI), probably corresponding to the labelling of the proteins in the subventral glands (see Supplementary Fig. S10C, D at *JXB* online). Surprisingly, the CBM2-bearing proteins were also localized in the vagina of adult females (55 DAI; Fig. 3G, H). Control experiments using the pre-immune serum instead of the anti-CBM2 revealed no background fluorescence (see Supplementary Figs S1I–J, U, V and S8C, D at *JXB* online).

The full length coding sequence of a new pectate lyase gene, *Mi-pel3* (accession number AY861685) was isolated. Similarly to the previously described Mi-PEL1 and Mi-PEL2 from *M. incognita* (Huang *et al.*, 2005), Mi-PEL3 belongs to family 3 pectate lyases. It has a 279 amino acid coding sequence with a 20 amino acid predicted signal peptide for secretion. To analyse the similarity relationship between those genes, a phylogenetic analysis of RKN pectate lyases and their closest homologues in bacteria was performed. It was found that the protein Mi-PEL3 is in a phylogenetic cluster distinct from Mi-PEL1 and Mi-PEL2, suggesting that *Mi-pel1*, *Mi-pel2*, and *Mi-pel3* have significantly diverged in *Meloidogyne* spp. (see Supplementary Fig. S11A at *JXB* online). In pre-parasitic juveniles, *Mi-pel3* is specifically transcribed in the subventral oesophageal glands

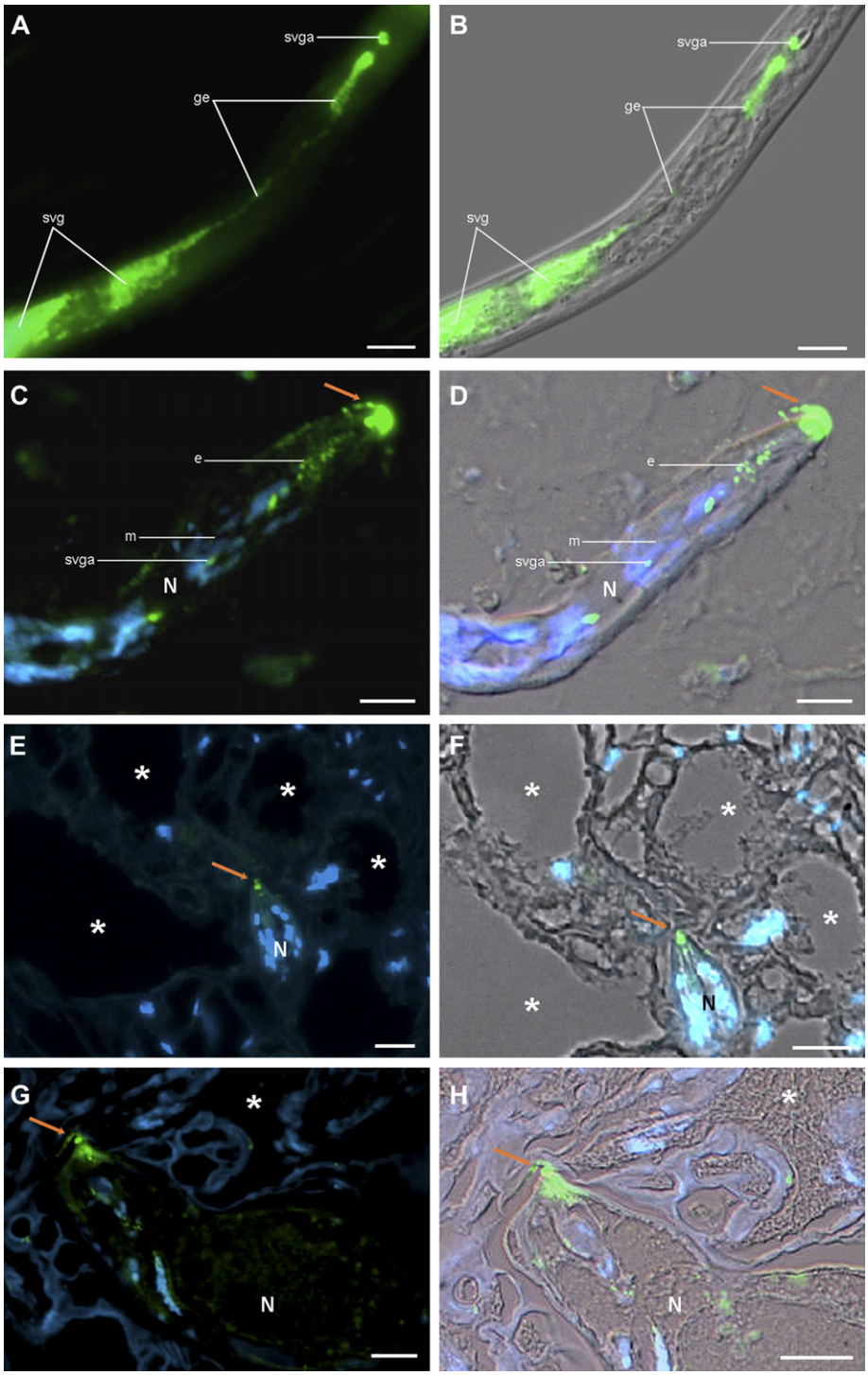

Fig. 2. Immunodetection of the Mi-ASP2 protein (green) within pre-parasitic juveniles of *Meloidogyne incognita* and during parasitism of roots of *Arabidopsis thaliana*. (A, B) Localization of Mi-ASP2 in the subventral glands (svg), subventral gland extensions (ge), and subventral gland ampullae (svga) of pre-parasitic juveniles; (C, D) root containing a migrating nematode (7 DAI), showing the secretion of Mi-ASP2 protein into root tissue around the head of the nematode (arrow); (E, F) gall containing a sedentary nematode (14 DAI), displaying Mi-ASP2 accumulated at the tip of the stylet (arrow) near the giant cells; (G, H) gall containing a sedentary nematode (21 DAI), displaying Mi-ASP2 accumulated at the tip of the stylet and along the giant cell wall (arrow). Micrographs C, E, and G show overlay images of Mi-ASP2 (in green) and DAPI-stained nuclei (in blue). Micrographs D, F, and H show overlays of images of Mi-ASP2 (green), DAPI-stained nuclei (blue), and differential interference contrast (grey) to better visualize plant and nematode tissues. e, esophagus. m, metacorpus. svga, sub-ventral gland ampulla. N, nematode. *, giant cells. Bars=10 μm.

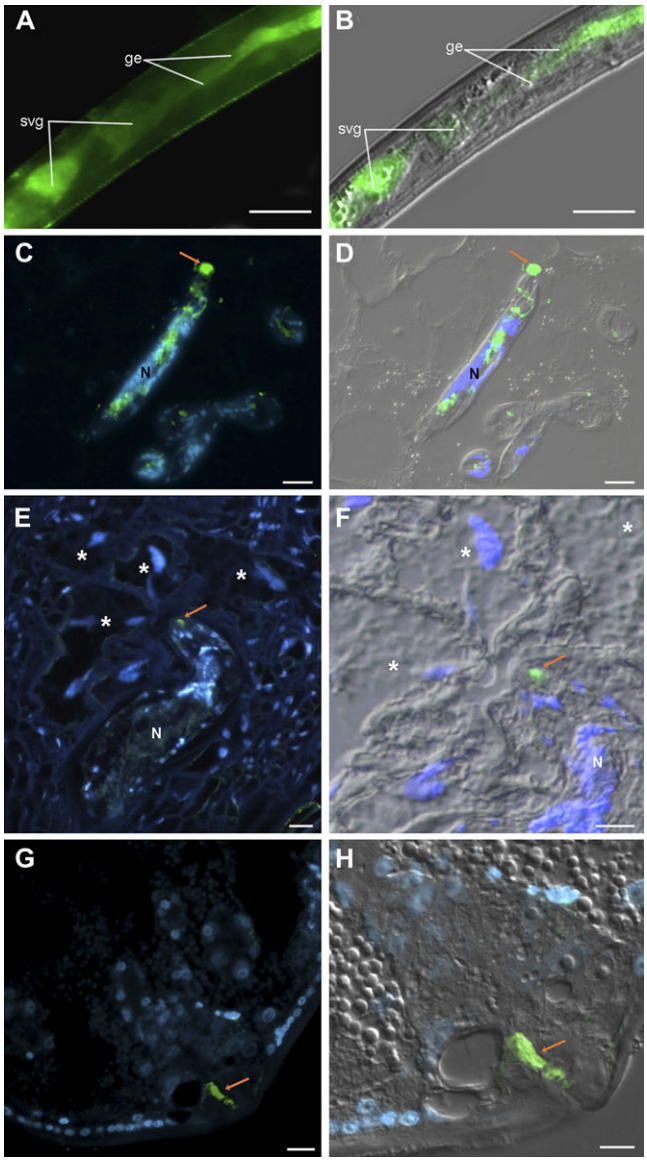

Fig. 3. Immunodetection of CBM2-bearing proteins (green) within pre-parasitic juveniles of *Meloidogyne incognita* and during parasitism of roots of *Arabidopsis thaliana*. (A, B) Localization of CBM2-bearing proteins in the subventral glands (svg) and subventral gland extensions (ge) of pre-parasitic juveniles; (C, D) root section containing a migrating nematode (7 DAI), showing the localization of CBM2-bearing proteins in the subventral glands of the nematode, and secreted into root tissue around the head of the nematode (arrow); (E, F) gall containing a sedentary nematode (14 DAI), displaying CBM2-bearing proteins accumulated at the tip of the stylet (arrow) near the giant cells; (G, H) localization of CBM2-bearing proteins accumulated in the vagina of adult females (55 DAI). Micrographs C, E, and G are overlay images of CBM2-bearing proteins (green) and DAPI-stained nuclei (blue). Micrographs D, F, and H are overlays of images of CBM2-bearing proteins (green), DAPI-stained nuclei (blue), and differential interference contrast (grey) to better visualize plant and nematode tissues. N, nematode. *, giant cells. Bars=10 µm.

(see Supplementary Fig. S12 at *JXB* online). In order to localize Mi-PEL3 secretion *in planta*, a synthetic peptide was designed to raise antibodies. The peptide was strongly conserved in four pectate lyase genes from *M. incognita* and shared only two and eight amino acids with Mi-PEL1 and Mi-PEL2, respectively (see Supplementary Fig. S11B at *JXB* online). On a western blot, the antiserum strongly reacted with a protein of the expected 30 kDa molecular weight (see Supplementary Fig. S6C at *JXB* online). Immunolocalization in pre-parasitic J2 whole mounts showed that Mi-PEL3 was localized within the subventral oesophageal glands, along their cytoplasmic extensions, and in the ampullae (Fig. 4A, B), suggesting its possible secretion during host plant infection by the nematode. Immunolocalization on sections of infected roots (7 DAI) revealed that the protein is secreted by the nematodes during migration along the roots (Fig. 4C, D). Although secretion of Mi-PEL3 was mainly observed in nematodes migrating along the vascular tissue, in some sections (14 DAI) it was also localized at the tip of the stylet of sedentary nematodes during the early stages of giant cell development (Fig. 4E, F). In contrast to cellulases, the presence of the protein was not observed in mature females, further supporting previously reported absence of transcription at this stage (Huang *et al.*, 2005). Control experiments with the pre-immune serum revealed no background fluorescence (see Supplementary Figs S1M–P and S8E, F at *JXB* online).

The 6D4 effector accumulates at the cell wall of feeding cells

The 6D4 protein was previously localized in the secretory granules of both subventral and dorsal oesophageal glands of J2s and in the granules of the dorsal gland of adult females dissected from roots (Davis *et al.*, 1992), suggesting its secretion in the plant host. The experiments using infected root sections (7 DAI) did not show any label using anti-6D4 during juvenile nematode migration. However, it was observed that 6D4 is abundantly secreted during both early (14 DAI) and late parasitic sedentary stages (21 and 30 DAI; Fig. 5A–F). At these stages the 6D4 protein was secreted *in planta* and accumulated adjacent to the nematode's head and along the walls of giant cells within the apoplasm (Fig. 5). Control experiments using pre-immune serum showed no background on infected root sections of all stages of gall development (see Supplementary Fig. S1Q–T at *JXB* online).

Discussion

A large body of literature has reported the identification of nematode candidate effectors based on three criteria: (i) the expression of the proteins in a secretrory organ such as amphids or oesophageal glands; (ii) the presence of a predicted signal peptide for secretion; and (iii) the expression of the corresponding genes during infection (reviewed in Caillaud *et al.*, 2008; Davis *et al.*, 2008; Bellafiore and Briggs, 2010). However, only few studies reported the actual secretion *in planta* of nematode proteins

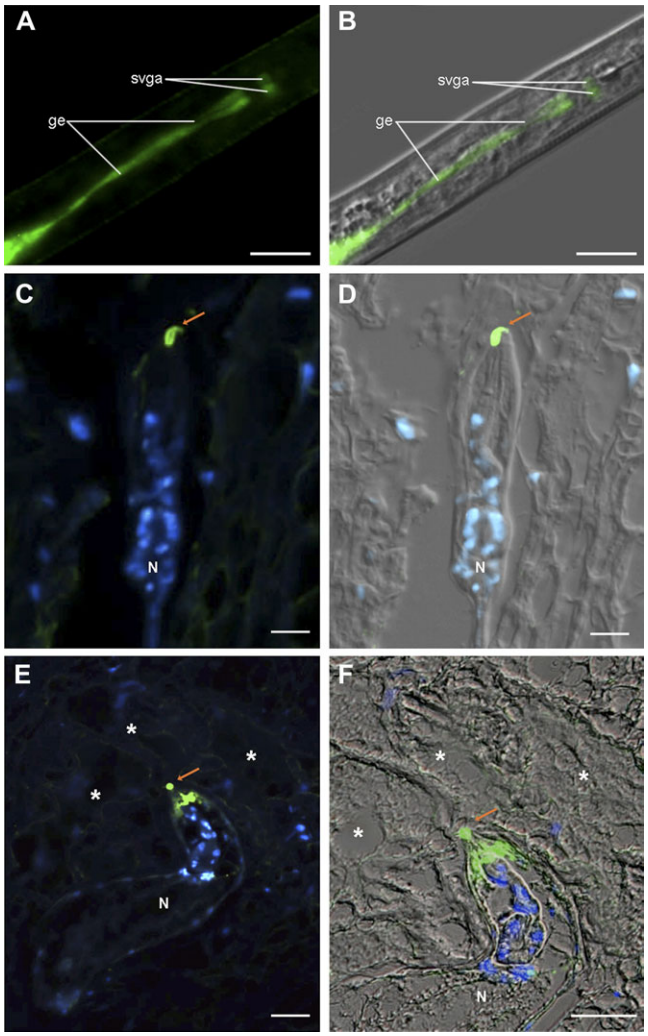

Fig. 4. Immunodetection of the Mi-PEL3 protein (green) within pre-parasitic juveniles of *Meloidogyne incognita* and during parasitism of roots of *Arabidopsis thaliana*. (A, B) Localization of Mi-PEL3 in the subventral gland extensions (ge) and subventral gland ampullae (svga) of pre-parasitic juveniles; (C, D) localization of Mi-PEL3 in root sections at the tip of the stylet of the nematode (arrow) during migration (7 DAI); (E, F) gall containing a sedentary nematode, showing Mi-PEL3 accumulated at the tip of the stylet (arrow) near the giant cells (14 DAI). Micrographs C and E are overlays of images of Mi-PEL3 (green) and DAPI-stained nuclei (blue). Micrographs D and F are overlays of images of Mi-PEL3 (green), DAPI-stained nuclei (blue), and differential interference contrast (grey) to better visualize plant and nematode tissues. N, nematode. *, giant cells. Bars=10 μm.

(Wang *et al.*, 1999; Doyle and Lambert, 2002, 2003; Jaubert *et al.*, 2005; Zhang *et al.*, 2006). Here the validity of the criteria generally used to predict *in planta* secretion of nematode proteins is confirmed as the secretion of five additional RKN proteins in the plant tissue during infection is shown (Table 1). In order to better characterize the destination compartments of RKN secreted proteins and the regulation of their secretion during parasitism, the secretion of the five proteins was monitored throughout parasitism by immunocytology on infected *Arabidopsis* root sections. The

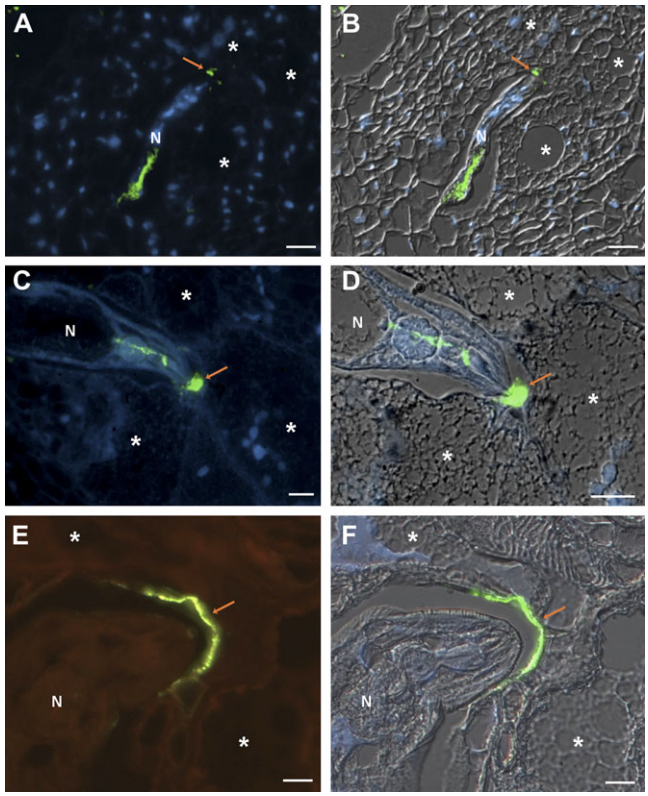

Fig. 5. Immunodetection of the 6D4 protein (green) during parasitism in roots of *Arabidopsis thaliana*. (A, B) Localization of 6D4 on the head of the nematode (arrow) during early stages of giant cell development (14 DAI); (C, D) gall containing a sedentary nematode displaying 6D4 secreted and accumulated at the head of the nematode, and along the giant cell wall (arrow) within the apoplasm (21 DAI); (E, F) gall containing a sedentary nematode (30 DAI), showing 6D4 accumulated near the giant cells (arrow). Micrographs A, C, and E are overlays of images of 6D4 (green) and DAPI-stained nuclei (blue). Micrographs B, D, and F are overlays of images of 6D4 (green), DAPI-stained nuclei (blue), and differential interference contrast (grey) to better visualize plant and nematode tissues. N, nematode. *, giant cells. Bars=10 μm.

immunolocalization method described here has been optimized for the fixation and embedding procedure and therefore efficiently preserves tissues from both the plant host and the pathogen. In addition, the major strength of the butyl-methyl-based embedding procedure lies in the fact that the plastic after polymerization can be dissolved, allowing much better antibody penetration and epitope accessibility and therefore increasing detection sensivity. Other methods based on paraffin embedding or cryosections result in poor morphology preservation and often elevated autofluorescence. An additional advantage of the method presented here is that it can be useful to localize effectors of other pathogens secreted during parasitism.

Nematode secreted proteins putatively involved in perception/recognition

MAP-1 was originally identified as the result of a comparative analysis of *M. incognita* near-isogenic lines

Table 1. Summary of the immunocytochemical analysis of secretion *in planta* of five *Meloidogyne incognita* effector candidates

Effector candidate	Secretory organ	Nematode parasitic stage		
		Migrating	Early sedentary	Late sedentary
MAP-1	Amphids	++	++	ND
Mi-ASP2	SubV glands	+++	+	ND
CBM2-bearing proteins	SubV glands	++	+	ND
	Vulva	–	–	++
Mi-PEL3	SubV glands	++	+	ND
6D4	SubV and D glands	ND	+++	+++

+, ++, and +++ show weak, strong, and very strong immunofluorescence detection, respectively; ND, not detected; –, non-functional at this developmental stage; SubV glands, subventral oesophageal glands; D, dorsal oesophageal gland.

avirulent/virulent to the tomato *Mi-1* resistance gene and shown to be secreted by pre-parasitic, infective J2s (Semblat *et al.*, 2001). Based on these results, it was hypothesized that the protein could be involved in the early steps of recognition between the plant and the nematode. Here, it could be further demonstrated that the protein was also secreted during J2 migration in the root tissues and later by the early sedentary stages in the plant apoplasm, along the wall of giant cells. Such a location of the protein indicates that MAP-1 might have a role during induction of young giant cells. Except for homologous sequences from *M. javanica* (Adam *et al.*, 2009), a species very close to *M. incognita* from a phylogenetic point of view, no sequence similar to *map-1* was found in databases. An InterPro scan identified a Barwin-related endoglucanase domain (IPR009009) at the C-terminus of the *M. incognita* and *M. javanica* proteins, which has been considered as possibly involved in a common defence mechanism in plants (Adam *et al.*, 2009). Recently, it was shown that *map-1* belongs to a small gene family, and that variation in the number/arrangement of internal repeats in the genes was correlated with the (a)virulence of *M. incognita* near-isogenic lines (Castagnone-Sereno *et al.*, 2009). Therefore, the hypothesis put forward here is that the repetitive region of MAP-1 may be involved in physical interactions with plant ligands, along either the nematode path or the wall of giant cells, or both, and may play a role in the specificity of the plant–nematode interaction. Further functional studies are underway to evaluate this hypothesis.

Nematode secreted proteins involved in protein degradation

The phylogenetic relationships of Mi-ASP2 (and similar proteins found in *M. incognita*) with the large repertoire of aspartic proteases from other organisms strongly suggest that it is a member of an independent family of aspartic protease-like proteins that has no equivalent so far in eukaryotes. Therefore, one important question concerns the evolutionary significance of the emergence of the *Mi-asp2* and related genes in *M. incognita* and their biological function(s). In animal-parasitic helminths, aspartic proteases contribute to host specificity and host range via the degradation of specific host molecules, as a result of the co-evolution of parasite enzymes and their host protein substrates (Brinkworth *et al.*, 2000; Williamson *et al.*, 2003). In planta localization of Mi-ASP2 showed a high concentration of this protein outside the tip of the stylet and along the nematode migration path in roots. This suggests the involvement of this aspartic-like protease during the very first steps of nematode infection in plant roots, and corroborates the hypothesis that *Mi-asp2* is a member of the *M. incognita* secretome. Furthermore, during the sedentary phase Mi-ASP2 had been detected close to the nematode's head and in the apoplasm along the giant cells. Assuming that *Mi-asp2* indeed encodes an active aspartic protease, a role for the protein in pre-digestion of peptidic nutrients is unlikely since J2s do not feed during migration, and sedentary stages feed from the cytoplasm of giant cells. The present observations rather suggest that the degradation of host proteins into smaller peptides in the plant apoplasm might be a key element for the successful parasitic lifestyle of the nematode. It was recently demonstrated that the phytopathogenic bacteria *Pseudomonas syringae* delivers protease effector proteins into the plant cell to suppress defence pathways, and that the plant plasma membrane is the primary site for subcellular localization of these effectors (Dowen *et al.*, 2009). Up to now, it has not been possible to localize Mi-ASP2 in the cytoplasm of (giant) cells. However, the possibility that an additional role for the protein may occur in the plant cell cannot be excluded.

Nematode secreted proteins involved in plant cell wall modification

Two sera were used to finely localize cell-wall modifying enzymes throughout parasitism. A serum raised against *M. incognita* CBM2 recognized nematode cellulases and expansins under denaturing conditions. The second serum was specific to a subfamily of *M. incognita* pectate lyases. With both sera, secretion of the proteins was observed from the stylet in front of the nematode head during the intercellular migration of J2 within the root. These results were in concordance with previous studies that showed the secretion by *M. javanica* migrating J2s of a pectate lyase into the plant extracellular matrix (Doyle and Lambert, 2002), similar to the secretion of cellulases by CNs during intracellular migration (Wang *et al.*, 1999). The reaction of the anti-CBM2 serum on male sections is in accordance with a role for CBM2-bearing proteins in migration within the plant tissue since males also migrate to exit the root. Interestingly, with both sera, immunolabelling was observed at the tip of the stylet of sedentary juveniles and in contact with the wall of young giant cells. However, the low intensity of the signal at this stage suggested that secretion of cell wall-modifying proteins by sedentary stages could result from

residual activity of the subventral oesophageal glands rather than being effectively involved in cell wall modifications associated with giant cell formation. In addition, a new potential role for CBM2-bearing proteins during late sedentary stages was shown. The presence of CBM2-bearing proteins in the vagina of adult females suggests that cell wall-modifying proteins could be secreted by females for the breakdown of root cells in order to create an adequate space for the development of its pear-shaped body. Alternatively, females could secrete cell wall-modifying proteins for egg laying. Orion and Franck (1990) have observed the formation of a canal ending at the root surface for extrusion of eggs. Although the work presented here on *A. thaliana* did not allow the observation of such canals, CBM2-bearing proteins secreted by females could possibly participate in the formation of the extrusion canal on thick plant roots.

Nematode proteins secreted in the nematode feeding site

Although its function in the interaction is still unknown, the 6D4 protein was of particular interest because it is abundantly produced by the subventral oesophageal gland of pre-parasitic J2s and in both the subventral and dorsal glands of parasitic stages, suggesting a role throughout parasitism in plant roots (Davis *et al.*, 1992). Surprisingly, secretion of 6D4 was not observed during migration, indicating that all proteins synthesized in the subventral glands are not simultaneously secreted. Instead, 6D4 is abundantly secreted during sedentary stages and accumulates in the root intercellular space and at the plant cell wall near the nematode's head. Noticeably, 6D4 secretion could be observed along the cell walls of young and mature giant cells. This result suggests that 6D4 activity could be associated with giant cell formation and maintenance.

Destination compartments for nematode secreted proteins

The *in planta* immunocytological analyses of five nematode proteins secreted from three distinct secretory organs of the nematode (amphids as well as subventral and dorsal…) all show the plant apoplasm as an important compartment where these proteins are delivered. During J2 migration through the plant root, secretion was observed in the intercellular space of proteins involved in cell wall modification (Mi-PEL3, CBM2-bearing proteins) or protein degradation (Mi-ASP2) and one amphidial secretion (MAP-1). In fungi and oomycetes, the intercellular space is known as the destination compartment for small cysteine-rich proteins and for effectors involved in pathogen protection from plant hydrolytic enzymes (reviewed by Hogenhout *et al.*, 2009). Consistent with intercellular migration within the plant root of RKNs, the extracellular space thus appears as an important interface between the host and the parasite. In addition, it was observed that nematode proteins actively secreted by sedentary stages (MAP-1, 6D4) accumulate at the plant cell wall during giant cell formation and maintenance, suggesting that the plant cell wall may be an important interface for feeding cell formation. Interestingly, the same accumulation at the wall of giant cells in the vicinity of the nematode was observed for a calreticulin secreted by *M. incognita* sedentary stages (Jaubert *et al.*, 2005). So far, no RKN secreted protein has been localized inside plant cells, including giant cells. The minute amount of secreted products and hypertrophy of giant cells might result in effector dilution when injected into cells, preventing their detection by direct immunocytology. Alternatively, modifications in the conformation of nematode proteins addressed to intracellular compartments might prevent recognition by specific antibodies. Several bodies of evidence suggest that nematode proteins are most possibly injected into plant cells. Indeed, during giant cell induction and feeding, the nematode inserts its stylet through the cell wall of selected parenchyma cells apparently without injury to the plasma membrane. The plasma membrane becomes invaginated around the stylet and a small pore is formed at the stylet orifice (Williamson and Hussey, 1996; Sobczak *et al.*, 1999). The stylet is thus considered to act as a syringe for the injection of secretory products and the withdrawal of nutrients from the plant cell cytoplasm. In addition, current (and still limited) knowledge of the plant targets of RKN candidate effectors and of the subcellular localization of these proteins when overexpressed in plant cells suggests a role inside the plant cells (Tytgat *et al.*, 2004; Huang *et al.*, 2006; Elling *et al.*, 2007; Hewezi *et al.*, 2009; Rehman *et al.*, 2009; Sacco *et al.*, 2009; Patel *et al.*, 2010; Wang *et al.*, 2010). A method is presented that finely demonstrates that proteins localized in the nematode glands are ultimately secreted into the host possibly inducing normal plant cells to dedifferentiate into feeding cells. Although the fine localization of nematode secreted products *in planta* is achievable, a next step will be to localize minute amounts of secretions in intracellular compartments.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Control images of immunodetection of Mi-ASP2, MAP-1, CBM2-bearing proteins, Mi-PEL3, and 6D4 using pre-immune sera in place of the primary antibody, in roots of *Arabidopsis thaliana* infected with *Meloidogyne incognita.*

Figure S2. Nucleotide and deduced amino acid sequences of the *Meloidogyne incognita* aspartyl protease-like *Mi-asp-2* cDNA.

Figure S3. Alignment of the predicted Mi-ASP2 protein with the cathepsin D-like Mi-ASP1 aspartic protease from *Meloidogyne incognita* (accession DQ360827).

Figure S4. Phylogeny of Mi-ASP2 from *Meloidogyne incognita* and its homologues.

Figure S5. Localization of *Mi-asp2* transcripts in *Meloidogyne incognita* pre-parasitic second-stage juveniles by *in situ* hybridization.

Figure S6. Immunoreactivity of the Mi-ASP2, CBM2, and Mi-PEL3 antisera against proteins from total *Meloidogyne incognita* J2 homogenate under denaturing conditions.

Figure S7. Control images of whole mount of J2s labelled with Mi-ASP2, CBM2-bearing proteins, and Mi-PEL3 in *Meloidogyne incognita* pre-parasitic juveniles.

Figure S8. Multiple sequence alignment of *Meloidogyne incognita* CBM2 modules.

Figure S9. Immunodetection of CBM2-bearing proteins (green) during parasitism in roots of *Arabidopsis thaliana*.

Figure S10. Phylogenetic analysis and multiple sequence alignment of pectate lyases.

Figure S11. Localization of *Mi-pel3* transcripts in *Meloidogyne incognita* pre-parasitic second-stage juveniles by *in situ* hybridization.

Table S1. The 100 best hits of the BlastP search against non-redundant protein databases at the NCBI site using the Mi-ASP2 sequence as query.

Table S2. Results of the BlastP search against the *Meloidogyne incognita* protein set using the Mi-ASP2 sequence as query.

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