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Identification and localisation of chitinases induced in the roots of potato plants infected with the potato cyst nematode *Globodera pallida*

Siyavosh RAHIMI*, Denis J. WRIGHT** and Roland N. PERRY*

**Entomology & Nematology Department, IACR-Rothamsted, Harpenden, Hertfordshire AL5 2JQ, UK and **Department of Biology, Imperial College of Science, Technology & Medicine, Ascot, Berkshire SL5 7PY, UK.*

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Summary - The electroblotting of extracts of potato roots infected with the potato cyst nematode, *Globodera pallida*, and their labelling with a polyclonal antibody against chitinases revealed the induction and increases in intensity of several chitinase bands with molecular masses in the range 18-80 kDa. The immunolabelling of sections of infected roots with the antibody demonstrated the extracellular localisation of chitinases and their concentration in the cortex, endodermis and pericycle layer. The role of these chitinases in the context of plant defences against pathogen attack is discussed. © Orstom/Elsevier, Paris

Résumé - ***Identification et localisation des chitinases induites dans les racines de plants de pomme de terre infestés par le nématode à kyste de la pomme de terre* Globodera pallida** - L'analyse par électro-transfert d'extraits provenant de racines de pomme de terre infestées par le nématode à kyste *Globodera pallida* et leur marquage par un anticorps polyclonal contre les chitinases a révélé l'induction et l'augmentation de l'intensité de plusieurs bandes correspondant aux chitinases et ayant une masse moléculaire s'étageant de 18 à 80 kDa. L'immuno-marquage par l'anticorps de sections de racines infestées a démontré la localisation extracellulaire des chitinases et leur concentration dans le cortex, l'endoderme et le péricycle. Le rôle de ces chitinases dans le contexte de défense de la plante contre les attaques d'agents pathogènes est discuté. © Orstom/Elsevier, Paris

Keywords : chitin, chitinases, *Globodera pallida*, potato cyst nematode, potato plants.

Chitinases in plants appear to have an important function in early embryo development (de Jong *et al.*, 1992). Chitinases have been detected in woody and herbaceous plants, including several important crop species (Boller *et al.*, 1983), and are involved in hormonal and developmental regulation (Shinshi *et al.*, 1987; Lotan *et al.*, 1989). Stresses in plants caused as a result of wounding, physiological stresses, attack by plant pathogens and treatment with ethylene and several chemicals and elicitors can induce marked increases in chitinase activity both locally or systemically (Métraux & Boller, 1986; Boller 1988; Broglie *et al.*, 1989; Collinge *et al.*, 1993). The type of chitinases most extensively studied in plants are endo-chitinases (E.C.3.2.1.14) which randomly hydrolyse internal β-1,4-linkages of chitin-releasing oligosaccharides of N-acetyl-D-glucosamine (Boller, 1985; Collinge *et al.*, 1993). Endo-chitinases are involved in the hypersensitive or systemic resistance response to microbial attack (Métraux & Boller, 1986; Boller, 1987; Pan *et al.*, 1992). Chitin, the natural substrate for chitinase, has not been reported from plants (Boller, 1987, 1988) but it is a major component of the cell walls of many fungal pathogens (Wessels & Sietsma, 1981; Wessels, 1993) and chitinase has been shown to attack the cell walls of these fungi *in vitro* (Young & Pegg, 1982; Mauch *et al.*, 1988; Broglie *et al.*, 1991). *In vivo* analysis of transgenic plants expressing high levels of constitutive chitinases demonstrated that, compared with their non-transgenic counterparts, these plants were more resistant to fungal pathogens and the development of disease symptoms was delayed (Broglie *et al.*, 1991; Benhamou *et al.*, 1993; Samac & Shah, 1994). The fungal-inhibitory action of chitinases was further enhanced when the enzyme was used in combination with β-1,3-glucanases (Broekaert *et al.*, 1988; Broglie *et al.*, 1991).

A potential but indirect role of chitinases in plant pathogen interactions is as elicitors of defence reactions. They are responsible for the release of oligosaccharides from the walls of fungi and plant cells which, in turn, stimulate the accumulation of compounds such as phytoalexins, extensin, proteinase inhibitors and lignin in the host plant as part of a defence mechanism (Darvill & Albersheim, 1984; Boller, 1987; Ryan, 1988). Another function attributed to chitinases is their involvement in the inactivation of the lipo-oligosaccharide signal molecules produced by certain *Rhizobium* strains (Roche *et al.*, 1991) which are responsible for the induction of root hair deformations, cortical cell divisions and nodule development in the roots of legume hosts (Truchet *et al.*, 1991).

Plant chitinases can be divided into at least three classes based on their amino acid sequence and cellular localisation (Payne *et al.*, 1990; Shinshi *et al.*, 1990). Class I chitinases are basic isoforms, localised in the central vacuole, containing a catalytic domain and a cysteine-rich domain similar to rubber hevein that may serve as an oligosaccharide binding site. Class II chitinases are acidic isoforms with a similar amino acid sequence to class I but they lack the hevein domain and are usually found in extracellular fluid of leaves (apoplastic compartment). Class III chitinases are also located extracellularly but show no homology with class I or class II chitinases. This class includes the lysozyme/chitinases from papaya, *Hevea*, *Parthenocissus*, *Rubus* and the chitinase of cucumber (Métraux *et al.*, 1989). A fourth class has been proposed by Collinge *et al.* (1993) which includes the basic sugar beet chitinase IV, the basic rape chitinase chB4 and the acidic bean PR4 chitinase.

Although research using antibodies has demonstrated the localisation of chitinases in roots of plants infected with a fungal pathogen (Benhamou *et al.*, 1990), no work has been reported on the immunolocalisation of chitinases in roots or other parts of plants infected with plant parasitic nematodes. In the present paper, we report investigations on the occurrence of chitinases in roots of potato plants infected with the potato cyst nematode (PCN), *Globodera pallida* pathotypes Pa1 and Pa2/3, and their localisation in the infected roots using a polyclonal antibody raised against chitinases.

Materials and methods

Unless stated otherwise, all the chemicals used were from Sigma Chemical Co. Ltd. UK.

Potato cultivars, experimental treatments and protein extraction

Two potato cultivars were used, cv. Désirée, which is susceptible to all pathotypes of *G. pallida*, and the experimental clone P55/7, with the H_2 gene from *Solanum multidissectum* conferring resistance to *G. pallida* Pa1 only (Dunnett, 1961); there are no commercial cultivars with complete resistance to *G. pallida*. Potato tubers were cut into small cubes (approximately 27 cm^3) each with a single sprout, left to heal for 30 min and then planted singly in 12 cm diameter plastic pots in 1:1 mixture of autoclaved sand:loam and kept in a glasshouse (18°C minimum temperature). Cysts of *G. pallida* Pa1 and Pa2/3 from stock cultures established on differential potato cultivars were regenerated on cv. Désirée and used after 6 months storage at 4°C. Thirty dry cysts were incorporated into the soil at the time of planting potato tuber pieces with five replicates per treatment. The majority of juveniles (J2) from these cysts hatched within 2 to 3 weeks and the number of nematodes per g of root which had invaded in each treatment was determined (Rahimi *et al.*, 1993). It was consistently 20% of cyst contents.

To extract proteins from roots, 5-10 g of roots were washed in several changes of distilled water (DW), blotted dry and homogenised using a pestle and mortar. The extract was passed through cheese cloth, centrifuged at 17 500 *g* and stored at -20°C until required. Each extraction was replicated a minimum of five times. Prior to analysis, extracts were centrifuged at 40 000 *g* for 30 min at 4°C.

Electrophoretic separation and electroblotting

The extracts from roots were separated using 7% SDS-PAGE electrophoresis (Laemmli, 1970) and electroblotted onto polyvinyline difluoride (PVDF) membrane as described previously (Rahimi *et al.*, 1996) and incubated in the primary antibody against chitinases diluted (1:3000; v/v) in 5% (w/v) Marvel in phosphate-buffered saline containing 0.05% (w/v) Tween (PBST). The polyclonal antibody (a gift from Dr. E. Kombrink) had been raised against chitinases purified from potato leaves treated with the elicitor derived from *Phytophthora infestans*.

Seed sterilization

Seeds of potato cv. Désirée were placed in a glass tube which had a sieve (60 µm pore size) attached to one end to retain the seeds. Seed sterilization (Sijmons *et al.*, 1991) was performed by immersing the tube in 70% ethanol for 2 min followed by immersing for 5 min in sterilized distilled water (DW). The seeds were left to dry overnight in a flow cabinet before being stored at 4°C in sealed sterile containers.

Growing sterilised potato seeds

The culture medium was modified from Knop (1860) according to Sijmons *et al.* (1991). The medium was autoclaved (15 min at 120°C and 10^5 Pa) and then cooled to 50°C in a flow cabinet: 20-25 ml was poured into individual Petri dishes (9 cm diameter). After the media had solidified, ten to twelve sterilised seeds, obtained from cv. Désirée, were transferred into each Petri dish using fine sterile forceps. The Petri dishes were sealed using strips of Parafilm and kept in growth chambers at 18°C. Incubation with nematode eggs was performed 7-10 days after seeds had been sown.

Inoculation with nematode eggs

Approximately 100 µl of cysts of *G. pallida* Pa1 were placed in 1.5 ml Eppendorf tubes and soaked in double-distilled water (DDW) overnight. Cysts were transferred to an aluminium plate and a piece of glass rod was used to crush the cysts and release the eggs

into 60 ml of DDW in a 100 ml measuring cylinder which was vortexed to separate the eggs and hatched juveniles from the cyst walls. The suspension was passed into a beaker through a sieve (60 µm pore size) to retain cyst walls and other debris. The suspension of eggs and J2 was passed through a 10 µm sieve, resuspended in 10 ml of DDW and pipetted into a conical-bottomed centrifuge tube and spun at 1000 *g* for 30 s. The liquid was poured out without disturbing the pellet of nematodes, replaced with 10 ml of 45% (w/v) sucrose solution, vortexed and centrifuged for 30 s which brought the nematode eggs to the top of the solution and pelleted the debris at the bottom. The fraction containing the nematode eggs was immediately transferred into the 10 µm sieve, washed with DDW to remove sucrose solution, resuspended in 10 ml of DDW and transferred to a flow cabinet. Sterile syringes (10 ml) were used to draw up first 5 ml of nematode eggs and then 5 ml of 0.2% (w/v) solution of $HgCl_2$ (disinfectant), shaking them several times to mix the solutions. After sterilization with $HgCl_2$ for 4 min, the suspension in the syringe was gently forced out through a sterile filter holder containing a sterile disc filter membrane (5 µm pore size). The sterile nematode eggs were retained in the filter membrane. The syringe was filled with sterile DDW by removing the holder and reattaching it, and the nematode eggs were washed by a gentle flow of water. This was repeated three times before the syringe was filled with air which was forced out of the syringe partially to dry the eggs. The filter membrane was removed using sterile forceps, blotted gently and left in a sterile Petri dish. A flamed scalpel blade was used to cut the membrane into four equal pieces and each portion was used to inoculate a plate. The Petri dishes containing potato plantlets were unsealed and a sterile scalpel blade was used to score the surface of the agar in "tramlines" away from the roots. The Petri dishes were then inoculated by rubbing the eggs stuck on the surface of a piece of membrane against the tramlines; in total, 30 plates were inoculated. The Petri dishes were sealed and placed in growth chambers at 18°C. After 7-10 days, the eggs hatched and J2 started to invade the roots. Controls consisted of Petri dishes containing non-infected plants.

Preparation of roots for immunolabelling with antibody

All the non-infected and infected roots for analysis were selected from plants of the same age. At least five replicates were used for each treatment. When the potato roots had been invaded and J2 had initiated development of the feeding site (syncytium), pieces of roots containing the syncytium and tissue above and below the syncytium were cut into 1 mm portions and placed in modified plastic tubes (1.0 × 0.5 cm) with polyester voile (25 µm mesh size) stuck on either end. This allowed infiltration of the chemicals into the roots. The procedure for fixation was modified from Karnovsky (1965). The tubes were placed immediately in 30 ml sterile tubes containing fixative (0.1% [v/v] glutaraldehyde and 2.5% [w/v] paraformaldehyde in 0.05 M sodium cacodylate buffer $Na(CH_3)_2AsO_2.3H_2O$; pH 7.2) for 4 h. The tubes fitted into a rotator which could be placed in a refrigerator or a freezer as required. The fixative was replaced with three washes of 0.025 M sodium cacodylate, pH 7.2 for 15 min at 4°C. The roots were dehydrated by placing the tubes in a series of ethanol solutions (v/v) of 30% at 4°C, 50, 70, 90, 95 and 100% rotating at –18°C, for 30 min each. The roots were infiltrated by replacing half of the volume of pure ethanol in the tube with acrylic resin (LR White, medium grade; The London Resin Co. Ltd., Hampshire, UK) and rotating at -18°C for 2 h followed by replacement of half of this solution with an equal volume of LR White resin and rotating again for 2 h. This step was repeated a third time. The contents of the tube were poured out and filled with pure LR White resin for 2 h at -18°C before emptying the solution and filling with fresh resin and leaving it rotating at –18°C overnight. The resin solution was then replaced with fresh LR White resin and left for 1 h. Finally, the pieces of roots were embedded in gelatin capsules size 00 (Agar, UK) filled with resin and left to polymerise at 60°C for 24 h. The capsules were left at room temperature for another 24 h to harden.

Sectioning root tissue and immunolabelling of root sections

Roots were sectioned using an ultracut microtome (Reichert-Jung, Austria) from a minimum of five replicates of infected and non-infected roots. The sections were cut 200-250 nm in thickness, placed on microscope slides with eight to ten wells (ICN, UK) and heated to 50°C for 1 h to ensure that the sections adhered to the wells. A quantity (30 µl) of 0.01% (w/v) sodium borohydrate was pipetted into each well, left for 10 min then washed thoroughly with DDW; excess water was shaken off. The sections were blocked for 1 h by adding 30 µl of 3% (w/v) BSA in PBS containing 0.02% (w/v) sodium azide. This was washed off with DDW and the sections were then labelled for 2 h or overnight with 10 µl of the primary antibody against chitinases diluted 1:10 or 1:50 in 3% BSA/PBS. The antibody was washed off by a quick rinse in DDW followed by immersing the slides in PBST (0.05% Tween 20) for 10 min and two changes of DDW for 5 min each; excess water was shaken off. The sections were labelled for 1 h with 10 µl of secondary antibody (anti-rabbit IgG, FITC conjugate) diluted 1:35 with 3% BSA/PBS. The secondary anti-

body was washed off and the slides left to dry for 10 min before a solution of glycerol/PBS (Citifluor; Agar, UK) and cover-slips were added. Specificity controls involved replacing the primary antiserum with a solution of 3% BSA in PBS or a dilution of non-immune rabbit serum prepared in 3% BSA/PBS. The sections were viewed under a fluorescent microscope (Olympus BH2; wavelength 500-580 nm) and photographs (Tri-X 400, Kodak or XP2 400, Ilford) were taken immediately.

Results

Electrophoretic separation and electroblotting

The electroblotting of the root extracts from P55/7 (Fig. 1A) grown in pots with 30 cysts of *G. pallida* Pa1 (track I) and Pa2/3 (track II) revealed the presence of two bands with chitinase activity with molecular masses of approximately 28 and 60 kDa which were absent in the non-infected treatment (track III).

The cv. Désirée showed a similar pattern of chitinase activity to that of P55/7 (Fig. 1B). Infection with *G. pallida* Pa1 (track I) induced two chitinase bands with molecular masses of approximately 26 and 60 kDa which were absent in the non-infected treatment (track III). The infection with Pa2/3 (track II) only induced a chitinase band of 60 kDa.

In addition, several other bands (not arrowed) also showed increases in intensity in the extracts from infected plants of both the cultivars.

Immunolabelling of root sections

Transverse sections from non-infected roots (controls) demonstrated that, when the primary antibody against chitinases was replaced with a solution of 3% BSA/PBS, no binding was visible (Fig. 2A). When the primary antibody was replaced with a dilution of 1:10 non-immunised rabbit serum (Fig. 2B), weak fluorescence was observed on the edges of the root sections and within the cells. Similarly, only weak fluorescence of the root tissue was apparent when the antibody against chitinases was used (Fig. 2C, D).

Sections from roots infected with *G. pallida* Pa1 showed clear labelling. The immunolabelling of roots 2 weeks after invasion by *G. pallida* Pa1 showed marked induction of chitinases (Fig. 3). The first set of sections (Fig. 3A, B) were from areas of infected roots several cm away from the nematode body and were probed with 1:10 dilution of the non-immunised rabbit serum or the primary antibody against chitinases. Whilst probing with non-immunised rabbit serum showed only very weak labelling of root tissue (Fig. 3A), probing with the primary antibody resulted in strong labelling in the cortex, endodermis and pericycle layer (Fig. 3B). Probing with non-immunised rabbit serum showed weak labelling of root tissue and only the nematode fluoresced strongly (Fig. 3C). By contrast, probing with the primary antibody resulted in strong labelling of root sections (Fig. 3D-F). The binding appeared to be only extracellular.

Longitudinal sections of infected roots were analysed (antibody dilution, 1:50) and sections, both at lower (Fig. 4A) and higher (Fig. 4B) magnification, clearly indicated the extracellular localisation of chitinases in the infected roots.

Discussion

Results from electroblotting experiments revealed two bands of chitinases induced in the roots of potato plants (P55/7 and cv. Désirée) infected with *G. pallida* pathotypes Pa1 and Pa2/3. One of these chitinase bands had a molecular mass of approximately 60 kDa and the other chitinase band had a molecular mass in the range 26-28 kDa. The chitinase bands could not be associated with resistance to *G. pallida*; cv. Désirée

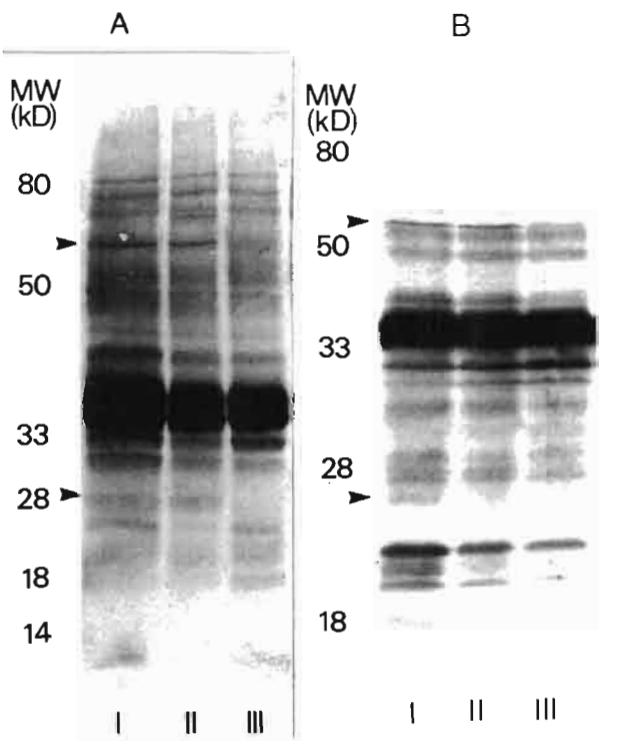

Fig. 1. *Electroblotting of the root extracts from 4-week-old plants probed with 1:3000 dilution of the polyclonal antibody against chitinases. A: Clone P55/7 from pots containing 30 cysts of* Globodera pallida *Pa1 or Pa2/3 (tracks I and II, respectively); track III is from non-infected extracts; B: Cv. Désirée from pots containing 30 cysts of* G. pallida *Pa1 or Pa2/3 (tracks I and II, respectively; track III is from non-infected extracts).*

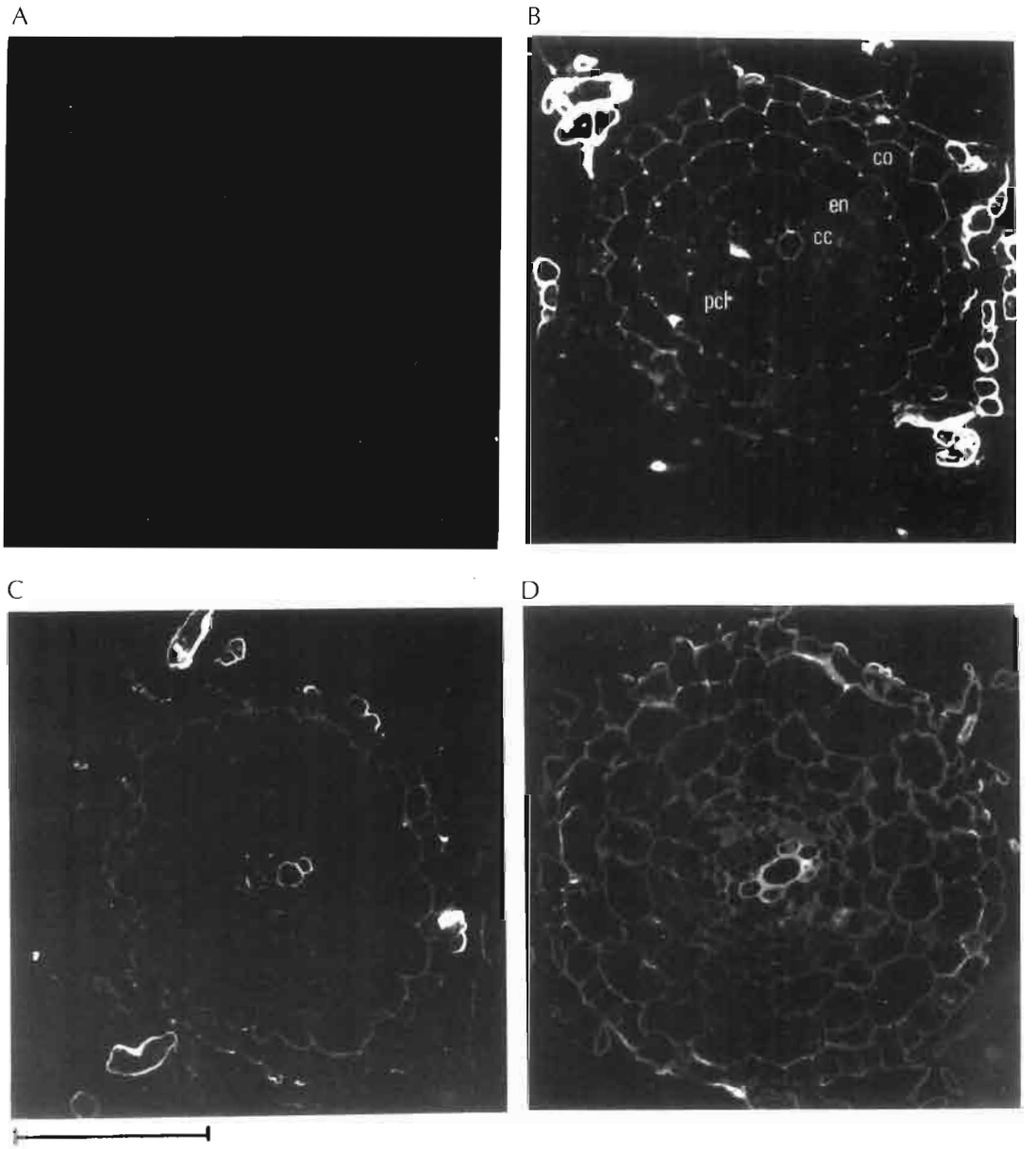

Fig. 2. *The immunolabelling of transverse sections (200 nm thick) of non-infected root. The sections were labelled with primary antibody against chitinases or a substitute followed by labelling with 1:35 dilution of secondary antibody (anti-rabbit IgG, FITC conjugate). A: The primary antibody was replaced with 3% solution of BSA/PBS; B: The primary antibody was replaced with 1:10 dilution of non-immunised rabbit serum; C, D: The sections were labelled with 1:10 dilution of antibody against chitinases. (Abbreviations: cc: central cylinder; co: cortex; en: endodermis; n: nematode, pci: pericycle layer; Scale bar = 150 μm).*

is susceptible to both pathotypes of *G. pallida*, whereas P55/7 is susceptible to Pa2/3 but resistant to Pa1. Therefore, the immunolabelling experiments were only done on cv. Désirée infected with *G. pallida* Pa1. There were also no differences in the timing of appearance of these chitinases after nematode invasion by Pa1 in P55/7 (incompatible interaction) and by Pa1 in cv. Désirée (compatible interaction) (Rahimi, 1994). It has also been shown that in P55/7, the exoglucanase and β-D-glucosidase activities were only significantly increased after inoculation with *G. pallida* Pa 2/3 (Rahimi *et al.*, 1996). A similar pat-

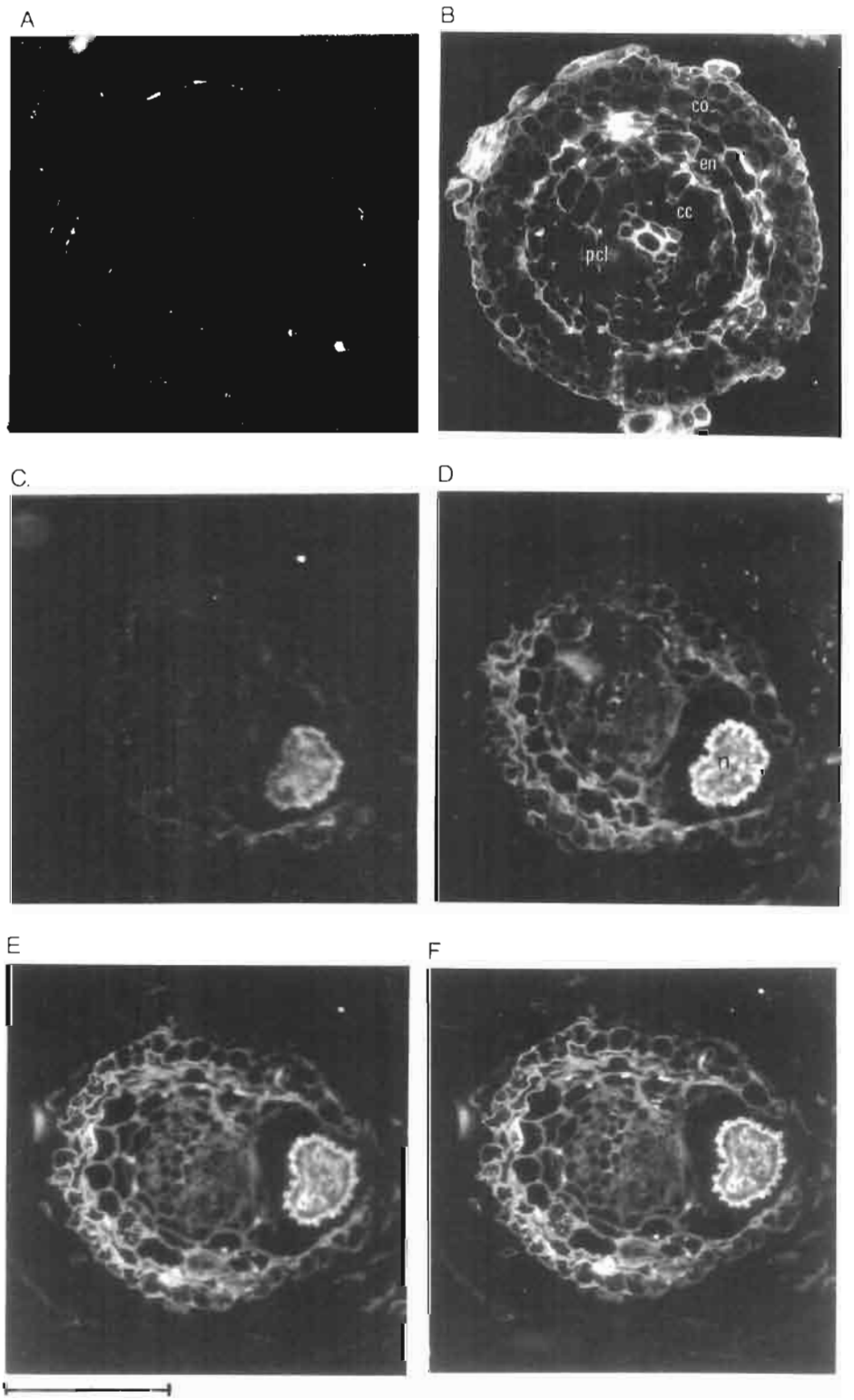

Fig. 3. *The immunolabelling of transverse sections (200 nm thick) of root infected with* Globodera pallida *Pa1. The sections were labelled with 1:10 dilution of non-immunised rabbit serum (A, C) or 1:10 dilution of the primary antibody against chitinases (B, D-F) followed by labelling with 1:35 dilution of secondary antibody. A, B: The sections were taken from areas of roots away from the nematode's head; C - F: The labelling of sections of root from the region where the nematode was embedded. (Abbreviations: see legend to Fig. 2; Scale bar = 150 µm).*

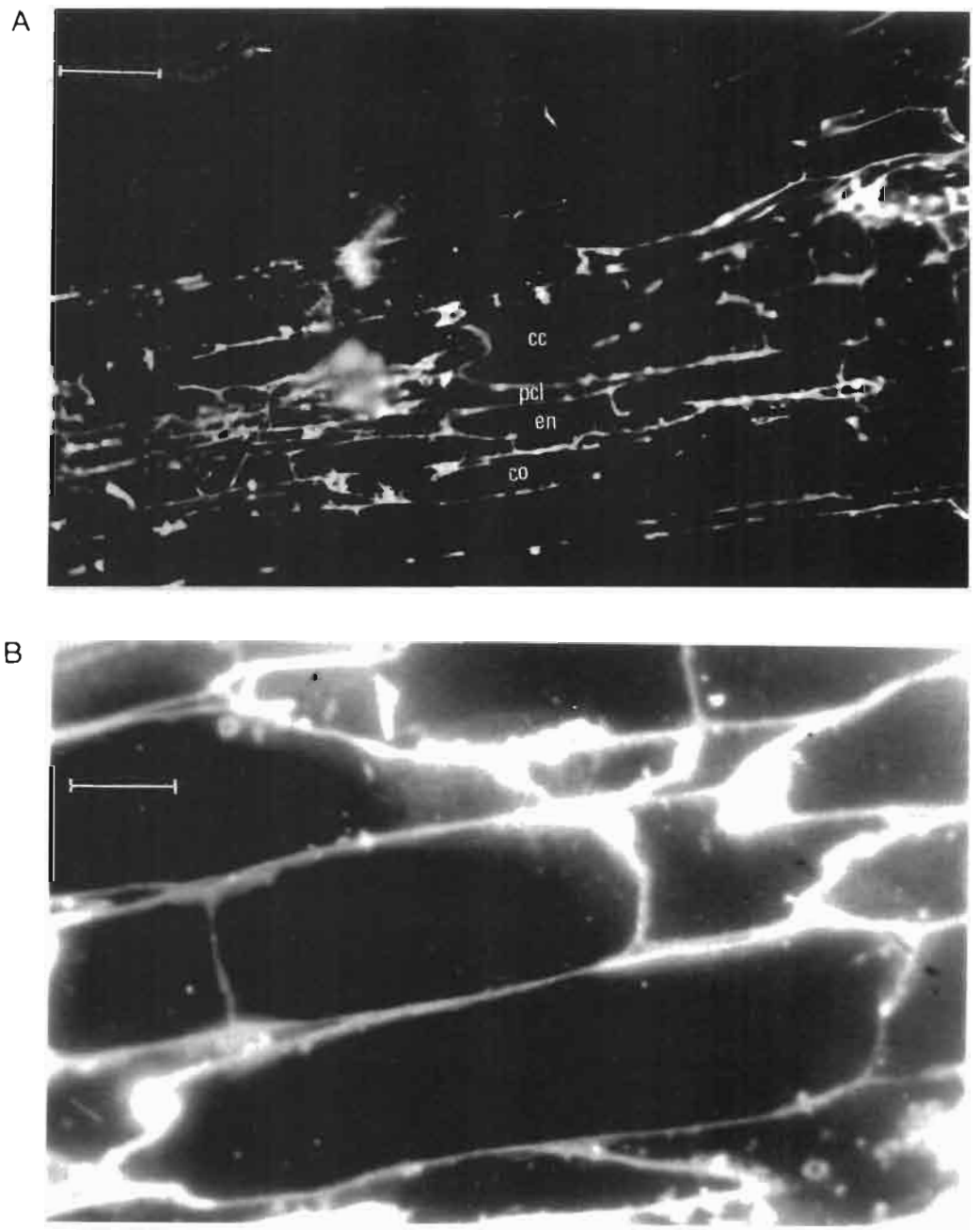

Fig. 4. *Longitudinal sections (200 nm thick) of root infected with* Globodera pallida *Pa1 and labelled with 1:35 dilution of secondary antibody. (Abbreviations: see legend to Fig. 2; Scale bars: A = 15 μm; B = 8 μm).*

tern of pathogenesis-related (PR) protein production in P55/7 was observed by Rahimi *et al.* (1993) where the susceptible interaction resulted in the accumulation of a greater number of PR proteins than the resistant interaction with Pa1. Together, these results indicate that increased activity of β-1,3-glucanase, β-D-glucosidase and chitinase does not relate directly to resistance to *G. pallida* but are more likely to be part of a general plant response to pathogen invasion.

The immunolabelling of sections from roots of cv. Désirée infected with *G. pallida* Pa1 showed strong binding of the polyclonal antibody to several areas.

The labelling appeared to be in the cortex, endodermis and pericycle layers. Both the transverse and longitudinal sections showed that the antibody bound mainly to chitinases that were located extracellularly. Very weak binding also was apparent in the central cylinder of both the infected and the non-infected sections. Sections of nematodes fluoresced strongly when either non-immunised rabbit serum or the primary antibody were used; this is likely to be the result of non-specific binding.

PR proteins appeared in leaves and roots of potato plants after infection with *G. pallida* (Rahimi *et al.*, 1993) and several of these new leaf proteins were β-1,3-glucanases which increased significantly only in intercellular extracts of leaves and leaf homogenates (Rahimi *et al.*, 1996). In contrast, when the extracts from leaves and roots of nematode infected plants were analysed for increased levels of chitinases using enzyme assays (Rahimi, 1994), only the extracts from the infected roots showed increases in chitinase activity of which exochitinases were significant. The present research confirms that the majority of chitinases induced in potato roots invaded with *G. pallida* are exochitinases. Whether these classes of chitinases are produced intracellularly in response to nematode invasion and are then transported to the extracellular spaces or are produced and located extracellularly is uncertain but analysis of roots at different times after infection (results not shown) indicated no intracellular location of chitinases. It is probable that the type of chitinases localised in potatoes infected with *G. pallida* belong to class III chitinases which are extracellularly localised and are possibly basic in nature. Clearly, there may be other groups of chitinases induced in potato roots by *G. pallida* which our antibody failed to detect due to its binding specificity.

Several studies have shown that various preparations of chitinases can be used successfully to control plant parasitic nematodes both *in vitro* and in soil (Miller & Sands, 1977; Mercer *et al.*, 1992), although the mode of action is unknown. For chitinases to be involved directly in plant defence reactions, they require a substrate, chitin, in the pathogen. Although chitin is a major structural component of nematode egg shells, it is absent in other life cycle stages, including J2, of nematodes (Bird & Bird, 1991). Thus, it is unlikely that the class of chitinases identified and localised in the present work is involved directly in plant defence reactions against nematode attack. The chitinases may be involved in other pathways resulting in resistance or tolerance of potato plants to invasion of other pathogens. For example, chitinases may play a secondary function as signal molecules which elicit the induction of other PR proteins or metabolites which are involved in plant-defence reactions. After invasion of potato plants by *G. pallida* and the appearance of chitinases in roots, large amounts of new proteins including glucanases and glucosidases accumulated in the leaves (Rahimi *et al.*, 1996). It is possible that the accumulation of these new leaf proteins was triggered by chemical signals from the infected roots; chitinases may have an important function in triggering this process which may eventually impart resistance in potato plants to subsequent aerial or root infections. Chitinases in plants exhibit hormonal and developmental regulation (Shinshi *et al.*, 1987; Lotan *et al.*, 1989) and an increase in chitinase activity may have a hormonal or developmental function.

The present studies demonstrate the appearance of chitinases in roots after invasion with potato cyst nematode, *G. pallida*, and the localisation of these chitinases in infected roots. Further work is required to isolate and sequence these chitinases in order to confirm their classification and to define their function in infected roots.

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