Aska Goverse

Cyst nematode-induced changes in plant development

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ter verkrijging van de graad van doctor
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van de Wageningen Universiteit,
dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 15 oktober 1999
des namiddags te half twee in de Aula.

Cover: Early feeding cell development in tomato roots infected with the potato cyst nematode *Globodera rostochiensis*. This longitudinal section shows the head region of a second stage juvenile with its stylet inserted in the initial feeding cell.

Cover design: Aska Goverse and Hein Overmars.

Goverse, Aska

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Thesis Wageningen – with references – with summary in Dutch
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Stellingen

1. Onder invloed van wortelexudaten van de aardappel secreteren pre-parasitaire juvenielen van de plantenparasitaire nematodensoort *Globodera rostochiensis* kleine peptide(n) die de proliferatie van zowel tabaksprotoplasten als humane bloedcellen co-stimuleren.
dit proefschrift

2. De vorming van syncytia door aardappelcystenaaltjes gaat vermoedelijk gepaard met een auxine-geïnduceerde ethyleenproduktie, hetgeen resulteert in de lokale stimulatie van celwand-afbrekende enzymen.
dit proefschrift

3. De morfologie van door cystenaaltjes geïnduceerde voedingscellen wordt gekenmerkt door een zogenaamde "cortical bridge" via welke het syncytium zich uitbreidt naar de vaatbundel (Sembdner, 1963; Jones and Northcote, 1972). De abnormale ontwikkeling van voedingscellen in aanwezigheid van de auxinetransportremmer *N*-(1-naphthyl)phtalamic acid (NPA) vormt een sterke aanwijzing dat deze polariteit mede wordt bepaald door een radiale auxine gradiënt.
dit proefschrift

   Sembdner (1963) *Nematologica* 9: 55-64.
   Jones and Northcote (1972) *J. Cell Sci.* 10: 789-809.

4. *Gland secretions originating from the esophageal glands of the nematode may be deposited outside the plasma membrane and interact with a membrane receptor or injected directly into the cytoplasm of the recipient cell through a perforation in the plasma membrane at the stylet orifice (Williamson and Hussey (1996)* The Plant Cell *8 : 1735-1745).*
   De voorspelde cytoplasmatische lokalisatie van onlangs gekloneerde resistentiegenprodukten gericht tegen diverse sedentaire plantenparasitaire nematoden (Milligan *et al.*, 1998; Van der Vossen *et al.*, 1999) ondersteunt de hypothese dat voor de inductie van een voedingscel secreties direct in de plantencel worden geïnjecteerd.

   Milligan *et al.* (1998) *The Plant Cell* 10: 1307-1319.
   Van der Vossen *et al.* (1999) Proceedings of the Keystone Symposium *Interactions and intersections in plant signaling pathways*, Coeur d'Alene Id, USA, p54.

5. Het behoeft geen hydrologische kennis om te voorzien dat het aanstellen van grote aantallen promovendi in combinatie met een beperkte doorstroomsnelheid binnen het huidige universitaire stelsel de vorming van een stuwmeer met jonge doctors in de hand werkt en het weglekken van wetenschappelijke kennis via een zogenaamde braindrain stimuleert.

6. De wetenschappelijke verdieping die opgedaan wordt bij het vervaardigen van een proefschrift gaat vaak gepaard met een tijdelijke bewustzijnsvernauwing van de auteur.

7. Het proefschrift is niet langer de kroon op het levenswerk van een academicus, maar de basis voor een werkzaam leven in de wetenschap.

8. Geluk zit in een heel klein tentje.

Stellingen behorende bij het proefschrift getiteld: "Cyst nematode-induced changes in plant development", door Aska Goverse.

Wageningen, 15 oktober 1999

Ter herinnering aan Loe van der Voet

CONTENTS

CHAPTER 1

General introduction

N.A. Cobb (1914): "The nematodes, poor beasts, do not furnish hides, horns, tallow or wool; they are not fit for food, nor do they produce anything fit for food; neither do they sing or amuse us in anyway; nor are they ornamental - in fact, when they are displayed in museums the public votes them hideous."

Plant-parasitic nematodes

Most members of the phylum Nematoda are transparent, vermiform organisms of microscopic size which live in various habitats on earth including arctic soils (Powers *et al.*, 1998) and deep-sea sediments (Jensen, 1992). The majority of this large phylum consist of free living nematodes that feed on bacteria, fungi or other nematodes. An example is the bacterivorous nematode *Caenorhabditis elegans*; a well-known model organism for molecular and cellular research in higher eukaryotes. Just a small percentage of all nematodes has a parasitic life-style. They have evolved sophisticated means to infect completely unrelated organisms, such as animals and plants. This requires special adaptations to parasitism, which is reflected by differences in morphology and a life cycle which is synchronised with the life cycle of the host. For years, these parasitic nematodes have encountered ample attention from scientists, since they cause serious problems to the health of millions of people, livestock and agricultural crops world wide.

Plant-parasitic nematodes in the order Tylenchida are characterised by a robust hollow stylet that can be protruded to pierce the plant cell wall: the major barrier that these nematodes have to overcome in order to penetrate the plant. This stylet is a small straw of about one micron in width with a circular lumen of 0.5 µm or even 0.25 µm at the tip (Bird, 1969; Ellenby and Wilson, 1969). Plant-parasitic nematodes have evolved various strategies to infect their host plants. Ectoparasites, like *Tylenchorhynchus dubius*, live in the rhizosphere and feed from epidermal and root hair cells. At the other hand, endoparasitic nematodes enter the plant in search for an appropriate food source. This group consists of migratory endoparasites like *Pratylenchus penetrans* and sedentary endoparasites like cyst (*Heterodera* and *Globodera* spp.) and root knot nematodes (*Meloidogyne* spp.), which become immobile and complete their life cycle inside the plant.

Cyst nematodes

Cyst nematodes are obligatory plant-parasitic nematodes that feed - in contrast to root knot nematodes - on a small host range. The potato cyst nematode species *Globodera rostochiensis* and *G. pallida* can only successfully reproduce on the Solanaceous species potato, tomato and egg plant (Evans and Stone, 1977). The potato cyst nematode originates from the Andes region in South America and is introduced in Western Europe in the late 19th century. These parasites were successfully spread throughout Europe together with potato breeding material. Despite the slow propagation rate of potato cyst nematodes, they became one of the major pests in potato crops resulting in major annual yield losses. Potato cyst nematodes are the causing agents of the disease better known in the Netherlands as "aardappelmoeheid".

At the end of the growing season, cysts filled with progeny are left in the soil in anticipation of new host plants. This persistent structure, which is the remaining cuticle of the dead parent female, can contain up to 600 eggs filled with dormant second stage juveniles. The infective

larvae will hatch in response to root exudates (Perry, 1987). Subsequently, they migrate through the soil towards the root system. Once the infective juvenile reaches the epidermis of the root, it enters the plant preferentially in the differentiation and elongation zone of the root by perforating cells using a combination of intensive stylet thrusting and enzymatic softening of the cell walls. Then, the nematode migrates intracellularly through the cortex in search for an appropriate feeding site.

Upon selection of the initial feeding cell, the infective juvenile becomes immobile and carefully punctures the cell with its stylet. The nematode stylet remains inserted for a couple of hours, which is the so called preparation period, just before the nematode starts to feed (Steinbach, 1973, Wyss and Zunke, 1986; Wyss, 1992). In case of the potato cyst nematode, a fully differentiated cortex cell is modified into a high metabolically active cell which is characterised by small secondary vacuoles, dense cytoplasm, numerous organelles and an enlarged nucleus (Cole and Howard, 1958; Sembdner, 1964; Rice *et al.*, 1985). The feeding cell expands towards the vascular bundle by progressive cell wall dissolution, which starts from plasmodesmata in the initial feeding cell (Grundler *et al.*, 1998). This results in a multinucleate syncytium which extends longitudinally along the root axis in close contact with the xylem. Cell wall protuberances are formed exclusively in vicinity of the xylem vessels to facilitate the uptake of large quantities of solutes by the feeding nematode (Jones and Northcote, 1972). The biotrophic cyst nematodes fully depend on this transfer-like cell for their development and reproduction.

Upon repeated cycles of feeding, the immobile second stage juvenile moulds into two successive parasitic juvenile life stages (J3 - J4). For cyst nematodes, sex is determined epigenetically in early nematode development by the size (Trudgill, 1967) and the nutrient composition of the feeding cell (Grundler *et al.*, 1991). The development of females requires the uptake of large amounts of nutrients from the infected root – up to 4 times their body volume per day (Sijmons *et al.*, 1991) – to support the extensive growth of the female body. Interestingly, it has been demonstrated that infective juveniles of the beet cyst nematode *H. schachtii* develop into males when the initial feeding cell is selected in the pericycle of the small crucifer *Arabidopsis thaliana*. Females develop in case a procambial cell is selected as the starting point for syncytium formation (Sobczak *et al.*, 1997). Cyst nematodes show a high degree of sexual dimorphism resulting in extensive swollen adult females, which burst out of the root, and vermiform adult males which are attracted in order to fertilise the eggs.

Nematode secretions

In both animals and plants, nematode secretions are considered to play an essential role in the intimate relationship between the parasite and its host. In a compatible interaction, the production and release of secretions in specific developmental life stages enables the nematode to manipulate host cell metabolism for its own benefit. This is examplified by the induction of a nurse cell by excretions/secretions (E/S) of the animal parasite *Trichinella spiralis* in infected muscle cells (Lee *et al.*, 1991) and the suppression of the immune response of the host by E/S of the filarial nematodes *Brugia malayi*, *Nippostrongylus braziliensis* and *Toxocara canis* (Allen and MacDonald, 1998). For obligatory plant-parasitic nematodes, it is assumed that secretions of the infective juvenile are injected into the parasitised cell resulting in the development of a feeding cell (reviewed by Williamson and Hussey, 1996).

In plant-parasitic nematodes, secretions are produced by several gland structures like the excretory pore, the rectal and amphidial glands. These structures are observed in most nematode species including free-living nematodes and as such, it is likely that they have a general role in nematode physiology. However, plant-parasitic nematodes are characterised by the presence of three oesophageal glands, *viz.* one dorsal gland and two subventral glands (Fig. 1A), which are most likely adaptations to plant parasitism. These glands are large single cells, in which secretory proteins are produced and stored depending on the developmental life stage of the nematode. The gland lobe is packed with secretory granules which can be transported via the gland extension into the ampulla from where the secretions are released into the oesophageal lumen by endocytosis. The

dorsal gland ends just behind the nematode stylet, whereas the subventral glands empty into the lumen just behind the metacorpal pump chamber; a structure which is involved in nematode feeding.

To elucidate the role of oesophageal gland secretions in the plant-nematode interaction, a lot of effort has been made to identify salivary gland proteins produced by cyst nematodes (Fig. 1B; for review Hussey, 1989). For a long time, research in this field is seriously hampered by the microscopic size and the biotrophic nature of these endoparasites. Based on the anatomy of the infective juvenile, it was speculated that the dorsal gland secretions were released into the plant, whereas the subventral gland secretions were involved in food digestion (Wyss and Zunke, 1986; Wyss, 1992). The use of monoclonal antibody technology (Mab) resulted in the detection of oesophageal gland proteins in different developmental life stages of the soybean cyst nematode *H. glycines* (Atkinson and Harris, 1986). Using a different set of Mabs, it was demonstrated that infective juveniles of *H. glycines* secrete subventral gland proteins *in vitro* upon exposure to the serotonin analogue 5-methoxy-DMT-oxalate (Goverse *et al.*, 1994) supporting a possible role for subventral gland proteins in plant parasitism. In addition, a large panel of Mabs was raised against subventral gland antigens of the potato cyst nematode *G. rostochiensis* and *G. pallida*, which showed cross-reactivity with the subventral glands of *G. tabacum* and *H. glycines* (De Boer *et al.*, 1996). Further characterisation of these proteins and their encoding genes resulted in the identification of the first animal-produced endoglucanases, which are involved in cell wall degradation during root penetration and intracellular migration (Smant *et al.*, 1998; Wang *et al.*, 1999). No nematode signalling molecules are identified yet, which are involved in feeding cell induction and/or maintenance.

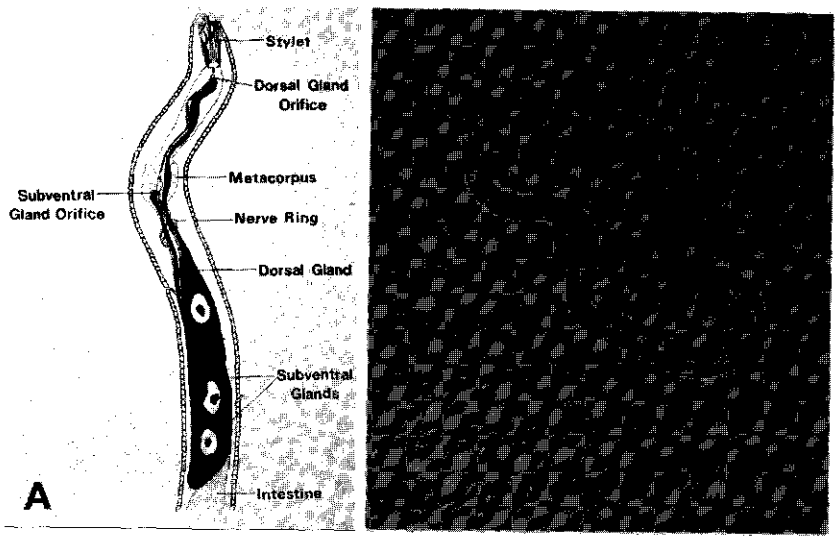

Fig. 1A. *Infective juveniles of cyst nematodes are characterised by the presence of two esophageal glands: one dorsal gland and two subventral glands.* ***B.*** *Stylet secretions produced by a pre-parasitic second stage juvenile of the potato cyst nematode* Globodera rostochiensis *upon exposure to potato root diffusate.*

Outline of this thesis

This thesis describes a first attempt to investigate the biological activity of cyst nematode secretions on plant cell proliferation and the molecular mechanisms underlying feeding cell development in plant roots upon cyst nematode infection.

To investigate the role of nematode secretions in feeding cell development, the *in vitro* induction and collection of putative nematode signalling molecules is needed. Chapter 2 describes the specific induction of nematode secretions from infective juveniles of the potato cyst nematode *Globodera rostochiensis* upon exposure to potato root diffusate (PRD). Using this host stimulus, relatively large quantities of naturally-induced secretions could be collected from infective juveniles. In Chapter 3, we describe the detection of an oligopeptide(s) in nematode secretions, which stimulate(s) the proliferation of plant cells using a bioassay based on tobacco leaf protoplasts.

The analysis of early changes in gene expression in response to cyst nematode infection is strongly supported by the use of an *in vivo* reporter gene system. Chapter 4 describes the use of the green fluorescent protein (GFP) to monitor the transcriptional regulation of respectively the viral CaMV 35S and the bacterial TR2' promoter in early feeding cell development in transgenic potato roots infected with *G. rostochiensis*. Unfortunately, the use of GFP in combination with the more subtle *Arabidopsis* cell cycle promoters *cycB1;1* and *cdc2a* was complicated by inadequate expression levels necessary for proper GFP detection in infected potato roots. This obstacle was overcome by using the firefly luciferase gene *luc* as an *in vivo* reporter gene (Chapter 6).

For decades, it is hypothesised that phytohormones could be involved in syncytium formation. In Chapter 5, a molecular genetic approach was used to study the role of auxin in cyst nematode-induced feeding cell development. The infection of the auxin-insensitive tomato mutant *diageotropica* and several *A. thaliana* auxin response mutants suggest that cyst nematodes manipulate the local auxin balance upon early feeding cell induction.

Chapter 6 gives an overview of cell cycle activation by plant-parasitic nematodes; especially cyst and root knot nematodes, and links the possible role of nematode secretions and auxin in feeding cell formation.

CHAPTER 2

Potato root diffusate-induced secretion of soluble, basic proteins originating from the subventral esophageal glands of potato cyst nematodes

Geert Smant, Aska Goverse, Jack W.P.G. Stokkermans, Jan M. De Boer, H. (Rikus) Pomp, Jacoline F. Zilverentant, Hein A. Overmars, Johannes Helder, Arjen Schots, Jaap Bakker

Phytopathology 87: 839-845 (1997)

ABSTRACT

In pre-parasitic juveniles (J2) of *Globodera rostochiensis*, six proteins with molecular weights of 30, 31a/b, 32, 39, and 49 kDa were recognized on western blots by a monoclonal antibody (MGR48) specific for the subventral esophageal glands. All these subventral gland proteins (*svps*) focused in the basic range (pI 6.8-8.6) of an immobilized pH gradient. Western blotting showed that the *svps* were present in pre-parasitic and in parasitic J2, and not in following juvenile stages and adult females. Minor *svp* quantities were also observed in adult males. Immunogold labelling of pre-parasitic (J2) showed that the *svps* were localized in the rough endoplasmic reticulum and secretory granules of the subventral esophageal glands. Potato root diffusate triggered the secretion of the *svps* through the stylet and 5-methoxy-N,N-dimethyltryptamine-hydrogen-oxalate (DMT) was shown to have only a quantitative, additional effect. The forward flow of the *svps* through the metacorporal pump chamber was confirmed by the presence of *svps* in the circular lumen of the esophagus (procorpus), as established by immunoelectron microscopy. Our data provide conclusive evidence that secretory proteins of the subventral glands of *G. rostochiensis* can be secreted through the stylet and support the hypothesis that the subventral esophageal glands play an important role in the early events of this nematode-plant interaction.

INTRODUCTION

Cyst nematodes, *Heterodera* and *Globodera* spp., have evolved highly specialized and complex feeding relationships with their host plants. After penetration behind the growing root tips, the second-stage juveniles (J2) migrate intracellularly toward the vascular cylinder and become immobile after the induction of an initial syncytial cell opposite a protoxylem pole. Cells adjacent to the initial syncytial cell are incorporated into the expanding syncytium by partial dissolution of the cell walls. The central vacuole of the syncytial cells is divided into small secondary vacuoles and the proliferation of mitochondria, Golgi-bodies, plastids, and endoplasmic reticulum implicates a dramatic enhancement of cell metabolism (Jones, 1981; Jones and Northcote, 1972; Melillo *et al.*, 1990; Rice *et al.*, 1985). The expansion of the syncytium is accompanied by the activation of the promoters of the cell cycle genes *Arath;cycB1;1* and *cdc2a* (Niebel *et al.*, 1994) and increased synthesis of DNA (Endo, 1971).

Nematode esophageal gland secretions are thought to play an important role in the host-parasite interaction (Hussey, 1989; Jones, 1981). Cyst nematodes have three large esophageal gland cells, one dorsal and two subventral. The dorsal gland has a long cytoplasmic extension that terminates in an ampulla, a reservoir for secretory granules. The ampulla is connected, via a sclerotized duct with an elaborate valve, with the esophageal lumen just behind the stylet knobs (Endo, 1984; Endo, 1987). The release of the secretions at the base of the stylet indicates that passenger molecules from the secretory granules formed in the dorsal gland can probably be secreted through the stylet and, as a consequence, can be involved in the host-parasite interaction.

The direction in movement of the secretions released from the two subventral glands is less clear and the biological role of the secretions has been the subject of speculation. The subventral esophageal glands terminate in ampullae within the esophagus located posterior to the metacorporal pump chamber (Endo, 1984). It has been argued that the metacorporal pump chamber of cyst nematodes, and also of root-knot nematodes, acts as an unidirectional valve which precludes forward flow (Doncaster, 1971; Wyss, 1992; Wyss and Zunke, 1986). It has therefore been suggested that subventral gland secretions are solely transported posteriorly and involved in intracorporal digestion (Doncaster, 1971) or the mobilization of lipid reserves (Wyss and Grundler, 1992).

Various artificial agents are used to obtain stylet secretions from plant-parasitic nematodes *in vitro* (Curtis, 1996; Davis *et al.*, 1994; Goverse *et al.*, 1994; McClure and Von

Mende, 1987). For example, the serotonin analogue, 5-methoxy-N,N-dimethyltryptamine-hydrogen-oxalate (DMT), induces stylet secretions, which are precipitated at the lip region (Curtis, 1996; Goverse *et al.*, 1994). Also secretions from the subventral glands have been observed in these precipitates. Using monoclonal antibodies it was shown that antigens from the subventral glands of pre-parasitic J2 of *Heterodera glycines* are present in DMT-induced stylet secretions (Goverse *et al.*, 1994). Comparable results for subventral esophageal gland proteins were obtained with *Meloidogyne incognita* using resorcinol as the inducing agent of stylet secetions (Davis *et al.*, 1994). However, the biological relevance of chemically-induced stylet secretions remains to be established.

Detailed information on the nature of stylet secretions of plant-parasitic nematodes is essential for understanding the complex feeding behavior of plant-parasitic nematodes. However, the minute size, the long life cycle, and the obligate biotrophy of plant-parasitic nematodes has hampered the isolation of substantial quantities of stylet secretions required for biochemical analysis and characterization (Gheysen and Van Montagu, 1995). In the last decade research on stylet secretions focused on generating monoclonal antibodies against the secretory granules within the esophageal glands. Various panels of monoclonal antibodies have been raised against stylet secretions of *H. glycines* (Goverse *et al.*, 1994), *Heterodera avenae* (Curtis, 1996), *Globodera rostochiensis* (De Boer *et al.*, 1996), and *M. incognita* (Davis *et al.*, 1992; 1994). However, biochemical information on the corresponding antigens is scarce. So far the most detailed information on secretory proteins comes from a monoclonal antibody specific for the dorsal esophageal glands of pre-parasitic J2 and adult females of *M. incognita*. This monoclonal antibody recognizes a large molecular weight glycoprotein (Mr >212,000) as established by its slow electrophoretic migration in a SDS-PAGE gel and positive staining with periodic acid-Schiff reagent (Hussey *et al.*, 1990).

To study the secretory pathway of proteins from the subventral esophageal glands of *G. rostochiensis*, we used a monoclonal antibody (MGR48) recognizing six subventral gland proteins (*svp*s). The secretion of these proteins through the stylet was stimulated by potato root diffusate and their precipitation at the lip region was shown to be pH dependent. In addition it is shown that the synthesis of these secretory proteins is under stringent developmental regulation.

RESULTS

Electrophoretic characterization of subventral gland proteins

SDS-extracted proteins from homogenized pre-parasitic J2 were separated by SDS-PAGE. The monoclonal antibody MGR48 identified three protein bands (*svp*31, 39, and 49) on western blots both under reducing (Fig. 1, lane 1) and non-reducing conditions (data not shown). Presumably due to a different homogenization procedure as compared to De Boer *et al.* (1996), *svp*30 could not be detected on one dimensional western blot herein. Oxidation of carbohydrate side chains with a range of periodic acid concentrations (1-50 mM) did not have any effect on the binding pattern of MGR48 on western blot (Fig. 1; lane 2).

Six protein spots were detected with MGR48, when protein extracts of pre-parasitic J2 were separated with two dimensional electrophoresis (IPG-Dalt) and electroblotted on PVDF-membrane (Fig. 2). *Svp*30 focused at pH 7.6, whereas *svp*31 focused as two isoelectric point variants, pI 7.6 and 7.9. A new protein species (*svp*32), which was not separated on one dimensional western blots before (De Boer *et al.*, 1996), appeared at pI 6.8 and 32 kDa. *Svp*39 focused at pI 7.3 in the applied pH range and *svp*49 at pI 8.7.

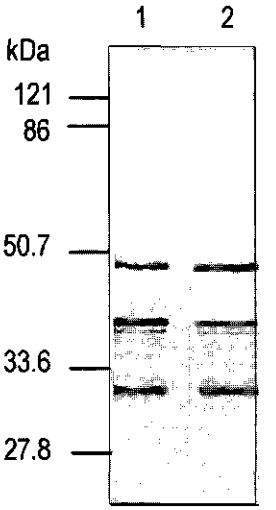

Fig. 1. Western blot of SDS-extracted proteins from pre-parasitic J2 of Globodera rostochiensis *after incubation in periodic acid. The experimental lane (2) was treated with 50 mM periodic acid in sodium acetate buffer (pH 4.5) to allow oxidation of carbohydrate side-chains. As compared to the control lane (1), no reduction in labelling intensity was observed using MGR48.*

Secretion of subventral gland proteins

Immunogold labelling experiments on thin sections of aldehyde-fixed pre-parasitic PRD-hatched J2 with MGR48 revealed clearly a specific concentration of gold particles within the matrix of secretory granules of the subventral esophageal gland cells (Fig. 3A). MGR48 also bound within the lumen of rough endoplasmic reticulum surrounding the nucleus of the subventral gland cells (Fig. 3B). Localization in smaller subcellular organelles such as Golgi body was not possible due to poor preservation of the ultrastructure.

Substantial quantities of precipitated stylet secretion from pre-parasitic J2 were obtained upon exposure of the J2 to 6.5 mM 5-methoxy-N,N-dimethyltryptamine-hydrogen-oxalate (DMT). In an indirect immunofluorescence assay, MGR48 labelled the DMT-induced stylet secretions (data not shown). No Coomassie Brilliant Blue stained secretions or excretions from the amphids, excretory/secretory pore, or rectal glands were observed in DMT assays. While neither any secretory granule movement in the esophageal glands nor metacorpal activity was observed, the use of DMT was strongly correlated with stylet thrusting.

Indirect immunofluorescence with MGR48 was used to compare DMT-treated and non-treated pre-parasitic PRD-hatched J2. A more granular and less intense labelling of the subventral esophageal glands was observed in DMT-treated J2 suggesting a decrease in gland content (data not shown). Immunogold electron microscopy of DMT-exposed J2 confirmed these observations. Both the dorsal and subventral esophageal glands contained considerable fewer secretory granules as compared to the control nematodes. Secretory granules were found stacked in the extension and ampulla regions of the gland cells. Fusion of individual secretory granules to more

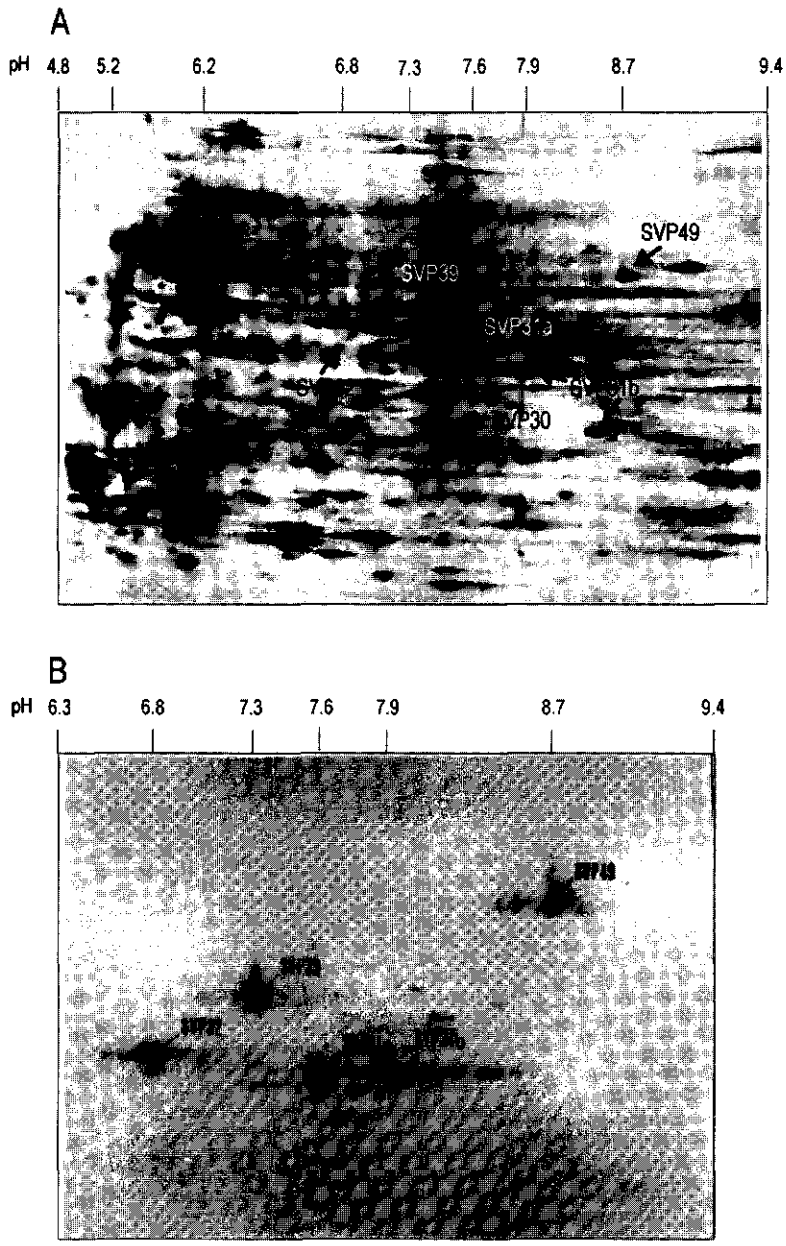

Fig. 2. *Two dimensional gel electrophoresis (IPG-Dalt) (**A**) and western blotting (**B**) of urea/Nonidet P-40- extracted proteins of* Globodera rostochiensis. *Isoelectric focusing was done in a immobilized pH gradient (pH range is 3 to 10). In the second dimension, a linear SDS-polyacrylamide gradient (8-18%) was used. The part of the western blot that showed labelling with MGR48 is depicted.*

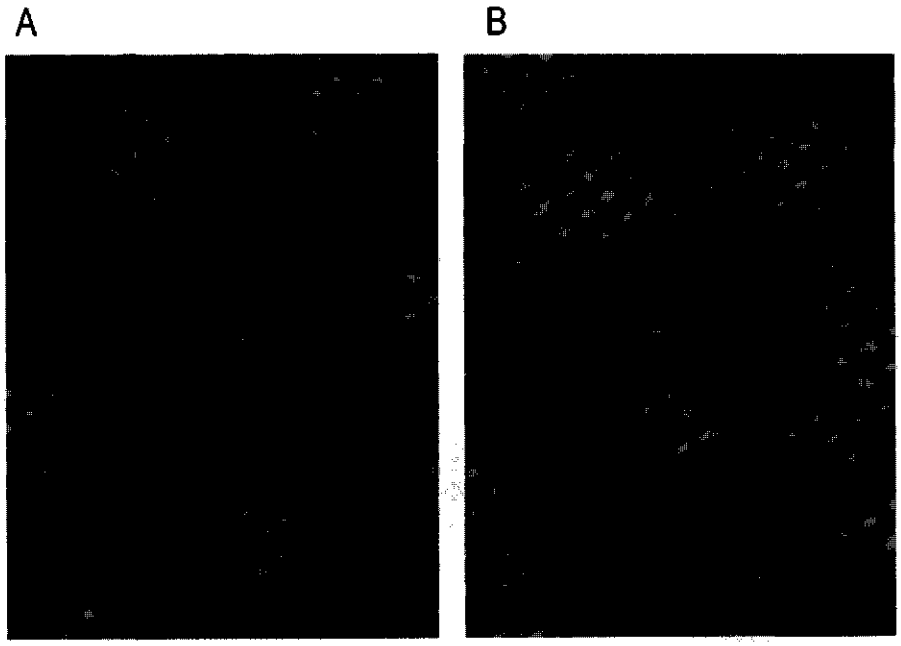

Fig. 3A and B. *Immunogold labelling of the subventral esophageal gland cell of a J2 of* Globodera rostochiensis *with MGR48. The gold particles localize to the rough endoplasmic reticulum (ER) surrounding the nucleus (N) and the dense core of the secretory granules (SG) Bar = 400 nm.*

translucent structures was observed among the typical homogeneous electron-dense granules in the cell body of the dorsal esophageal gland (Fig. 4).

The precipitation of stylet secretions observed at the lip region upon exposure to DMT appeared to be pH dependent and not due to the effect of DMT itself. DMT tends to acidify the solution (pH is 3.5 to 4 in distilled water at room temperature) and no particulates were observed when more alkaline buffers (pH> 5.5) were used. These observations were confirmed by incubation of PRD-hatched J2 in various acidic buffers (pH<5.5) without DMT. All acidic buffers used resulted in the precipitation of secretions at the stylet tip.

Testing the effect of different hatching conditions showed that, apart from stimulation of hatching, potato root diffusate also triggers the secretion of *svp*s. Incubation of tap-water-hatched J2 in standard pore water did not result in the secretion of *svp*s at a detectable level on dot blots using MGR48 (Fig. 5A). In contrast, substantial quantities of *svp*s were detected on dot blot when PRD-hatched J2 were used.

DMT supplemented to the incubation fluid (standard pore water) resulted in an additional effect on secretion of *svp*s. DMT stimulated secretion of *svp*s by tap-water-hatched J2. Secretion of *svp*s was further increased if PRD-hatched J2 were used instead of tap-water-hatched J2. Exposure of J2 to DMT did not harm the nematodes irreversibly, as they were still able to infect potato root and develop into adult females (data not shown).

One dimensional western blotting revealed that three *svp*s (31, 39 and 49 kDa) were present in standard pore water when PRD-hatched J2 were used. Among these proteins *svp*31 was predominant (Fig. 5B).

Developmental regulation of *svp*

The developmental regulation of *svp*s throughout the nematode's life cycle was determined by western blot analysis (Fig. 6). The protein content of each nematode extract was estimated and equal protein quantities of each developmental stage were analyzed. The *svp*s were neither detectable in fresh eggs containing young J2 nor in dry eggs containing J2 in diapause. Maximum immunodetection of *svp*s occurred in homogenates from pre-parasitic J2, PRD-hatched from dried cysts which had passed the diapause. The staining intensity of *svp*31/32 decreased in parasitic J2, while in this stage no substantial decrease was observed for *svp*39 and *svp*49 (Fig. 6). None of the three *svp*s could be detected in other juvenile stages and adult females. A minor quantity of *svp*49 was observed in adult males.

The size of the subventral esophageal glands changes throughout the life cycle, and the total protein content increases in the later life stages. For example, the total protein content of adult females is about 300 times higher as compared to pre-parasitic J2. To exclude the possibility that the apparent absence of *svp*s in other stages than J2 was due to dilution from increasing total protein contents, an additional SDS-PAGE gel was heavily overloaded with protein extracts of parasitic stages. No specific changes were detected on the western blot as compared to Figure 6.

Fig. 4. *Electron micrograph of the dorsal esophageal gland cell in* Globodera rostochiensis *after treatment with 6.5 mM 5-methoxy-N,N-dimethyltryptamine-hydrogen-oxalate (DMT). Translucent structures (arrows) appear among the typical electron-dense secretory granules (SG) of the dorsal esophageal gland. Bar = 400 nm.*

Immunogold labelling of parasitic J2 *in planta*

Potato roots infected with J2 were used to study the secretion of the subventral gland proteins during the early events of plant parasitism. The impermeability of the cuticle usually prevents infiltration of LR Gold resin in parasitic J2 inside plants tissue. As a consequence the nematodes shrink after polymerization of the resin and soft tissue in the nematode shows morphological distortion. However, in some cases the cuticularized lumen of the esophagus remained well preserved in thin sections. Serial sections of the esophagus showed immunogold labelling of *svp* antigens within the circular lumen of the esophagus anterior to the metacorpal pumpchamber (procorpus) (Fig. 7). Using MGR48 the labelling intensity was not strong, but very specific to esophageal lumen both in cross (Fig. 7A) and longitudinal (Fig. 7B) sections.

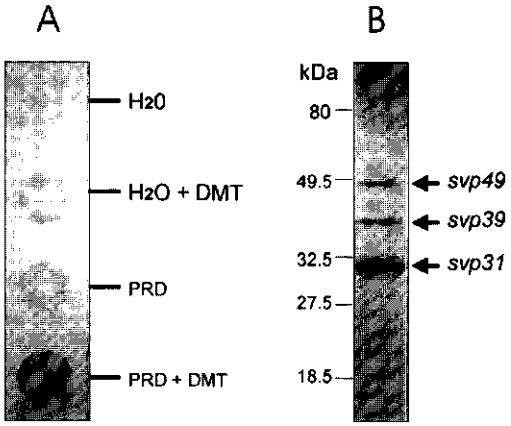

Fig. 5. Concentrated solubilized stylet secretions of Globodera rostochiensis *tested with MGR48 on western blot and dot blot.* ***A.*** *A dot blot of concentrated incubation fluids of J2 either hatched in tap-water (H2O) or potato root diffusate (PRD) after 24 hr in buffered standard pore water.* ***B.*** *Western blot of concentrated incubation fluid of potato root diffusate-hatched J2 after an incubation period of 24 hr in standard pore water. The 31 kDa protein band is predominating antigen in stylet secretions. The addtion of 5-methoxy-N,N-dimethyltryptamine-hydrogen-oxalate to a final concentration of 4 mM is indicated as +DMT.*

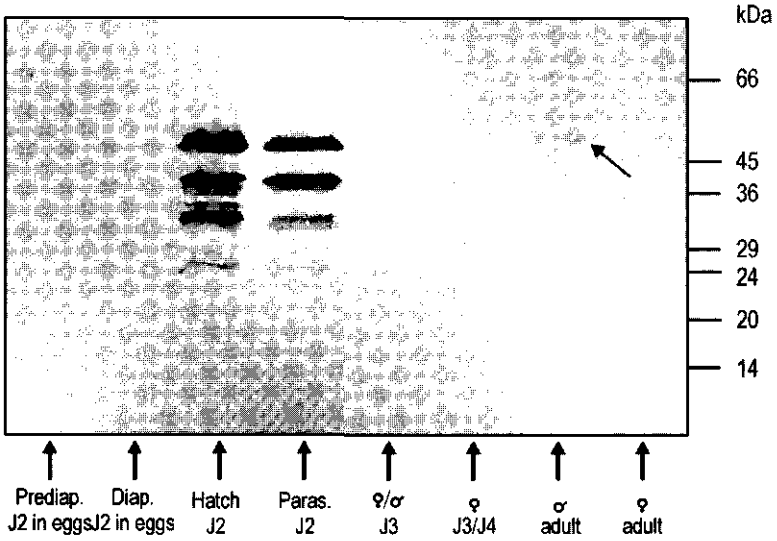

Fig. 6. Western blot of SDS-extracted proteins from various life stages of Globodera rostochiensis. *Immunodetection was done with MGR48. Prediap.= eggs containing young J2; diap. J2 = eggs containing J2 in diapause; hatch. J2 = freshly in potato root diffusate hatched J2; para. J2 = parasitic J2 isolated from infected potato roots; ♂ / ♀ J3 = isolated third stage parasitic juveniles, males and females; J3/J4 = mixed sample of isolated third and fourth stage parasitic female juveniles; ♂ adult = isolated adult males; ♀ adult = isolated adult females. The minor signal detected in adult males is indicated by an arrow.*

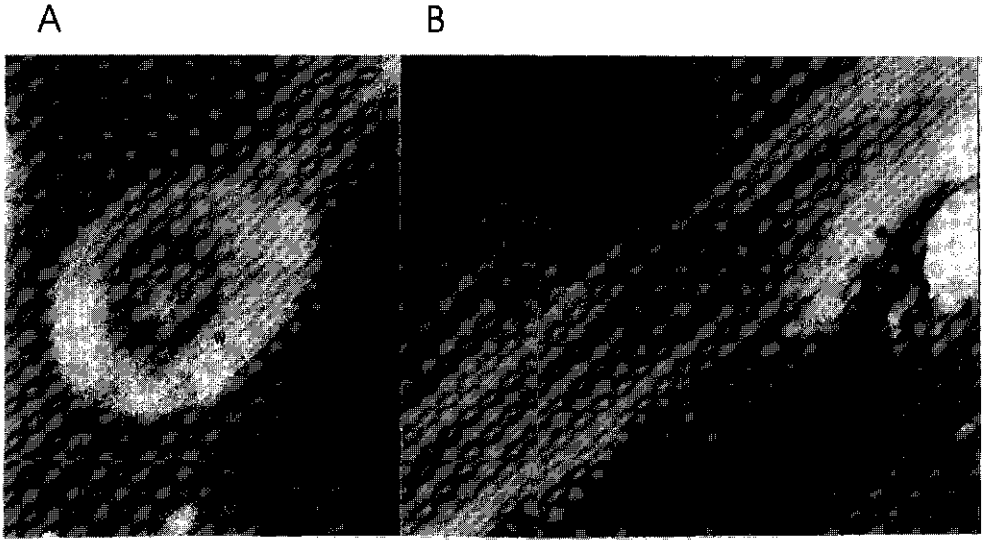

Fig. 7. *Electron micrograph of a cross (**A**) and longitudinal section (**B**) of the circular lumen of the esophagus in parasitic* Globodera rostochiensis *J2* in planta. *Immunogold labelling (arrow) with MGR48 shows specific localization in the circular lumen (el) of the esophagus (procorpus; w = cuticularized esophageal wall).*

DISCUSSION

The subventral esophageal glands of the potato cyst nematode *G. rostochiensis* contain basic proteins (*svp*). On two dimensional electrophoresis five out of six *svps* focused in the basic part of the immobilized pH gradient. Oxidation of carbohydrate epitopes using periodic acid is a means to detect whether a monoclonal antibody is directed to a carbohydrate moiety of a glycoprotein (Woodward *et al.*, 1985). Since periodic acid oxidation did not change the binding pattern of MGR48 on western blot, the epitope of MGR48 is not of carbohydrate nature. Therefore it is concluded that the epitope of MGR48 is either a conserved stretch of amino acid residues present in distinct protein species, or MGR48 recognizes successive stages of post-translational protein processing.

It has been argued (Wyss and Zunke, 1986) that the metacorporal pump chamber of beet cyst nematode *Heterodera schachtii* forms an anatomical barrier that precludes the secretion of subventral esophageal gland proteins into the plant. However, apart from stimulation of hatching, PRD stimulates the secretion of *svps* through the stylet of the potato cyst nematode *G. rostochiensis*. *Svp*31 was predominant in the collected stylet secretions and this finding could indicate that this protein is the final, biologically active form. During secretion *in vitro* no activity of the pump chamber was observed.

Immunogold labelling in the lumen of the esophagus (procorpus) in parasitic J2 *in planta* with MGR48 indicated also that *svps* of *G. rostochiensis* are transported anterior to the metacorpal pumpchamber. These data from pre-parasitic and parasitic J2 under relatively natural conditions suggest that *svps* are secreted through the stylet into plant tissue.

Under natural conditions a J2 has to migrate from the egg toward the roots of a host plant. Presumably, secretion of *svps* during this period would be non-functional. Our results could

suggest that pre-parasitic J2 which hatch in the vicinity of potato roots would start to secrete *svps* in the soil. However, in our experiments eggs were exposed to PRD at a high concentration, which is unlikely to exist in the soil unless in the immediate vicinity of the roots of a host plant. Therefore, we assume that pre-parasitic J2 do not secrete *svps* under natural conditions until the moment that a root of a host plant is reached.

Until now, exposure to 5-methoxy-N,N-dimethyltryptamine-hydrogen-oxalate (DMT) was the most obvious means to induce the secretion of esophageal gland proteins of cyst nematodes (Goverse *et al.*, 1994). In the case of potato cyst nematodes, *svp* containing stylet secretions can be induced in J2 from eggs hatched in PRD. DMT was shown to have an additional stimulatory effect on *svp* secretion. With respect to the observed forward flow of *svps*, our data are in accordance with previously published data on the flow of the chemically-induced secretion of *svps* in *H. glycines* (Goverse *et al.*, 1994) and *M. incognita* (Davis *et al.*, 1994). However, the observation that these stylet secretions precipitate at the lip region needs further investigation. For potato cyst nematodes (J2) we showed that the precipitation of stylet secretions does not occur above pH 5.5.

The expression of the *svps* in potato cyst nematodes is developmentally regulated. Interestingly, *svp*31 predominating in concentrated incubation fluids after incubating pre-parasitic J2 for 24 hours gives the lowest immunodetection in homogenates of parasitic J2. Most *svps* were detectable in pre-parasitic and in parasitic J2, and not in J3, J4 and females. Presumably, the *svps* have most likely a role in the early events of the plant-nematode interaction. From this perspective they may either be involved in cell wall breakdown during the migratory process (Steinbach, 1972) or in feeding site induction (Steinbach, 1973). The *svps* are apparently not involved in feeding-site maintenance. Remarkable is the increased expression of the *svps* in adult males. Contrary to J3, J4 and females, adult males are mobile and can leave the root. Expression of *svps* in adult males would favor the hypothesis that *svps* are involved in the migratory process. Our findings are in accordance with the accumulation of secretory granules in the subventral esophageal glands of males in *H. schachtii* males (Wyss and Zunke, 1986) and *H. glycines* (Baldwin *et al.*, 1977).

The isolation and functional analysis of genes encoding secretory proteins from the esophageal glands is one of the major challenges in plant nematology. Monoclonal antibodies provide in theory a suitable starting point to isolate genes. A monoclonal antibody directed against the esophageal glands was used to isolate a cDNA clone from a *M. incognita* expression library (Ray *et al.*, 1994). The cDNA sequence showed similarities with the rod portion of myosin heavy chain of various origins. It was hypothesized that the encoded protein has a role in the intracellular movement of secretory proteins, and is not involved in the host parasite interaction.

Our results indicate that, in contrast with previous assumptions (Wyss, 1992; Wyss and Zunke, 1986), secretions from the subventral esophageal glands of cyst nematodes probably play a direct role in the host-parasite interaction as concluded earlier for root-knot nematodes (Davis *et al.*, 1994). Analyzing various developmental stages strongly suggests, that the *svps* are involved in the early events of the interaction. Further purification and characterization of these *svps*[1] will without doubt be a major step forward toward the understanding of the interaction between plants and sedentary plant-parasitic nematodes.

[1] The analysis of *svp1*, *2* and *3* - that resulted in the identification of the first animal-produced endoglucanases - is extensively described in the thesis of G. Smant (1998).

MATERIALS AND METHODS

Nematodes

Globodera rostochiensis (Wollenweber) Skarbilovich pathotype Ro_1 was used in all experiments. Nematodes were reared and hatched as described previously (De Boer *et al.*, 1992; Roosien *et al.*, 1993). When diapause is completed, a minority of J2 will spontaneously hatch upon hydration in tap-water. The resulting J2 are indicated in this paper as tap-water-hatched J2. When eggs are incubated in potato root diffusate (PRD) (Clarke and Perry, 1977) the majority of the J2 will hatch. These J2 are named PRD-hatched J2.

Samples of parasitic J2 were collected from infected potato roots 10-13 days post-inoculation. Plant roots were cut into 1-cm pieces, and processed for 30 s in a blender, and juveniles were separated from root debris by sieving and centrifugation in a 35% (w/v) sucrose solution at 1,000 x *g* for 5 min. Mixed third-stage male and female juveniles (J3) were collected 16 days post-inoculation, whereas mixed 3rd and 4th (J4) stage females were collected 19 days post-inoculation. Adult males and adult females were obtained 25 days and 35 days post-inoculation, respectively, as described by De Boer *et al.* (1992). Eggs with young J2 were collected from females 2 months post-inoculation. Eggs containing J2 in diapause were collected from dry cysts.

Protein extraction and SDS-PAGE

SDS-extracted proteins from PRD-hatched J2 of *G rostochiensis* were obtained by homogenization in 10 mM sodium phosphate buffer pH 7.2 using a 2-ml Potter-Elvehjem homogenizer with a Teflon pestle. Samples were supplemented with one volume of SDS-sample buffer (125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 10% (v/v), 2-mercaptoethanol, 20% (v/v) glycerol, 0.05% (w/v) bromophenol blue). The samples were heated in boiling water for 5 min and centrifuged for 5 min at 14,000 x *g*. For non-reducing SDS-PAGE, 2-mercaptoethanol was omitted from the sample buffer. Protein concentrations were determined using the bicinchoninic acid assay with bovine serum albumin as a standard (Pierce, Rockford, USA). Analytical SDS-PAGE and immunodetection with MGR48 hybridoma culture supernatant was performed essentially as described by De Boer *et al.* (1992). Prestained SDS-PAGE standards (Biorad, Richmond, USA) were used in each run.

Two-dimensional gel electrophoresis

Freshly PRD-hatched J2 of *G. rostochiensis* were homogenized in 1% (v/v) Nonidet P-40 and 2% (v/v) 2-mercaptoethanol. Protein concentration was estimated according to Bradford (1976) using bovine serum albumin as standard. Shortly before isoelectric focusing (IEF) gel electrophoresis, five volumes of 8 M urea, 0.5% (v/v) Nonidet P-40, 2% (v/v) 2-mercaptoethanol, 2% (v/v) carrier ampholytes pH 3-10 (BioRad), 1 mM Pefabloc® (Boehringer Mannheim, GER) and a 0.025% (w/v) of bromophenol blue were added to the sample. This solution was thoroughly mixed, incubated for 1 h at room temperature and subsequently centrifuged for 10 min at 14,000 x *g*. In the first dimension 15 µg of a total protein extract was separated by IEF on an immobilized pH 3 to 10 gradient (Immobiline Dry Strips 3-10L, Pharmacia LKB Biotechnology, Upsala, Sweden). The immobilized pH gradient strips with the focused proteins were equilibrated for 15 min in freshly prepared 10 ml 50 mM Tris-HCl (pH 6.8), 4.17 M urea, 30% (v/v) glycerol, 1% (w/v) SDS, 16.2 mM dithiothreitol, and subsequently for 15 min in 10 ml 50 mM Tris-HCl (pH 6.8), 4.17 M urea, 30% (v/v) glycerol, 1% (w/v) SDS, 240 mM iodoacetoamide, with 0.025% (w/v) bromophenol blue, and seperated by second-dimension electrophoresis in an 8-18% (w/v) SDS-PAGE gradient gel (ExcelGel 110x245x0.5 mm, Pharmacia). The SDS-PAGE gel was used for semi-dry western blotting onto PVDF-membrane at 0.8 mA/cm^2 for 1 h.

Induction and collection of stylet secretions

Unless stated otherwise PRD-hatched pre-parasitic J2 were used for the induction of stylet secretions. For each replicate an estimated 20,000 pre-parasitic J2 were collected and exposed overnight to 4 ml of a test solution at 20°C.

To investigate the effect of DMT (5-methoxy-N,N-dimethyltryptamine-hydrogen-oxalate; Research Biochemicals, Natick, USA) and the pH of the incubation medium, DMT was dissolved to a final concentration of 6.5 mM in tap-water, 10 mM glycine buffer (pH 3.5 and 10), or 10 mM Tris-HCl buffer (pH 8.0). In control solutions DMT was omitted. When appropriate, 0.01% Coomassie Brilliant Blue G-250 (CBB G-250) was added to enhance the visibility and collection of stylet precipitates (Davis *et al.*, 1994; Goverse *et al.*, 1994).

To test the effect of hatching conditions on the production of stylet secretions, either tap-water-hatched or PRD-hatched J2 were incubated in standard pore water-based solutions. Standard pore water was used to simulate natural conditions for nematodes *in vitro*. Standard pore water contains 0.1 mM KCl, 0.2 mM NaCl, 0.35 mM $Ca(NO_3)_2$, 0.3 mM $Mg(NO_3)_2$, 0.3 mM NH_4NO_3 at pH 6 (Schouten and Van der Brugge, 1989). Standard pore water was supplemented with a 20 mM sodium phosphate solution (pH 6) to buffer the acidifying effect of DMT. In these experiments the final DMT concentration was 4 mM.

Two methods were used to collect stylet secretions. For immunofluorescence testing with MGR48, precipitated stylet secretions were collected with a micropipet (Gilson Medical Electronics, Villiers-le-Bel, France). Alternatively, soluble stylet secretions were collected by shaking the J2 suspension vigorously on a whirl mix followed by centrifugation at 10,000 x *g* for 5 min. The supernatant contained the solubilized stylet secretions and was checked for absence of J2 using a dissecting microscope. Secretions were either processed for denaturing western blotting or for native dot blotting.

Incubation fluids with solubilized stylet secretions were concentrated for western blotting at 4 C using a 1.5 ml microcentrifuge ultrafiltration unit with a molecular weight cut-off of 5 kDa (Ultrafree MC, Millipore Corp., Bedford, USA.). The samples were concentrated from 4 ml to a final volume of 10 µl per sample, and were supplemented with an equal volume SDS-sample buffer. After addition of the protease inhibitors Pefabloc® (1 mM), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (2.8 µM), $EDTA.Na_2$ (0.1 mM) and pepstatin A (0.15 µM), samples were stored at -20 C until usage.

Western and dot blotting

Semi-dry western blotting and immunodetection were performed as described by De Boer *et al.* (1996). For dot blot assays, 2 ml native solubilized stylet secretion was concentrated onto a nitrocellulose blotting membrane under mild vacuum (Schleicher and Scheull, Dassel, GER). Subsequently, the dot blot was left to dry and processed for immunodetection as described (De Boer *et al.*, 1996). In this experiment MGR48 hybridoma culture supernatant, diluted 1:40 in PBS-0.1% (v/v) Tween-20 (PBS, pH 7.2: 150 mM NaCl; 2.6 mM KCl; 8.1 mM Na_2HPO_4; 2.65 mM KH_2PO_4)., was used as the primary antibody. Control blots were obtained by omitting the primary antibody.

To detect whether the monoclonal antibody MGR48 is directed to a carbohydrate epitope, periodic acid treatment of western blots was performed according to Woodward *et al.* (1985). The control lanes were kept in 50 mM sodium acetate buffer (pH 4.5), while the experimental lanes were exposed to 1, 5, 10, 50 mM periodic acid in the sodium acetate buffer (pH 4.5) in the dark for 90 min. Control and experimental strips were then rinsed with sodium acetate buffer (pH 4.5) and incubated for 30 min in 50 mM sodium borohydride in PBS.

Indirect immunofluorescence microscopy

Indirect immunofluorescence microscopy of pre-parasitic J2 was performed similar to De Boer *et al.* (1996). Coomassie brilliant blue-stained stylet secretions were collected by micropipet and used for indirect immunofluorescence microscopy as described (Davis *et al.*, 1994; Goverse *et al.*, 1994) with following modifications. Stylet secretions were dried on #24 multiwell glass slides instead of using dialysis membranes and blocked with 10 µl 1% (w/v) bovin serum albumin in PBS for 10 min before antibody treatments.

Immunoelectron microscopy

G. rostochiensis pre-parasitic J2 were either fixed in 2% (w/v) paraformaldehyde and 1% (v/v) glutaraldehyde or in 2% (w/v) paraformaldehyde, and embedded in LR White (London Resin Co. Ltd., Basingstoke, England) (De Boer *et al.*, 1996). Thin roots of potato (*S. tuberosum* spp. *tuberosum* cv. Bintje) were inoculated with *G. rostochiensis* (30). Parasitic J2 were observed within infected pieces of thin translucent potato roots and fixed and embedded in LR Gold (London Resin Co. Ltd., Basingstoke, England) 5-7 days after inoculation (Van Lent *et al.*, 1990).

For immunogold labelling thin sections (50-80 nm) were collected on nickel square-mesh grids coated with 0.6% (w/v) Formvar. The labelling was performed on 30 µl drops at room temperature as follows: 20 min on 50 mM glycine in PBS (pH 7.2), 30 min on 0.2 % (v/v) bovin serum albumin-C (Aurion, Wageningen, NL) in PBS, 2 h on monoclonal antibody hybridoma supernatant (diluted 1:20 in 0.2% (v/v) bovin serum albumin-C in PBS), six washes of 5 min each with 0.2% (v/v) bovin serum albumin-C in PBS, 2 h on goat-anti-mouse IgG (H&L) conjugated with 10 nm colloidal gold (Aurion, Wageningen, NL) diluted 1:20 with 0.2 % (v/v) bovin serum albumin-C in PBS, four washes of 5 min each with 0.2% (v/v) bovin serum albumin-C in PBS, three washes of 5 min each with PBS, 5 min on 2% (v/v) glutaraldehyde (EM grade, Agar Scientific, Essex, UK) in PBS, and five 5 min washes with distilled water. Except for the colloidal gold conjugate solution, all solutions were filtrated before use (0.22 µm; Millipore Corp.). In control sections the primary antibody was omitted or replaced by an irrelevant monoclonal antibody of known specificity. Post staining was done with 2% (w/v) aqueous uranyl acetate and lead citrate (Reynolds, 1963).

CHAPTER 3

Naturally-induced secretions of the potato cyst nematode co-stimulate the proliferation of both tobacco leaf protoplasts and human peripheral blood mononuclear cells

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ABSTRACT

Naturally-induced secretions from infective juveniles of the potato cyst nematode *Globodera rostochiensis* co-stimulate the proliferation of tobacco leaf protoplasts in the presence of the synthetic phytohormones NAA and BAP. Using a protoplast-based bioassay, a low molecular weight peptide(s) (< 3 kDa) was shown to be responsible for the observed effect. This mitogenic oligopeptide(s) is functionally dissimilar to auxin and cytokinin and, in addition, it does not change the sensitivity of the protoplasts towards these phytohormones. In combination with the mitogen phytohaemagglutinin (PHA), cyst nematode secretions also co-stimulated mitogenesis in human peripheral blood mononuclear cells (PBMC). The stimulation of plant cells isolated from non-target tissue - these nematodes normally invade the roots of potato plants - suggests the activation of a general signal transduction mechanism(s) by an oligopeptide(s) secreted by the nematode. Whether a similar oligopeptide-induced mechanism underlies human PBMC activation remains to be investigated. Reactivation of the cell cycle is a crucial event in feeding cell formation by cyst nematodes. The secretion of a mitogenic low molecular weight peptide(s) by infective juveniles of the potato cyst nematode could contribute to the redifferentiation of plant cells into such a feeding cell.

INTRODUCTION

Cyst nematodes are highly specialized plant parasites that intervene in the developmental program of plant cells. Feeding site formation is induced by second stage (J2) pre-parasitic juveniles, which penetrate the rhizodermis preferentially in the elongation zone and migrate intracellularly through the cortex in search of a cell with high meristematic potential near the vascular bundle (Golinowski *et al.*,. 1996). Feeding site formation is not restricted to plant roots *per se*; cyst nematodes are able to induce feeding sites in stems and leaves of plants grown *in vitro* (Sijmons *et al.*, 1991; A. Goverse, unpublished observations). The competence of a cell to differentiate into a feeding cell seems to depend on its position - preferably a cell opposite to the protoxylem pole - and on developmental cues (Scheres *et al.*, 1997). Subsequently, this cell redifferentiates into a metabolically highly active transfer cell. In all cyst nematode-plant interactions, feeding cell induction is characterized by similar dramatic cytological changes. Partial cell wall dissolution results in the expansion of the feeding cell within the stele along the xylem. Subsequent fusion of the protoplasts results in a multinucleated cell complex, a syncytium, which can include up to 200 cells (Jones, 1981). Studies on the morphogenesis of feeding cells induced by the cyst nematodes *Heterodera glycines*, *H. schachtii* and *Globodera rostochiensis* (Endo, 1986; Golinowski *et al.*, 1996 and Sembdner, 1963, respectively) implicate that the ontogeny of feeding cells is accomplished by completion of a general developmental program irrespectively of the plant organ – root, stem or leaf – or the host plant.

Feeding cell proliferation coincides with a reactivation of the cell cycle (Gheysen *et al.*, 1997), which is a prerequisite for the development and maintenance of the feeding cell. In *Arabidopsis thaliana*, activity of the cell cycle genes *cdc2aAt* and *Arath;cycB1;1* was detected within a few hours after feeding cell induction by the beet cyst nematode *H. schachtii*. Cdc2aAt:*gus* was strongly expressed inside the feeding cell. Expression of *Arath;cycB1;1:gus* was weak inside the feeding cell, but strong in cells immediately surrounding the syncytium at the growing edges. Moreover, ^{3}H -thymidine incorporation was observed in dividing cells prior to incorporation into the feeding cell (Niebel *et al.*, 1996). The necessity of cell cycle activation for nematode development was shown by the application of oryzalin (inhibitor of mitosis) and hydroxyurea (inhibitor of DNA synthesis). Both resulted in a complete obstruction of feeding cell development in roots of *Arabidopsis* (De Almeida Engler *et al.*, 1999). In addition, DNA endoreduplication was observed in the enlarged nuclei of elongating feeding cells (Endo, 1971). The increase in copy number of essential genes could be functionally relevant in the metabolically highly active syncytium. It is assumed that cyst nematodes directly or indirectly induce the

reactivation of the cell cycle by initial mitotic stimulation of the precursor cell prior to incorporation of adjacent cells in the growing syncytium (Gheysen *et al.*, 1997). The intriguing question is how the infective nematode initiates these plant responses, thereby forcing a change in cell fate.

Nematode secretions are thought to play a key role in feeding cell development and maintenance (reviewed by Williamson and Hussey, 1996). Microscopic observations indicate that stylet secretions of the salivary glands are released during migration through the plant root and injected into the selected root cell by pre-parasitic J2 (Wyss and Zunke, 1986). Reduction of secretory granule density in the subventral esophageal glands corresponds with the preparation period during which the nematode stylet is inserted in the parasitised cell and no food ingestion takes place (Wyss, 1992). These results suggest that esophageal gland secretions comprise pathogenicity factors, including those involved in feeding cell induction. Recently, the first pathogenicity factors of cyst nematodes were cloned. Four genes encoding endogenous cellulases were isolated from the cyst nematode *H. glycines* and *G. rostochiensis*. These cellulases were demonstrated to be specifically expressed in the subventral glands of infective juveniles (Smant *et al.*, 1998). Secretions of pre-parasitic J2 of *G. rostochiensis* can be collected upon exposure to potato root diffusate (Smant *et al.*, 1997). These naturally-induced secretions include functional cellulases. The ease with which natural secretions can be collected potentially facilitates the detection of regulatory molecules involved in feeding cell initiation and development.

This paper focuses on the biological relevance of nematode secretions in relation to plant cell proliferation. A tobacco protoplast-based bioassay was used to monitor tobacco leaf protoplast proliferation in response to secretions of infective juveniles of the potato cyst nematode *G. rostochiensis*. In the presence of the phytohormones auxin and cytokinin, cell division activity of the protoplasts was significantly enhanced by secretions. This is the first report describing a mitogenic compound in plant-parasitic nematode secretions. It is demonstrated that the protoplast activating compound(s) is a small peptide(s). Remarkably, nematode secretions also stimulated cell proliferation in human peripheral blood mononuclear cells (PBMC) in the presence of the mitogen phytohaemagglutinin (PHA). These results implicate that nematode secretions are able to increase the proliferative response of both plant and mammalian cells.

RESULTS

Tobacco leaf protoplast proliferation assay.

Tobacco mesophyll protoplasts have the potential to divide *in vitro* (Nagata and Takebe, 1970), and protoplast-based assays have been used to study the effect of external stimuli (Takahashi *et al.*, 1989, Delbarre *et al.*, 1994, Matsubayashi and Sakagami, 1996). A tobacco leaf protoplast assay (Stickens and Verbelen *et al.*, 1996) was adapted to investigate the effect of nematode secretions on plant cell division. For each experiment protoplasts were isolated from the successive leaves of a 6-weeks-old *in vitro* plant. Protoplast proliferation was determined after 5 days of culturing and division activity was quantified by counting the number of dividing cells relative to the total number of cultured tobacco leaf protoplasts. Despite the rigorous standardization of plant growth and the protoplast isolation procedure, the cell division rate in standard medium supplemented with 1 µg/ml α-naphthaleneacetic acid (NAA) and 1 µg/ml 6-benzylaminopurine (BAP) ranged from 30% to 57% between the different protoplast batches (Fig. 1-6). However, provided that a single protoplast batch - protoplasts isolated from a single plant - was used as starting material, no significant differences were observed between replicates within an experiment (at $P > 0.1$; see Table 1-5). Hence, this assay is reliable and distinctive as long as a single split protoplast batch is used per experiment.

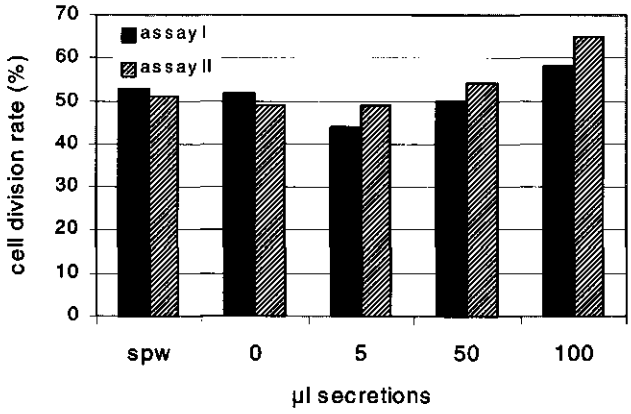

Fig. 1. Dose-responsive stimulatory effect of potato root diffusate-induced secretions of infective juveniles of Globodera rostochiensis *on the proliferative response of cultured tobacco protoplasts in the presence of 1 µg/ml NAA and 1 µg/ml BAP. A gradual, significant increase in the cell division rate was observed upon addition of secretions (at $P < 0.05$; see Table 1). As a control, 100 µl standard pore water (SPW) was added instead of nematode secretions. Data are expressed as the percentage of dividing cells relative to the total number of cells. The data presented are from two independent experiments (assay I and II).*

Specific stimulation of cell division activity of tobacco protoplasts by secretions of *Globodera rostochiensis*.

Embedded tobacco protoplasts were exposed to increasing quantities of naturally-induced secretions from infective juveniles (J2) of *G. rostochiensis* (5, 50 and 100 µl) in the presence of 1 µg/ml NAA and 1 µg/ml BAP. Figure 1 shows a gradual, significant increase in the cell division rate upon addition of secretions (at $P < 0.05$; see Table 1). Addition of 100 µl secretions - equivalent to the amount produced by 1000 secreting nematodes – resulted in a significant effect ($P < 0.001$ in all pairwise *t*-tests; data not shown). Addition of 5 µl and 50 µl secretions did not significantly affect the cell division rate.

To test whether standard pore water (SPW) - the solution in which secretions were collected - affected protoplast proliferation, cells were exposed to medium with a fixed BAP concentration (1 µg/ml) and a variable NAA concentration (0, 0.5, 1, 3 and 7 µg/ml) in the presence or absence of 100 µl SPW. SPW had no detectable effect on the division activity of the protoplasts in these treatments (Fig. 2).

Contribution of microbial contaminants could be excluded, since secretions were obtained from surface-sterilized pre-parasitic J2. To check whether the antibiotic treatment was adequate, *in vitro* tomato or potato plants were inoculated with J2. This resulted in normal nematode development without microbial contamination (data not shown). Hence, the observed effect on cell division activity was induced by a compound(s) derived from *G. rostochiensis*.

To exclude the possibility that potato root diffusate (PRD) – used for nematode hatching - was responsible for the observed mitotic stimulation of protoplasts, an excess of 100 µl PRD was added to the culture medium. At this concentration PRD did not affect cell division activity ($P > 0.1$ in both pairwise *t*-tests; data not shown).

Table 1. *Deviance table for the effect of five different concentrations of potato root diffusate-induced secretions of infective juveniles of* Globodera rostochiensis *on the cell division rate as tested in two independent protoplast batches.*

Source	d.f.[a]	Deviance	Deviance ratio[d]
Secretions	4	34.80	8.70 *[e]
Batch	1	2.39	2.39 n.s.
Residual	4	6.60 [b,c]	
Total	9	43.79	

[a] Degrees of freedom
[b] The residual deviance indicates the goodness of fit of the model.
[c] The residual deviance is below the chi-square threshold value (at $\alpha = 0.05$, d.f. = 4)
[d] The deviance ratio is used as a measure for the significance of the respective variate on the cell division rate.
[e] * $0.01 < P < 0.05$; ** $0.01 < P < 0.001$; *** $P < 0.001$; n.s. : non significant ($P > 0.1$)

To check whether the observed effect could be attributed to non-specific nematode compounds, different amounts of culture fluids collected from mixed life stages of the free living nematode *Caenorhabditis elegans* were tested. Culture fluids from *C. elegans* had an inhibiting effect on protoplast proliferation in all concentrations tested ($P < 0.005$ in all pairwise *t*-tests; data not shown).

These data demonstrate that the cell division activity of tobacco protoplasts is specifically enhanced by secretions of pre-parasitic J2 of *G. rostochiensis*. When protoplasts were exposed to secretions solely, no cell division was observed (data not shown). In the secretion concentration range tested, stimulation of protoplast cell division was only observed in the presence of both auxin and cytokinin. Addition of 100 µl secretions resulted in a significant increase in cell division rate (+10% ± 3% (mean ± SD)) and this quantity was used for further testing.

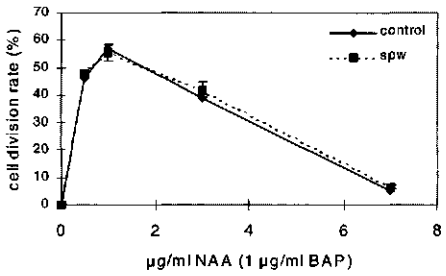

Fig. 2. *The proliferative response of tobacco leaf protoplasts in the absence (control) or presence of 100 µl standard pore water (SPW). SPW is normally used for collecting nematode secretions. Protoplasts were cultivated in medium supplemented with 1µg/ml BAP and variable NAA concentrations for 5 days. Proliferation was expressed as the percentage of dividing cells relative to the total number of cells. The data presented are from one representative experiment out of three with comparable results. For SPW, the mean ± SD of two independent counts are shown.*

Tobacco protoplast cell division is stimulated by an oligopeptide(s) released by infective juveniles of *G. rostochiensis*.

Proteolytic digestion of nematode secretions with the exoprotease Pronase E resulted in the inactivation of secretions (Fig. 3 and Table 2). Pronase E treatment of the secretions resulted in cell division rates which were significantly lower as compared to undigested controls (at $0.05 > P > 0.01$). Incubation of secretions for 3 hr at 37°C per se did not affect mitogenic activity of the secretions. The effectivity of the exoprotease Pronase E (1 µg/µl) treatment was checked by digestion of total J2 homogenate (1 µg/µl) at 37° C for 3 hr. Complete digestion of the proteins was observed after SDS-PAGE and Coomassie Brilliant Blue staining of the gel. Homogenate without Pronase E revealed the expected total protein pattern of J2 (data not shown).

The mitogenic activity of nematode secretions on cultivated tobacco leaf protoplasts was also lost by heat-inactivation (Fig. 3 and Table 3). The addition of heat inactivated nematode secretions resulted in cell division rates which did not differ significantly from the cell division rates observed for the control protoplast population exposed to 100 µl SPW (at $P > 0.1$).

The mitogenic stimulus was demonstrated to be present in the low molecular weight fraction (LMW < 3 kDa) of the secretions (Fig. 4). Large differences were found between the cell division rates supplemented with the low molecular weight fraction and the control treatments (at $P < 0.001$ in all pairwise t-tests). No significant activity was observed in the high molecular weight fraction (HMW > 3 kDa) which revealed cell division rates similar to the control treatments (at $P > 0.05$).

It is concluded that a low molecular weight peptide(s) released by pre-parasitic J2 of G. rostochiensis upon exposure to potato root diffusate is responsible for the observed enhanced cell division rate in this tobacco protoplasts assay.

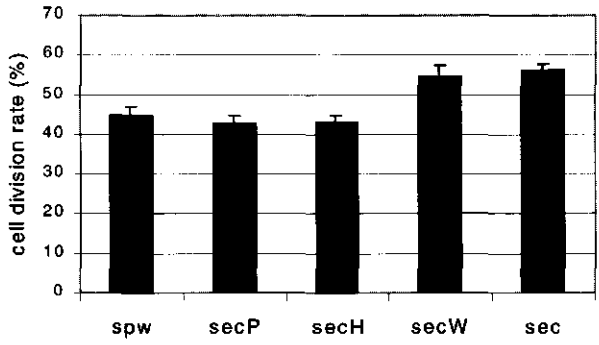

Fig. 3. Heat and Pronase E treatments of potato root diffusate-induced secretions of infective juveniles of Globodera rostochiensis *result in loss of mitogenic activity of secretions in cultured tobacco protoplasts. Within individual protoplast batches no significant difference was observed between the proliferative responses after the addition of 100 µl SPW and heat (secH) or Pronase E (secP) inactivated secretions (at $P > 0.1$). The effect of the addition of nematode secretions (sec) on the cell division rate is highly different from the control group (at $P < 0.001$; see Table 2 and 3). Similar significant stimulation of cell division activity was observed for secretions incubated for 3 hr at 37°C omitting Pronase E (secW). The data presented are from one representative experiment out of two with comparable results. Data of the treatments are expressed as the mean ± SD of triplicates.*

Table 2. *Deviance table showing the effect of pronase E on the mitogenic activity of secretions of infective juveniles of* G. rostochiensis *as tested in two protoplast batches and three independent replicates.*

Source	d.f.[a]	Deviance	Deviance ratio[d]
Secretions	2	70.17	35.09 ***[e]
Batch	1	10.01	10.01 **
Replicates	2	0.860	0.43 n.s.
Residual	12	14.03 [b,c]	
Total	17	95.07	

[a] Degrees of freedom
[b] The residual deviance indicates the goodness of fit of the model.
[c] The residual deviance is below the chi-square threshold value (at $\alpha = 0.05$, d.f. = 12)
[d] The deviance ratio is used as a measure for the significance of the respective variate on the cell division rate.
[e] * $0.01 < P < 0.05$; ** $0.01 < P < 0.001$; *** $P < 0.001$; n.s.: non significant ($P > 0.1$)

Table 3. *Deviance table showing the effect of heat treatment on the mitogenic activity of secretions of infective juveniles of* G. rostochiensis *as tested in two protoplast batches and three independent replicates.*

Source	d.f.[a]	Deviance	Deviance ratio[d]
Secretions	2	43.51	21.75 ***[e]
Batch	1	5.97	5.97 *
Replicates	2	2.19	1.10 n.s.
Residual	9	11.81 [b,c]	
Total	14	43.79	

[a] Degrees of freedom
[b] The residual deviance indicates the goodness of fit of the model.
[c] The residual deviance is below the chi-square threshold value (at $\alpha = 0.05$, d.f. = 9)
[d] The deviance ratio is used as a measure for the significance of the respective variate on the cell division rate.
[e] * $0.01 < P < 0.05$; ** $0.01 < P < 0.001$; *** $P < 0.001$; n.s.: non significant ($P > 0.1$)

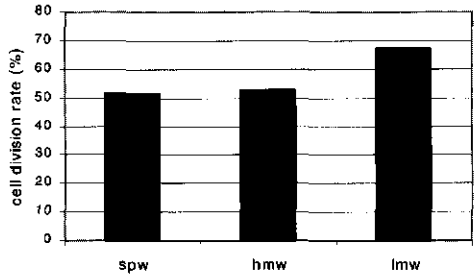

Fig. 4. A low molecular weight fraction (< 3 kDa) of potato root diffusate-induced secretions of infective juveniles of Globodera rostochiensis *harbors a mitogenic compound(s). The effect of the addition of the low molecular weight fraction (LMW) on the cell division rate is highly different from the high molecular weight fraction (HMW) as well as from the addition of 100 µl SPW (at P < 0.001). The data presented are from one representative experiment out of three with comparable results.*

Secretions of *G. rostochiensis* infective juveniles co-stimulate auxin/cytokinin-induced protoplast proliferation.

Tobacco protoplasts divide in the presence of both NAA and BAP and dose-response curves (*e.g.* Fig. 2) were used to obtain information about the nature of the active compound(s) in nematode secretions. Theoretically, nematode secretions could stimulate protoplast proliferation by changing the sensitivity of protoplasts towards auxin and/or cytokinin or by functionally replacing one of these phytohormones. An increased sensitivity of the protoplasts towards NAA would imply the curve in Fig. 2 to shift to the left; a reduction of the sensitivity of protoplasts should result in a shift to the right. Similar phenomena would be expected in the case that the sensitivity towards BAP was changed. Alternatively, if secretions could functionally replace NAA, an increase of the protoplast division rate would be observed at below optimal concentrations of NAA (< 1 µg/ml). The largest effect would then be observed in the range of very low NAA concentrations. Increasing the NAA concentration would lower the relative contribution of the secretions. At above optimal NAA concentrations (> 1 µg/ml), virtually no change in the shape of the curve would be expected. Similar changes can be foreseen when nematode secretions would functionally replace BAP.

Protoplasts were exposed to 100 µl secretions in the presence of suboptimal phytohormone concentrations. Addition of secretions resulted in a significant increase in cell division activity when protoplasts were cultured in low auxin concentrations (0, 0.05, 0.1, 0.5 and 1 µg/ml NAA) in combination with a fixed cytokinin concentration (1 µg/ml BAP) (Fig. 5 and Table 4). In a second assay, protoplast proliferation was also significantly enhanced by secretions in the presence of low cytokinin concentrations (0.01, 0.1, 0.25, 0.5, 0.75 and 1 µg/ml) and a fixed NAA concentration (1 µg/ml). This was based on calculated *P*-values for the *F*-tests of the mean deviance ratios (at $P < 0.001$ data not shown). Finally, the effect of secretions was investigated at above optimal auxin concentrations (3 µg/ml and 7 µg/ml NAA versus 1 µg/ml BAP). Addition of 100 µl secretions again resulted in a significant increase in cell division activity (at $P < 0.001$; Fig. 6 and Table 5).

As shown in Fig. 5 and 6, addition of nematode secretions resulted in an increase in protoplast cell division, which was apparently independent of the phytohormone concentration

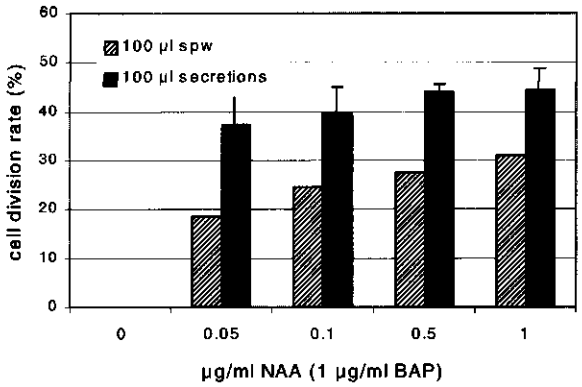

Fig. 5. *Co-stimulatory effect of potato root diffusate-induced secretions of infective juveniles of* Globodera rostochiensis *in the presence of 1 µg/ml BAP and variable low NAA concentrations. Cultured tobacco protoplasts do not proliferate in the absence of NAA, but cell division activity was significantly enhanced in the presence of 100 µl secretions as compared to the control (100 µl SPW; at $P < 0.001$; see Table 4). The data presented are from one representative experiment out of two with comparable results. Data of the additive effect of secretions are expressed as the mean ± SD of two independent counts.*

Table 4. *Deviance table showing the (interactive) effect of secretions of infective juveniles of* G. rostochiensis *in combination with four low concentrations of NAA on the proliferative response of cultured tobacco protoplasts as tested in two protoplast batches and two independent replicates.*

Source	d.f.[a]	Deviance	Deviance ratio[d]
Secretions	1	87.32	87.32 ***[e]
Batch	1	28.39	28.39 ***
Auxin	3	82.58	27.53 ***
Replicates	1	3.83	3.83 n.s.
Auxin×Secr	3	9.21	3.07 n.s.
Residual	18	24.92 [b,c]	
Total	27	236.25	

[a] Degrees of freedom
[b] The residual deviance indicates the goodness of fit of the model.
[c] The residual deviance is below the chi-square threshold value (at $\alpha = 0.05$, d.f. = 18)
[d] The deviance ratio is used as a measure for the significance of the respective variate on the cell division rate.
[e] $0.01 < P < 0.05$; ** $0.01 < P < 0.001$; *** $P < 0.001$; n.s.: non significant ($P > 0.1$)

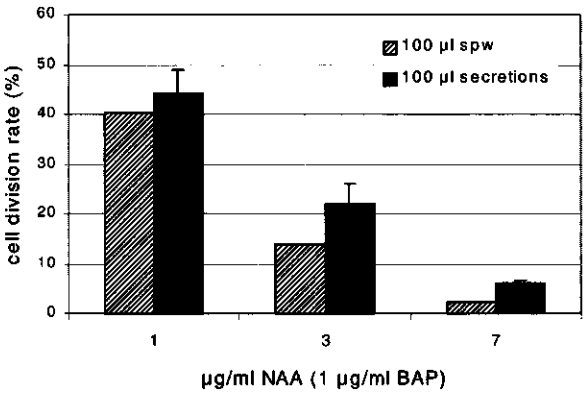

Fig. 6. *Co-stimulatory effect of potato root diffusate-induced secretions of infective juveniles of* Globodera rostochiensis *results in significant enhanced cell division rates in the presence of 1 µg/ml BAP and variable high concentrations auxin (3 µg/ml and 7 µg/ml NAA; at $P < 0.001$; see Table 5). Standard pore water (SPW) was used as a control treatment. The data presented are from one representative experiment out of two with comparable results. Data of the additive effect of 100 µl secretions are expressed as the mean ± SD of two independent counts. Note that the data presented in Fig. 5 and Fig. 6 were obtained with different protoplast batches and hence, they were analyzed separately.*

Table 5. *Deviance table showing the (interactive) effect of secretions of infective juveniles of* G. rostochiensis *in combination with three high concentrations of NAA on the proliferative response of cultured tobacco protoplasts as tested in two protoplast batches and two independent replicates.*

Source	d.f.[a]	Deviance	Deviance ratio[d]	
Secretions	1	19.81	19.81	**[e]
Batch	1	3.99	3.99	n.s.
Auxin	2	809.96	404.98	***
Replicates	1	3.22	3.22	n.s.
Auxin×Secr	2	7.49	3.74	n.s.
Residual	10	20.00 [b,c]		
Total	17			

[a] Degrees of freedom
[b] The residual deviance indicates the goodness of fit of the model.
[c] The residual deviance is just above the chi-square threshold value of 18.3 (at $\alpha = 0.05$, d.f. = 10)
[d] The deviance ratio is used as a measure for the significance of the respective variate on the cell division rate.
[e] * $0.01 < P < 0.05$; ** $0.01 < P < 0.001$; *** $P < 0.001$; n.s.: non significant ($P > 0.1$)

used. This implies that secretions do not functionally replace auxin/cytokinin or change the sensitivity of the protoplasts for these phytohormones. Statistical analysis of these data showed that there is indeed no interaction between the addition of secretions and the effect of either phytohormone on the cell division rate of cultured tobacco protoplasts. Mean deviance ratios for the interactive effect between secretions and respectively NAA (Table 4 and 5) or BAP were not significant (at $P > 0.1$) at both below optimal and above optimal phytohormone concentrations. Hence, it is concluded that secretions co-stimulate tobacco protoplasts proliferation in the presence of variable auxin and cytokinin concentrations.

Secretions of G. rostochiensis co-stimulate PHA-induced human peripheral blood mononuclear cell proliferation.

To test whether secretions could also stimulate cell division in a mammalian cell system, a proliferation assay was conducted using human peripheral blood mononuclear cells (PBMC). PBMC are a mixture of lymphocytes including monocytes and T cells and proliferation of these cells can be induced by the addition of various mitogenic compounds or antibodies that activate receptor complexes present at the cellular plasma membranes. Mitogenic stimulation of the cells is routinely determined by measuring ^{3}H-thymidine incorporation during DNA-synthesis.

Co-mitogenic activity of secretions was examined in the presence of the mitogen phytohaemagglutinin (PHA). Incorporation of ^{3}H-thymidine was significantly increased after addition of 25 µl secretions - an equivalent of the amount produced by 250 secreting potato cyst nematodes - compared to the response obtained in the presence of 25 µl SPW and 25 µl heat-inactivated secretions (Fig. 7). A strong enhancement of cell proliferation of approximately 44% was only detected for cells cultured in the presence of optimal concentrations of the mitogen PHA (25 µg/ml). No mitogenic activity of secretions was detected in the absence of PHA or in the presence of a suboptimal concentration PHA (5 µg/ml). These data suggest that nematode secretions only increase ^{3}H-thymidine incorporation when the upper limit of PHA stimulation is approached or reached. This experiment was repeated using monoclonal antibodies (mAbs) raised to the accessory molecules CD2 and CD28 present on the plasma membrane of T cells. Addition of secretions did not result in a significant increase in ^{3}H-thymidine incorporation, neither at optimal nor suboptimal concentrations of the stimulating anti-CD2 /anti-CD28 mAbs (data not shown).

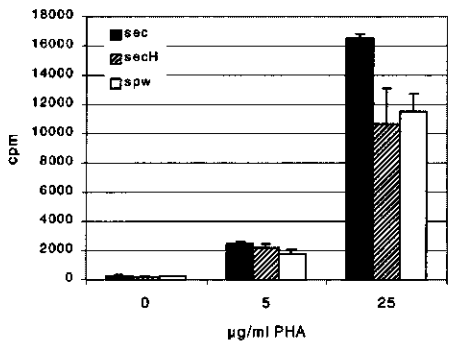

Fig. 7. *Co-stimulatory effect of 25 µl potato root diffusate-induced secretions (sec) of infective juveniles of* Globodera rostochiensis *results in a significant increase in ^{3}H-thymidine incorporation during PHA-induced PBMC proliferation, as compared to 25 µl standard pore water (SPW) or 25 µl heat-inactivated secretions (secH). The data presented are from one representative experiment out of two with comparable results. Data of the treatments are expressed as the mean ± SD of quadruplicates.*

DISCUSSION

In this paper, evidence is provided that pre-parasitic J2 of the potato cyst nematode *Globodera rostochiensis* release (a) low molecular weight peptide(s) upon stimulation by potato root diffusate. This oligopeptide(s) enhance(s) plant cell proliferation in the presence of the synthetic phytohormones NAA and BAP. It is demonstrated that nematode secretions neither change the sensitivity of the protoplasts towards NAA or BAP nor functionally replace these phytohormones. Hence, it is concluded that nematode secretions co-stimulate the proliferative response of tobacco leaf protoplasts. Since tobacco is a non-host for *G. rostochiensis,* the stimulation of cell division in tobacco protoplasts by naturally-induced secretions of the potato cyst nematode illustrates the activation of a general mechanism in plants. Moreover, nematode secretions co-stimulate mitogenesis in human peripheral blood mononuclear cells (PBMC) in the presence of the mitogen phytohaemagglutenin (PHA). Purification of the mitogenic compound(s) is in progress to elucidate whether similar activating principles are involved in both systems.

The role of peptide growth factors in animal and plant cell proliferation.

In animal systems, cells release and detect extracellular signaling molecules such as hormones, cytokines and growth factors. This is presumably the most significant way in which animal cells communicate. Oligopeptides play a major role in cell-cell communication and in this respect insulin, glucagon, interleukins and epidermal growth factors (EGF) can be named. A remarkable feature of these biologically active peptides is that all are formed by truncation of a larger polypeptide. Dozens of signaling peptides have been identified and the majority is recognized by specific receptors anchored in the plasma membrane of the recipient cell (reviewed by Hancock 1997). Mitogenic peptides play a major role in cell cycle control and re-activation of the cell cycle can be induced by these extracellular signal molecules (Hughes, 1992).

Oligopeptides are also involved in communication between filarial nematodes and their host. The presence of *Trichostrongylus colubriformis* in the small intestine of sheep and goats is associated with epithelial renewal. It has been demonstrated that excretory-secretory (ES) products from this nematode are responsible for this cell proliferation. ES products from fourth stage larvae of *T. colubriformis* stimulated HT29-D4 cell growth *in vitro*. This effect (partially) disappeared upon heat-treatment and trypsin digestion (Hoste *et al.*, 1995). This and other examples (Colina *et al.*, 1990; Robinson and Gustad, 1997; Deehan *et al.*, 1998) indicate that the presence of mitogenic peptides in ES products from animal parasitic nematodes may not be uncommon.

In plants only two kinds of mitogenic oligopeptides have been identified so far. Expression of *enod*40 in transgenic roots of *Medicago truncatula* resulted in extensive cortical cell divisions and bombardment of roots with an *enod*40 expression cassette resulted in dedifferentiation and cell division of cortex cells (Charon *et al.*, 1997). A second group are the phytosulfokines. PKS-α and PKS-ß – a sulfated pentapeptide and a tetrapeptide, respectively - induce the proliferation of *Asparagus officinalis* mesophyll protoplasts in cultures, which do normally not respond to any combination of phytohormones (Matsubayashi and Sakagami, 1996; Matsubayashi *et al.*, 1997). On the surface of rice cells, both high- and low-affinity specific saturable binding sites were demonstrated, which were not occupied by auxin or cytokinin. This suggests the activation of a novel signal transduction pathway by these oligopeptides in the presence of auxin and cytokinin, which is involved in plant cell proliferation (Matsubayashi *et al.*, 1997; 1999).

Animal peptides stimulating the proliferation of plant cells have - to the best of our knowledge - not been reported before. A mitogenic oligopeptide(s) released by infective juveniles of the cyst nematode *G. rostochiensis* upon exposure to potato root diffusate could be pathogenesis related. Whether this oligopeptide(s) is co-responsible for syncytium formation remains to be proven.

Nematode secretions co-stimulate plant cell and PBMC proliferation.
In animal systems, it is often observed that combinations of mitogenic growth factors stimulate cell proliferation. Co-stimulation is a term used for growth factors, which solely do not induce cell proliferation, but enhance the proliferative response of cells upon mitogenic stimulation (reviewed by Hughes, 1992). An example of co-stimulation is the effect of the amphiphilic peptide substance P in PBMC proliferation in the presence of suboptimal concentrations PHA. It is suggested that the neuropeptide substance P enlarges the proliferative response of PBMC to the mitogen PHA by increasing the intracellular Ca^{2+} concentration of the cells (Kavelaars *et al.*, 1993).

In this paper, we demonstrate that naturally-induced secretions from *G. rostochiensis* co-stimulate tobacco protoplast proliferation in the presence of auxin and cytokinin. Statistical analyses did not reveal an interaction between secretions and the concentrations of the phytohormones NAA or BAP on the proliferative response of tobacco protoplasts. This implies that nematode secretions neither change the sensitivity of the protoplasts towards NAA or BAP nor functionally replace these phytohormones. Similarly, nematode secretions co-stimulated PBMC proliferation in the presence of the mitogen phytohaemagglutinin (PHA) only. In both systems, nematode secretions increase the proliferative response of plant and mammalian cells given that the appropriate mitogens are present. As such, co-stimulation properly reflects the observed activity of potato cyst nematode secretions.

Specific co-stimulation of PHA-induced PBMC proliferation by mitogenic compounds of infective juveniles of *G. rostochiensis*.
Secretions of the potato cyst nematode activate human PBMC in the presence of the mitogen phytohaemagglutinin (PHA). PHA is isolated from the red kidney bean (*Phaseolus vulgaris*) and is a mixture of five tetrameric glycoproteins. This multivalent lectin stimulates the proliferation of PBMC by cross-linking receptors and accessory molecules on the cell surface of T cells and monocytes (reviewed by Geppert, 1992). This elicits the production of growth factors – respectively the interleukins IL-1 and IL-6 by monocytes, and IL-2 by T cells - , which finally results in the activation of intracellular signal transduction pathways involved in proliferation of these cells (Kishimoto, 1994). Thus, nematode secretions probably stimulate the production of interleukins by the T cells and/or the monocytes.

Specific activation of the T cell population can be achieved by using the two stimulating monoclonal antibodies anti-CD2 and anti-CD28, which bind to the corresponding CD2 and CD28 accessory molecules only present at the T cell surface (reviewed by Fletcher and June, 1992). Secretions were not able to co-stimulate PBMC proliferation in the presence of anti-CD2/anti-CD28. This result indicates that nematode secretions are not able to induce the production of interleukins in T cells and that the observed proliferative response is the result of interleukin production by the monocytes. Alternatively, nematode secretions stimulate parallel pathways in T cells, which are solely induced in the presence of PHA. Since a large number of specific agonists and antagonists is available in combination with several mitogens, the use of standard PBMC proliferation assays will greatly facilitate the elucidation of signal transduction mechanism(s) underlying the co-stimulation of PBMC by naturally-induced nematode secretions.

Mode of action of mitogenic oligopeptides from infective juveniles of *G. rostochiensis*.
Mitogenic secretions of the infective cyst nematode might be involved in feeding cell development. In potato and tomato roots, differentiated cortex cells opposite to the protoxylem poles are preferred for feeding cell initiation by *G. rostochiensis* (Jones and Northcote, 1972; A. Goverse, unpublished observations). Induction of feeding cells coincide with the reactivation of the cell cycle by the parasitic nematode (Gheysen *et al.*, 1997). It remains to be proven whether mitogenic peptides released by infective cyst nematodes are (co)responsible for the redifferentiation of these cells. It is remarkable to see that in roots of leguminous plants cortical cells in a similar position are susceptible to *Rhizobium* Nod factors (mitogenic lipo-chitin oligosaccharides). These LCOs

mediate re-entry of the cell cycle during nodule formation (Libbenga and Harkes, 1973; Yang *et al.*, 1994).

The mitogenic compound(s) present in cyst nematode secretions co-stimulate cell proliferation at extremely low concentrations. The secretions of a single infective juvenile suffice to increase cell division activity significantly in a pool of 160 PBMC or 200 tobacco leaf protoplasts. This peptide(s) could interact extracellularly with receptors anchored in the plasma membrane. Alternatively, this low molecular weight compound could act intracellularly bypassing the plasma membrane. The latter was demonstrated for amphiphilic oligopeptides such as the wasp venom peptide mastoparan (Higashijima *et al.*, 1990) and the neuropeptide substance P (Mousli *et al.* 1990a), which act by direct activation of the G-protein complex (Mousli *et al.*, 1990b). During feeding cell induction, the nematode carefully protrudes its stylet through the cell wall. Hence, nematode secretions may be deposited between the plasma membrane and the cell wall or secretions may be released directly into the cytosol through a perforation in the plasma membrane at the stylet orifice (Williamson and Hussey, 1996). So from its biology it can not be deduced whether cyst nematode secretions act directly on cytosolic compounds or interact with membrane receptors present in tobacco protoplasts.

In summary, it is concluded that secretions of infective juveniles of the potato cyst nematode *Globodera rostochiensis* comprise a mitogenic peptide(s), which is capable to intervene in signal transduction pathways involved in plant and animal cell proliferation. Though by no means proven, mitogenic compound(s) in secretions could be co-responsible for feeding cell development upon nematode infection. The detection of such key intermediates, which might be involved in communication between parasite and host, will contribute to the understanding of feeding cell induction and development by sedentary plant-parasitic nematodes. It supports the hypothesis that manipulation of plant cell development by cyst nematodes is preceded by initial mitogenic stimulation of the cell. As a next step we will identify this secreted oligopeptide(s) and investigate its role in feeding site induction.

MATERIALS AND METHODS

Nematode secretions.

Secretions were collected from pre-parasitic second stage juveniles (J2) from the potato cyst nematode *Globodera rostochiensis* Ro1 Mierenbos basically according to Smant *et al.*, (1997). Thousands of dry cysts were rehydrated in tap-water on 100 µm nylon sieves for 7 days at 20°C. Large numbers of pre-parasitic J2 were obtained by hatching the nematodes in 0.22 µm filter-sterilized potato root diffusate (PRD) for 3 days. The nematode suspension was transferred to conical glass tubes and mixed with freshly prepared 70% (w/v) sucrose in a 1:1 ratio. A layer of 1 ml tap-water was placed onto the (35%) sucrose solution and the J2 were collected by centrifugation at 1,000 × *g* for 5 min. The J2 were washed three times with tap-water by inverting the glass tube.

Pre-parasitic J2 were surface sterilized by incubating the nematodes successively in a cocktail of 0.5% (w/v) streptomycin sulphate-penicillin G (20 min), 0.1% (w/v) ampicillin-gentamycin (20 min), sterile tap-water (5 min) and 0.1% (v/v) chlorhexidin-digluconate followed by three washing steps of 5 min in sterile tap-water. Finally, surface sterilized juveniles were concentrated and suspended in sterile standard pore water (Schouten and Van der Brugge, 1989) supplemented with 15 mM NaCl (SPW). Juveniles were placed in sterile glass petri dishes in a final density of approximately 10,000 J2/ml SPW and incubated overnight at room temperature. The suspension was transferred into sterile glass tubes to concentrate the juveniles by centrifugation at 1,000 × *g* for 2 min. Supernatant containing secretions released by the nematodes was removed and stored in 1 ml aliquots at –80°C. The protein content of the supernatant was too low to be quantified using the method of Bradford (1976). Therefore, the concentration was

expressed as the amount of secretions produced overnight by the number of pre-parasitic J2 per µl SPW.

Different developmental life stages of the free-living nematode *Caenorhabditis elegans* strain Bristol N2 were obtained from a stock culture kindly provided by H. Van Luenen (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Nematodes were collected, surface sterilized and incubated as described above for *G. rostochiensis*. Culture fluid was obtained by incubating the nematodes in SPW omitting 15 mM NaCl to circumvent osmotic stress for this nematode species.

Fractionation of secretions.

Secretions obtained from surface-sterilized infective juveniles of *G. rostochiensis* were separated into two molecular weight fractions: proteins with a molecular mass of 0-3 kDa or larger than 3 kDa, respectively. Microcon microcentrator units with a membrane cut-off of 3 kDa (Amicon Inc., Beverly MA, USA) were rinsed with sterile SPW before adding sterile secretions. Low molecular weight products were obtained by centrifugation of the microcentrator at 14,000 × *g*. High molecular weight products were retained on the 3 kDa cut-off membrane and were washed with an excess of SPW. Finally, they were taken up in sterile SPW corresponding to the original sample volume.

Inactivation of secretions.

Inactivation of secretions was achieved by heat treatment or proteolytic digestion. Secretions were heat-inactivated by incubation at 100°C for 10 min. Proteolytic digestion was performed by complete digestion of sterile secretions in SPW using 1 µg/µl of the exoprotease Pronase E (Sigma, St. Louis MO, USA) for 3 hr at 37°C. Pronase E was removed by centrifugation at 14,000 × *g* using a Microcon microcentrator unit with a membrane cut-off of 3 kDa (Amicon Inc., Beverly MA, USA). As a control, secretions were incubated under the same conditions in the absence of Pronase E.

Proliferation assay with tobacco leaf protoplasts.

For each assay, protoplasts were isolated according to Denecke *et al.* (1989) from successive leaves of a single 6 weeks old plant of *Nicotiana tabacum cv Samsun NN*. This plant was grown *in vitro* on Murashige & Skoog's culture medium containing 3% sucrose with a regime of 16 hr light. Leaves were incubated in TEX-enzyme solution 1.2% (w/v) cellulase Onozuka R10 (Yakult Honsha Co. Ltd., Tokyo, Japan) and 0.5% (w/v) macerozyme R10 (Yakult Honsha Co. Ltd., Tokyo, Japan) omitting phytohormones for 20 hr at 28°C. Protoplasts were collected over a 80 µm nylon sieve and concentrated in 1 ml W5 medium by centrifugation at 500 × *g* for 5 min. To determine the number of protoplasts, the cells were transferred to a Falcon 14 ml polystyrene tube (Becton Dickson Labware, Lincoln Park, NJ, USA) into a final volume of 10 ml W5 solution. After 30 min protoplasts were washed twice with W5 solution by centrifugation at 300 × *g* for 5 min. Protoplasts were resuspended in TEX-medium to a final concentration of 2×10^5 protoplasts per ml. Protoplasts were embedded in a so called liquid-thin-layer culture (Stickens and Verbelen 1996; Potrykus and Shillito 1986). For each treatment 1 ml of the protoplast suspension was carefully immobilized in 6 cm disposable petri dishes by addition of 1 ml 1.2% (w/v) SeaPlaque agarose (Duchefa, Haarlem, The Netherlands) in TEX-medium at 37°C. The thin agar layer was covered with 2 ml TEX medium supplemented with the synthetic auxin NAA and cytokinin BAP. Unless indicated otherwise, the final NAA and BAP concentrations were 1 µg/ml (discounting the volume of the solidified culture medium). Aliquots of secretions were added to the TEX-hormone solution. In controls, an equal volume of SPW was added instead of secretions. Petri dishes were wrapped with Parafilm and incubated for 5 days in the dark at 24°C.

Microscopic observations of cell division rate.
Embedded tobacco protoplasts were observed using an inverted light microscope (Leitz, Wetzlar, Germany). The cell division rate was determined for each treatment by counting the number of dividing cells relative to the total number of cells. Per treatment several spots were randomly selected throughout each petri dish, and all the vital cells present in that particular location were examined (200 × magnification). Protoplasts were classified either as dividing or non-dividing.

Statistical analysis.
To estimate the systematic effects of multiple variables on a counted response variable in the form of a ratio of counts, regression analyses were carried out by the use of generalized linear models (McCullagh and Nelder 1989). The explanatory variables such as replicate, protoplast batch, auxin concentrations, presence/absence of secretions and plant hormone × secretion interactions were fitted to the cell division rate by means of logistic regression models with the variance proportional to the binomial variance and a logit-link function. The deviance was used as a measure for goodness of fit for the model. The model was accepted when the residual deviance did not exceed the chi-square test value (McCullagh and Nelder 1989). The deviance ratio is used as a measure for the significance of the respective variate on the cell division rate. *F*-tests for the mean deviance ratios were used to assess the treatment effects. Pair-wise differences between treatment means on the logit scale were assessed using *t*-tests. All statistical analyses were performed using Genstat 5 version 3.2 (Genstat 5 Committee, Payne *et al.*, 1987).

Proliferation of human peripheral blood mononuclear cells.
Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood, obtained from the National Bloodbank (The Netherlands), by centrifugation over Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) density gradients (1.077 g/cm^3) at 1000 × *g* for 20 min (Kavelaars *et al.*, 1995). Cells (4 ×10^4 per well) in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 5% foetal calf serum (FCS) were stimulated to proliferate with 25 µg/ml phytohaemagglutinin (PHA; Murex Diagnostics S.A., Chatillon, France) in a final volume of 150 µl. PHA is a component in aqueous extracts of seeds of *Phaseolus* spp.. In addition, cells were cultivated in the presence of suboptimal concentrations PHA (5 µg/ml) or in the absence of PHA. Cells were incubated at 37°C and 5% CO_2 for 3 days. An excess of ^{3}H-thymidine (1 µCi) was added to the cells 16 hr before the end of the culture. Cells were harvested by the use of an automated cell harvester (Skatron, Lier, Norway) and incorporation of ^{3}H-thymidine was determined using a scintillation counter. Alternatively, cells were stimulated to proliferate with anti-$CD2_1$ plus anti-$CD2_2$ monoclonal antibody (mAb; 1:8000) in combination with anti-CD28 mAb (1:1000; CLB, Amsterdam, The Netherlands). Mitogenic activity of naturally-induced nematode secretions of pre-parasitic J2 from *G. rostochiensis* was determined in the presence of above mentioned concentrations mitogen by including 25 µl sterile secretions in medium of the cells. Specificity of the response was determined by the addition of 25 µl SPW and 25 µl heat-inactivated secretions. Each treatment was replicated four times in two independent proliferation assays using PBMC from different donors.

CHAPTER 4

In planta monitoring of the activity of two 'constitutive' promoters, CaMV 35S and TR2', in developing feeding cells induced by *Globodera rostochiensis* using GFP in combination with confocal laser scanning microscopy

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ABSTRACT

Under the control of either the constitutive CaMV 35S or the mannopine synthase TR2' promoter, the Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria*, was expressed in transgenic potato (*Solanum tuberosum*) plants. Confocal laser scanning microscopy (CLSM) was applied to observe GFP *in planta* and, subsequently, to investigate promoter activity in developing feeding cells upon potato cyst nematode (*Globodera rostochiensis*) infection. Both the CaMV 35S and the TR2' promoter were strongly up-regulated in young feeding cells in less then 4 days upon infection by *G. rostochiensis* whereas the GFP level in the surrounding tissues remained low. Optical sectioning revealed intense green fluorescence in the dense cytoplasm of the entire syncytial cell, including the most distal cell. Furthermore, GFP was observed within the digestive system of the feeding nematode, showing that proteins with an apparent molecular weight of 32 kDa can be taken up by parasitic juveniles of *G. rostochiensis*. Provided CLSM is used, GFP was shown to be a powerful tool that allows *in vivo* monitoring of gene expression inside young developing feeding cells. Finally, the transcriptional regulation of the CaMV 35S and TR2' promoter in plant-nematode interactions is discussed.

INTRODUCTION

Cyst nematodes are obligatory plant parasites with limited host ranges that include economically important crops such as potato, sugarbeet and soybean. After penetration of the rhizodermis and intracellular migration, the second stage juvenile selects an initial syncytial cell (ISC) within the vascular cylinder (Golinowski *et al.*, 1996). The juvenile carefully punctures the cell wall with its stylet and its tip remains extended in the ISC for the so-called preparation period. During this period, which takes about 2.5-5 and 6-8 hours for *Globodera rostochiensis* and *Heterodera schachtii*, respectively (Steinbach, 1973; Wyss and Grundler, 1992), no metacorpal activity is observed. It is assumed that secretions from the subventral glands are released at the feeding site during this period (Smant *et al.*, 1997). After the nematode starts feeding, breakdown of cells walls between the ISC and adjacent cells commences resulting in the formation of a primary syncytium. Subsequently, more cells within the vascular cylinder are incorporated and a mature multinuclate syncytium develops, which is, among others, characterised by high metabolic activity, disintegrated vacuoles and extensive cell wall ingrowths. Parasitic second stage juveniles (J2) and later developmental stages are immobile and fully depend on a properly functioning syncytium (Hussey, 1989).

To unravel the molecular mechanisms underlying feeding site induction, a reporter system for gene expression is indispensable. GUS (ß-glucuronidase; (Jefferson *et al.*, 1987), was the first enzyme used for this purpose either to investigate alterations in promoter activity or to reveal new nematode responsive promoter sequences by using promoter tagging studies. A number of strong constitutive promoters, such as 35S (from CaMV), *RolA-D* (from *Agrobacterium rhizogenes*), nopaline synthase, y-TIP and T-*cyt* promoter (from *A. tumefaciens*) were screened for their activity in nematode-induced feeding cells using promoter - *gus* constructs. In feeding cells of *Heterodera schachtii* induced in *A. thaliana* roots, these constitutive promoters were shown to be silenced completely (Goddijn *et al.*, 1993). On the other hand, promoter activation of cell cycle promoters *cdc2a* and *Arath;cycB1;1* was found in early stages of syncytium induction in the same plant-nematode interaction. Inside the syncytia, strong *cdc2a* - *gus* expression was seen 2-3 days post infection (dpi). Predominantly at the growing edge of the syncytium, early *Arath;cycB1;1* - *gus* activity was observed (Niebel *et al.*, 1996). Recently, a number of *H.schachtii* -responsive elements were isolated from an *A. thaliana* promoter trap collection including line NC0728 and ARM1. Using these promoters, *gus* expression was seen as soon as 5 h and 4 days post infection, respectively (Barthels *et al.*, 1997).

Because of the destructive preparation of plant tissue and the requirement of exogenous substrate, the use of *gus* as a reporter gene has its limitations. The Green Fluorescent Protein (GFP), originally found in the jellyfish *Aequorea victoria*, is a suitable alternative and allows the continuous monitoring of gene expression *in vivo* without the addition of exogenous substrates or co-factors (Prasher *et al.*, 1992; Chalfie *et al.*, 1994). Urwin *et al.* (1997) were the first to evaluate the value of GFP (27 kDa) as a nondestructive reporter system in plant-nematode interactions. The activity of the CaMV 35S promoter was analysed in *Arabidopsis thaliana* infected with *H. schachtii* and *Meloidogyne incognita*. The powerful CaMV 35S promoter resulted in *gfp* expression throughout the entire root system, which hampered direct monitoring promoter activity in the feeding cells by using epifluorescence microscopy. Evidence for *gfp* expression in syncytia was obtained 7 days after inoculation by showing progressive down-regulation of the CaMV 35S promoter. The decline of *gfp* expression made the outline of the syncytia visible as a negative, orange red fluorescent image in a green fluorescent background.

In this paper, we demonstrate that confocal laser scanning microscopy (CLSM) is a powerful tool to monitor *gfp* expression directly in expanding syncytia induced by *G. rostochiensis* in potato. Preliminary studies using standard epifluorescence microscopy, revealed that *gfp* expression in syncytia is masked by strong autofluorescence of various cell types of the root tissue. By scanning optical sections with CLSM, this problem was overcome and it could be shown that two 'constitutive' promoters, *viz.* CaMV 35S and TR2', are specifically upregulated in the syncytia of *G. rostochiensis*. From the presence of high concentrations GFP in the digestive system of feeding nematodes, it can be concluded that the feeding tubes of *G. rostochiensis*, which functions as a molecular sieve, allows the uptake of proteins with an apparent molecular weight of 32 kDa. The observed discrepancies with other studies on the model plant *Arabidopsis thaliana* are discussed.

RESULTS

GFP expression in transgenic potato plants

Gfp expression was checked by dot-blotting total protein extracts followed by exposure to a polyclonal serum against recombinant GFP. For western blot analysis, three and four of the best expressors were selected from plants harbouring the CaMV 35S- and TR2'-mGFP4 fusion constructs, respectively. For that, protein extracts of leaves and roots were examined by non-reduced SDS-PAGE followed by western blotting. Immunodetection was performed by using a polyclonal serum against recombinant GFP. This resulted in the detection of a single protein band (Fig. 1). *Gfp* was expressed as a monomer with an apparent MW of 32 kDa (35S-mGFP4), whereas, due to the extended N-terminus, TR2'-mGFP4 gave rise to a monomer of 33 kDa. GFP was present in both leaves and roots. Protein expression levels varied between 0.1-1.0 %.

GFP fluorescence in transgenic potato plants

Epifluorescence microscopy revealed that both *gfp* constructs were functionally expressed in potato plants. GFP was observed in the cytoplasm and in the nucleus irrespective the cell type examined or the construct used. Detection of GFP fluorescence in shoots and roots was in accordance with the GFP expression patterns determined by western blot analysis. A GFP expression level of at least 0.5% was required for convenient epifluorescence microscopic observations.

In leaves of potato plants harbouring the CaMV 35S-mGFP4 fusion construct fluorescence was observed in guard cells, epidermal cells, parenchyma cells, mesophyll cells and trichomes. For the TR2' promoter, GFP fluorescence was only observed in guard cells of potato leaves (data not shown).

In roots of potato plants, background fluorescence seriously hindered the observation of GFP fluorescence irrespective the filter combination used. Autofluorescence was primarily emitted by xylem vessels, cell walls, epidermal and necrotic cells and meristematic tissue. No specific GFP

fluorescence could be observed. The masking of GFP by high background fluorescence of the roots could be circumvented when confocal laser scanning microscopy (CLSM) was used instead of standard epifluorescence microscopy. In optical sections of potato roots harbouring the CaMV 35S-mGFP4 construct, GFP fluorescence was detected in the cytoplasm and nucleus of meristematic cells of the vascular cylinder and in cortical cells (data not shown). For the TR2' promoter, no GFP fluorescence could be observed in the root.

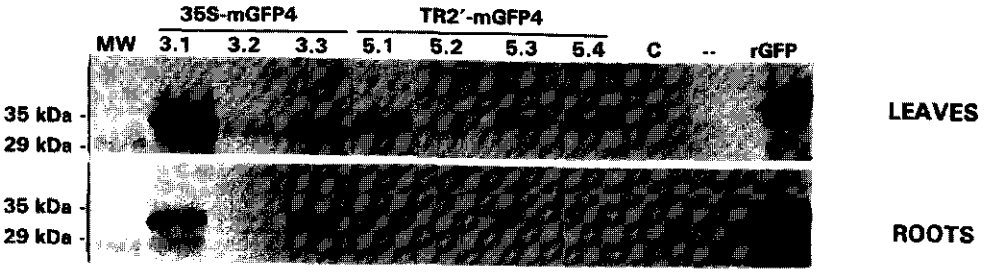

Fig. 1. Western blot analysis of GFP expression in leaves and roots of transgenic potato plants (Solanum tuberosum *6487-9) harbouring the CaMV 35S-mGFP4 or the TR2'-mGFP4 construct. For non-reducing SDS-PAGE, 7 µg total soluble proteins were loaded per lane. GFP was detected using a polyclonal serum against recombinant GFP. Abbreviations: rGFP = recombinant GFP; C = protein sample of a control potato plant.*

CaMV 35S and TR2' promoter activity in feeding cells induced by *Globodera rostochiensis*

The response of the constitutive promoters CaMV 35S and TR2' upon infection with the potato cyst nematode *G. rostochiensis* was determined by CLSM in the transgenic potato line 3.1 and 5.1, respectively. Non-transgenic potato roots were infected, too, and were used as a control for autofluorescence in feeding cells. During the onset of parasitism (2, 4, 8, 11 and 13 days post infection) *gfp* expression was observed in the roots.

Scanning images of feeding cells in the roots of non-transformed plants resulted in the detection of minor autofluorescence in all cell walls. Major fluorescence was observed in xylem elements, necrotic cells at the site of penetration and along the migratory pathway, and in the thickened cell walls surrounding the feeding cell (Fig. 2A). No fluorescent structures were observed inside the feeding cell. These observations did not change during further development of the feeding cell. Moreover, no green or greenish autofluorescence could be detected inside the parasitic nematode.

In transgenic potato plants, a strong GFP signal could be observed in feeding cells induced by immobile and feeding second stage juveniles (4 days post infection). Bright GFP fluorescence was restricted to the dense cytoplasm. Secondary vacuoles replacing the central vacuole were observed as dark spheres. Cells adjacent to the initial feeding cell are incorporated by cell wall dissolution resulting in fusion of the protoplasts creating a multinuclear syncytium.
Changes in GFP fluorescence during syncytium development showed that the fusion of the protoplasts occurred acropetally and basipetally along the stele taking the initial syncytial cell as a point of reference. In root tissue surrounding the nematode feeding site, GFP related fluorescence was absent, in case of TR2' or very low, in case CaMV 35S was used. These patterns did not change during parasitism.

Similar bright GFP fluorescence was visible in the feeding cells of the female life stages J3 (Fig. 2D, 2E and 2F) and J4. Scanning images revealed that the major part of the feeding cell is located within the stele next to the vascular system (Fig. 2G and 2H). Variation in shape and

volume of the feeding cells seems to depend on the location of the initial cell. Intense GFP fluorescence in the syncytial cells allows the detection of even the most distal cell, which is typically located adjacent to a xylem vessel (Fig. 2I). Irrespective whether *gfp* was preceded by CaMV 35S or the TR2' promoter, the *gfp* expression patterns in the syncytium and its immediate vicinity were virtually identical.

GFP is ingested by feeding potato cyst nematodes during the onset of parasitism

In contrast to the scanning images of non-transgenic control roots, bright green fluorescence was present in the intestine of nematodes feeding on transgenic roots. This phenomenon occurred in all parasitic stages (J3; Fig. 2B and 2C). Most likely, GFP was extracted from the cytosol via the feeding tube, which functions as a molecular sieve. These results implicate that feeding cell and nematode development were not hampered by exposure to relatively high concentrations of GFP.

DISCUSSION

We have shown that scanning optical sections with CLSM allows continuous monitoring of early gene expression patterns in developing syncytia in *gfp*-transformed plants. This is illustrated by the specific upregulation of the two 'constitutive' promoters CaMV 35S and TR2' in young syncytia. Since the early events in expanding syncytia are crucial for the establishment of feeding cells, the reporter gene *gfp* in combination with CLSM is a valuable tool in elucidating the underlying molecular mechanisms of feeding cell induction and maintenance.

From our observations it is clear that GFP is neither interfering with the development of the feeding cell nor the nematode. Normal growth of the nematode, including moulting, is achieved within four weeks. Interestingly, strong GFP fluorescence was observed in the digestive system of feeding juveniles of *G. rostochiensis*. Cyst nematodes take up nutrients from the cytoplasm using a so called feeding tube, which is attached to the nematode stylet and functions as a molecular sieve excluding the uptake of high molecular weight compounds from the cytoplasm. The use of fluorescent dextrans revealed that the beet cyst nematode *H. schachtii* retracts solutes from the cytoplasm with a maximum molecular size between 20 and 40 kDa (Böckenhoff and Grundler, 1994). Urwin *et al.* (1997) detected GFP in the root knot nematode *Meloidogyne incognita* on Western blot, but not inside adult females of *H. schachtii*. Therefore, the exclusion limit was suggested to be at most 28 kDa. The presence of GFP in the feeding stages of *G. rostochiensis* indicates that the feeding tubes of this nematode allows the uptake of larger proteins as compared to *H. schachtii*. Furthermore, the strong fluorescence of the digestive system suggests that potato cyst nematodes ingest GFP at a relative high rate and that the removal from the digestive system, either by degradation or defecation, occurs at a relatively slow rate.

Promoters that are switched on in syncytia are valuable tools to engineer host plant resistance. In potato the activity of the CaMV 35S and the TR2' promoter was enhanced in feeding cells induced by *G. rostochiensis*. GFP signals in developing syncytia were far stronger than in the cortex, vascular cylinder or endodermis. Since the formation of the GFP chromophore is a redox-sensitive process (Heim *et al.*, 1994), changes in the intensity of GFP-related green fluorescence could be due to modifications in the physiological conditions of particular cells, too. Our results are in striking contrast with the activity of promoter-*gus* constructs in feeding cells of the model plant *A. thaliana*. Various strong promoters, such as CaMV 35S, *RolA-D* (from *Agrobacterium rhizogenes*), nopaline synthase, y-TIP and T-*cyt* promoter (from *A. tumefaciens*), were reported to be silenced completely in feeding cells induced by *H. schachtii* and *M. incognita* (Goddijn *et al.*, 1993). In a more detailed study, Urwin *et al.* (1997) reported a progressive down-regulation of the CaMV 35S promoter in feeding cells upon infection of *gfp*-transformed *A. thaliana* with *H. schachtii*, whereas a strong fluorescence was observed throughout the whole root system. The discrepancy with our data can not been explained by differences in the promoter sequence, since

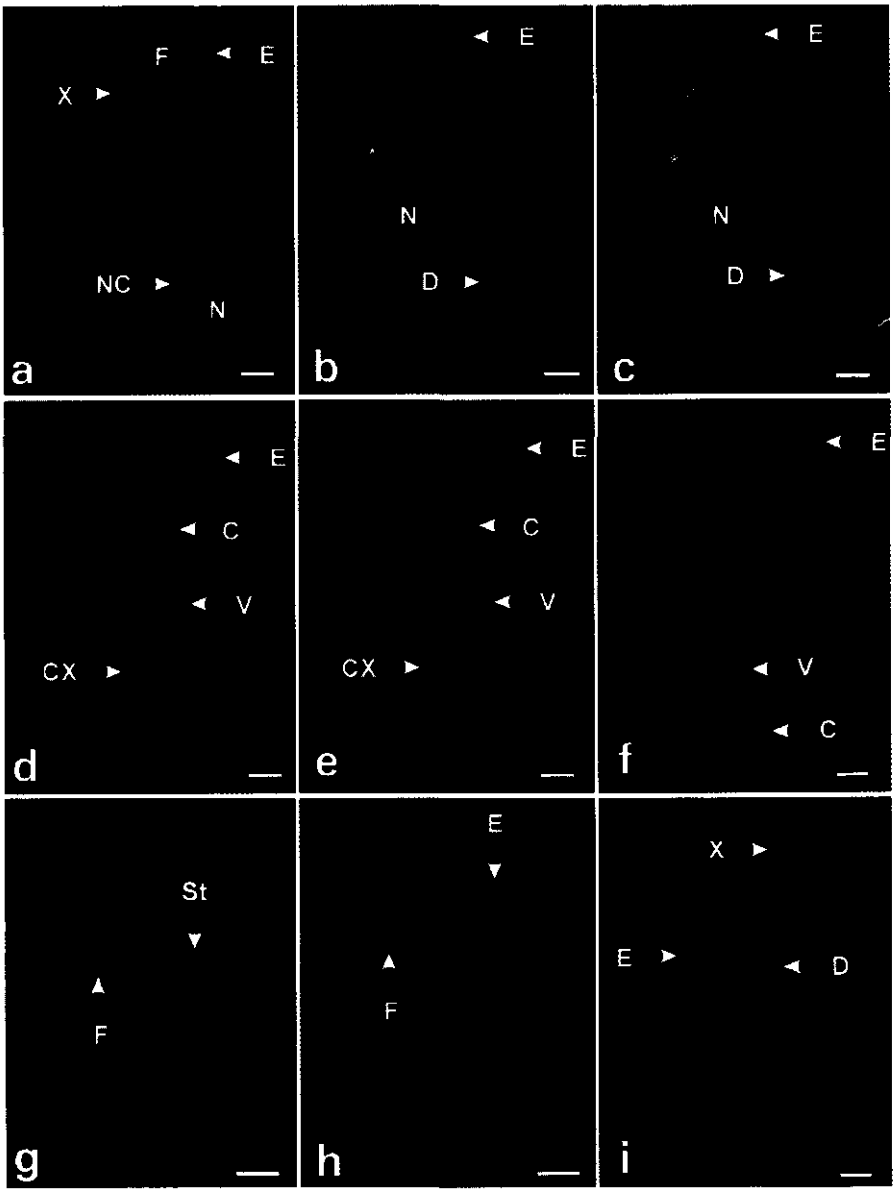
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Fig. 2. [←] *Merged CLSM images of in planta GFP expression in feeding cells induced by the potato cyst nematode* Globodera rostochiensis *in untransformed (**A**) and transgenic (**B-I**) potato roots (200x). GFP expression is controlled by either the CaMV 35S (**B-F**) or the TR2' promoter (**G-I**). Green and red represent fluorescence emitted at 522 nm and wavelenghts longer than 580 nm, respectively. Bars: 50 µm. **A.** Nematode-infected root of a non-transgenic potato plant (dpi =13). The outline of the anterior part of the female juvenile (N; J3) is clearly visible and the position of the head is indicated by an asterisk. Xylem (X), necrotic cells (NC) and epidermal cells (E) strongly autofluoresce. Neither inside the feeding cell (F), nor in the nematode (N) autofluorescence was observed. **B** and **C.** Two optical sections of a nematode-infected transgenic potato root (dpi = 13). The posterior part of the female juvenile (N; J3) is located outside the root. The red spots present inside the nematode are lipid granules and the position of the tail is indicated by an asterisk. GFP fluorescence is visible in the digestive system (D) of the nematode. This nematode is connected with the feeding cell of which a part is shown in **D, E** and **F. D, E** and **F.** Three successive optical sections of one infection site in a transgenic potato root (dpi=13). Bright GFP fluorescence is visible inside the whole feeding cell, which is located within the vascular cylinder. The central part of the syncytium, located below the nematode head, is shown here. GFP accumulated in the dense cytoplasm (C). The central vacuoles are replaced by secondary vacuoles (V). Faint GFP fluorescence is visible in the cortical cells (CX). Epidermal cells show low levels of red autofluorescence (E). **G.** Composition of a bright field and GFP fluorescence image of a feeding cell (F) induced in a transgenic root (dpi=8). The syncytium is located asymmetrically within the stele (St) of the root (partly shown here). No GFP background expression could be detected in adjacent tissues. **H.** Fluorescence image of the feeding cell shown in **G.** Expression of GFP in syncytia results in similar fluorescence patterns when induced in roots either harbouring the TR2' promoter or the CaMV 35S promoter (**D, E** and **F**). **I.** The most distal cells (D) of a developing feeding cell (dpi=11). Only cells adjacent to the xylem vessels (X) are incorporated. No green fluorescence was detected in the distal cells of syncytia in control roots (data not shown).*

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the CaMV 35S promoter construct used by Urwin *et al.* (1997) is identical to the one used in this paper.

Goddijn *et al.* (1993) suggested that the transcription factor ASF-1 is involved in the observed down-regulation of constitutive promoters in feeding cells as, except for T-*cyt* and *Rol*B-*Rol*C, the promoters used in their study have the *as*-1 recognition sequence of ASF-1 in common. According to this view, the recognition sequence of CaMV 35S may act in a different way in the interaction potato-*G. rostochiensis* . However, other observations suggest that down-regulation of the CaMV 35S promoter in developing feeding cells is unlikely. The CaMV 35S promoter is, similar to the TR2' promoter, auxin responsive (Landridge *et al.*, 1989; Leung *et al.*, 1991; Liu *et al.*, 1994) and auxin is required for the transition of the G_1 to S phase in the cell cycle (John *et al.*, 1993). Apart from this (though presumably not independent), the CaMV 35S promoter is reported to be preferentially active during the S phase of the cell cycle (Nagata *et al.*, 1987). Since endoreduplication of DNA in developing syncytia is accomplished by repeated G_1-S-G_2-G_1 cycles shunting the M phase (Niebel *et al.*, 1996), instead of down-regulation, an enhanced activity of the CaMV 35S promoter in developing syncytia is to be expected. The intriguing question remains what causes the observed discrepancy between the activity of the CaMV 35S promoter in the interaction *H. schachtii*-*A. thaliana* and *G. rostochiensis*-potato, which may implicate subtle differences in transcriptional regulation mechanisms.

By selecting plants with expression levels of at least 0.5% of the total soluble protein fraction, *gfp* expression could be monitored in optical sections of developing syncytia with CLSM. CLSM seems the most obvious means to circumvent the problems related to autofluorescence and enables direct monitoring of gene expression patterns *in planta* during the onset of parasitism.

Detecting early promoter activity in developing feeding cells can probably be improved by switching to improved *gfp* mutants resulting in higher quantum yields and regeneration efficiency (Chiu *et al.*, 1996; Crameri *et al.*, 1996; Pang *et al.*, 1996; Siemering *et al.*, 1996). It is expected that if these improved *gfp* variants are used in combination with CLSM, the expression patterns of more subtle, typical plant promoters can be monitored in developing feeding cells, as well.

MATERIALS AND METHODS

Plant material

Transgenic potato plants were obtained by introducing the vector pBIN35S-mGFP4, kindly supplied by Dr. J. Haseloff (MRC Cambridge, UK), and pBINPLUSTR2'-mGFP4 into *Agrobacterium tumefaciens* (strain LBA4404) for subsequently use in stem transformation of *Solanum tuberosum* 6487-9 (Van Engelen *et al.*, 1994). In pBINPLUSTR2'-mGFP4, derived from pBINPLUS (Van Engelen *et al.*, 1995), the transcription is controlled by the mannopine synthase promoter TR2' obtained from pCPO3 (Van Engelen *et al.*, 1994), and stopped by the nopaline synthase terminator Tnos. *Gfp* was amplified from pBIN35S-mGFP4 by using the 5'-end primer GGCGCAAGCTTCGAGCTCAGTAAAGGAGAAGAACTT and 3'-end primer GCCCGAATTCAGATCTGCTTATTTGTATAGTTCATCCAT (Isogen Bioscience bv; Maarssen, The Netherlands). The following PCR conditions were used: 5 min 94°C, 10 cycles of 1 min 94 °C, 5 min 50 °C, 3 min 72°C, and 10 min at 72°C. By the introduction of a *Sst*I and *Bgl*II site mGFP4 was cloned into the proper reading frame using the *Sst*I - *Bgl*II sites of the TR2' expression cassette. Translation was designed to start at the ATG codon present in the *Nco*I site of the MCS. Transgenic plants were regenerated from callus and selected for kanamycin resistance.

Protein extraction and analysis

Plant tissue was collected and homogenized in liquid nitrogen. Per g fresh weight, 2 ml TE (10 mM Tris-HCl, 1 mM EDTA pH 8) including 1 mM pefa-block was added. Chloroplasts were removed by centrifugation at 3,000 rpm for 5 min. Supernatant was collected and the protein concentrations were determined according to Bradford (1976). For dot-blot analysis 20 µg native soluble protein extract in TE was concentrated on nitrocellulose using a vacuum dot-blot apparatus (Schleicher and Schuell, Dassel, Germany). Blots were blocked in 5% dry milk (w/v) in PBS-0.1% (v/v) Tween (PBST) and incubated in 1% dry milk-PBST containing a polyclonal antibody against recombinant GFP (1:3000; Clontech Laboratories, Palo Alto, CA, USA). Detection was performed by an alkaline phosphatase conjugated secondary antibody (1:5000 Jackson ImmunoResearch Laboratories, West Grove, PA, USA). For non-reduced SDS-PAGE, 7 µg soluble protein was loaded with sample buffer onto 12% Ready Gels (BioRad Laboratories, Veenendaal, The Netherlands). Gels were semi-dry blotted onto PVDF membranes and GFP was detected as previously described for dot-blotting. In both blotting procedures 75 ng rGFP was used as a positive controle. Protein expression levels were determined on the basis of the total soluble protein concentration.

Infection with the potato cyst nematode Globodera rostochiensis

Primary transformants of potato were grown *in vitro* on 1.5 % agar plates containing 3.29 g/l Gamborg B5 medium with vitamines and 2% (w/v) sucrose (pH 6.2). Plates were incubated at 24°C with 16h light. Three week old roots were used for inoculation with sterile pre-parasitic juveniles (J2) of *Globodera rostochiensis* Ro1 Mierenbos. Dry cysts were surface sterilized and hatched according to Heungens *et al.* (1995). Subsequently, J2's were incubated in 0.2% (w/v) streptomycin sulphate for 20 min., in 0.2% (w/v) penecillin G for 20 min., in sterile tap-water for 10 min., and in 0.2% (v/v) chlorhexidin for 3 min. This treatment was followed by extensive washing in sterile tap-water. J2's were collected on a 5 µm filter and 5 µl nematode suspension

(about 50 J2) was used for infection. The J2 were either pipetted unto the root tip or under the agar layer near the root tip. Thereafter, plates were incubated in the dark at 18°C.

CLSM and epifluorescence microscopy of transgenic plants and nematode feeding cells
Tissue samples of transgenic potato plants were examined *in planta* on object slides. For epifluorescence microscopy the following filter sets were used: Blue BP450-490 RKP510 BP515-560; BP450-490 RKP LP515 and UV-violet BP355-425 RKP455 LP460 (Leitz). Confocal laser scanning microscopy (CLSM; MRC-600 BioRad) was performed using a 488 nm laser beam. Fluorescence was monitored simultaniously using a band pass filter (522 nm) and a long pass filter (580 nm). Images were merged and data were saved on disc.

CHAPTER 5

Auxin is involved in feeding cell induction by cyst nematodes

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This chapter has been submitted in a modified form

ABSTRACT

Infection of the extreme auxin-insensitive tomato mutant *diageotropica* (*dgt*) and a number of *Arabidopsis* auxin-response mutants with the potato cyst nematode *Globodera rostochiensis* and the beet cyst nematode *Heterodera schachtii* , respectively, strongly point at a role for auxin in early feeding cell development. Nematode development was significantly reduced in the single gene, recessive mutant *dgt* and was accompanied by abnormal feeding cell development. For the *Arabidopsis* auxin-insensitive mutants, only a significant reduction of the infection rate was observed for the mutant *axr2*. However, the majority of the mutants showed a reduction in feeding cell hypertrophy – like in *dgt* - and a reduction in nematode-induced lateral root formation. The induction of *gus* expression in expanding syncytia of the auxin-responsive *Arabidopsis* promoter trap line 5-1E1 upon infection with *H. schachtii* suggested that early feeding cell development is accompanied by a local auxin accumulation. In addition, the obstruction of polar auxin transport in the root either by the application of *N*-(1-naphthyl)phtalamic acid (NPA) or by the use of the *Arabidopsis* auxin efflux mutants *pin1-1/ttg1* and *eir1-1* resulted in a substantial reduction of cyst nematode infections. Moreover, abnormal feeding cell development was observed in the presence of NPA, which was accompanied by the disruption of radial feeding cell expansion, disorganized cell division and metaxylem formation.

INTRODUCTION

Cyst forming nematodes of the genera *Heterodera* and *Globodera* have the potential to induce the formation of feeding cells by refined intervention in the developmental program of the host plant. Pre-parasitic second-stage juveniles enter the root preferentially at the elongation or differentiation zone. Juveniles migrate intracellularly towards the vascular cylinder. Depending on the nematode-host combination, a differentiated or non-differentiated root cell is preferred as starting point for feeding cell induction (Magnusson and Golinowski, 1991 and literature cited therein). The morphology and ultrastructure of syncytia is basically the same irrespective of the nematode - host plant combination. Feeding cell formation is presumably initiated in response to signal molecules released by the infective juvenile (reviewed by Williamson and Hussey, 1996).

Very early in feeding cell development, cell wall openings between the initial syncytial cell and adjacent cells are formed. Initially, cell wall breakdown occurs by a gradual widening of plasmodesmata, later on large openings are created without the involvement of these natural cytoplasm bridges (Grundler *et al.* 1998). Progressive cell wall dissolution results in the expansion of the feeding cell within the stele along the xylem vessels. Subsequent fusion of the protoplasts results in a hypertrophied multinuclear cell complex, a syncytium, which can include up to 200 cells (Jones, 1981). The central vacuole of the cells is replaced by numerous small secondary vacuoles and the dense cytoplasm contains numerous organelles and enlarged nuclei. Extensive cell wall protuberances are formed at those parts of the syncytium that are in close contact with xylem elements. These protuberances greatly enlarge the plasma membrane surface thereby facilitating massive short-distance nutrient import which is essential for nematode development. The hypertrophic feeding cell is lined by a thickened cell wall to resist the osmotic pressure, which increases up to 9,000-10,000 hPa (Jones and Northcote, 1972, Böckenhoff and Grundler, 1994).

In case the starting point of syncytium formation is a pericycle or (pro)cambium cell, feeding cell establishment coincides with lateral root formation. This was observed for the oat cyst nematode (*Heterodera avenae*) on wheat (Grymaszewska and Golinowski, 1991), and the beet cyst nematode (*Heterodera schachtii*) on rape (Magnusson and Golinowski, 1991) and *Arabidopsis thaliana* (Sijmons *et al.*, 1994a). Lateral root formation is initiated in pericycle cells, which are arrested in the G_2 phase of the cell cycle. Feeding cell development is apparently accompanied by the stimulation of neighboring pericycle cells to reenter the cell cycle. This progression in the cell cycle is controlled by cyclin genes in combination with *cdc2* genes (Doerner, 1994). In young root

parts of *A. thaliana*, the cell cycle regulating protein kinase *cdc2a* was shown to be expressed all over the pericycle (Hemerley *et al.*, 1993). Niebel *et al.* (1996) detected *cdc2a-gus* expression upon cyst nematode infection not only in young but also in older root parts where *cdc2a* is normally not expressed. Exogenous application of auxin to roots not only resulted in a great raise in the number of lateral root initials, but also increased the expression of the mitotic cyclin gene *Arath;cycB1;1* (Doerner *et al.* 1996). Infection of *A. thaliana* roots with beet cyst nematodes resulted in strong *Arath;cycB1;1-gus* expression in very young synctia only. Apparently, lateral root initiation and syncytium formation have a number of characteristics in common. This is further illustrated by large scale screening of promoter trap lines, which resulted in a relatively high proportion of genes that was expressed both in lateral roots and in nematode feeding cells (Scheres *et al.*, 1997). Strong auxin-insensitive mutants such as the tomato mutant *diageotropica* (*dgt*) fail to form lateral roots and it would be interesting to see how parasitism is affected by this kind of response mutants.

In this paper, evidence is provided that auxin plays a prominent role in the early stages of feeding cell development by cyst nematodes. The role of auxin was demonstrated by using phytohormone mutants from tomato and *A. thaliana* inoculated with either potato cyst (*Globodera rostochiensis*) or beet cyst (*H. schachtii*) nematodes. *In vitro* the tomato mutant *dgt* was shown to be *de facto* resistant to potato cyst nematodes. Further evidence for the role of auxin was demonstrated by transcriptional activation of the auxin-responsive promoter trap line 5-E1 and disruption of feeding cell morphogenesis by application of the auxin transport inhibitor *N*-(1-naphthyl) phtalamic acid (NPA). The role of a localized disturbance of the auxin levels is discussed in relation to feeding cell formation by cyst nematodes.

RESULTS

Early feeding cell development is restricted in the auxin-insensitive tomato mutant *dgt*

Under *in vitro* conditions, the auxin-insensitive tomato mutant *diageotropica* (*dgt*) is *de facto* resistant to the potato cyst nematode *Globodera rostochiensis* (Fig. 1). Penetration and migration in the roots of the mutant *dgt* was normal, but the majority of the parasitic second stage juveniles were unable to develop into adult females (Fig. 2A). Only one female developed on *dgt* after the inoculation of approximately 50 *dgt* roots. Nematodes infect roots preferably just behind the root tip. The mutant *dgt* lacks lateral roots and therefore the number of available infections sites is reduced. To correct for this potential bias, a fixed number of nematodes (≈ 50) was added to an equal number of root tips from both *dgt* and the control Money Maker. Inhibition of nematode development was not the result of an abnormal morphology of the *dgt* roots. In cross-sections the roots of *dgt* and Money Maker were virtually identical. Only the number of xylem elements were slightly higher and somewhat compressed. This phenomenon has been described for the *dgt* shoot by Zobel (1974).

Under *in vivo* conditions, a similar though less pronounced effect was observed. Nematode development, expressed as the average number of cysts per plant, was significantly reduced by 71% in *dgt* roots (126±23 (mean ± SD)) as compared to the Money Maker control (441±110). Cysts formed on *dgt* were slightly smaller and the average number of eggs per cyst was substantially reduced by about 25% on *dgt* (167±4 (mean ± SD)) compared to Money Maker (224±0.4).To see whether a hampered feeding cell proliferation could be underlying the poor development of the nematode, infected roots of *dgt* and Money Maker were sectioned at different time intervals. In both *dgt* and Money Maker, syncytium induction started in the outer cortex and radial expansion towards the stele was observed at 3 and 6 days post inoculation (dpi). The parasitic J2 were found with their stylet still inserted into the initial syncytial cell. However, a complete degradation of the cytoplasmic content was observed in the majority of the feeding cells induced in *dgt* (Fig. 2C). Mitotic activity - which usually occurs in cells surrounding the developing syncytium in Money Maker – was also observed in *dgt*. Occasionally, poorly developed

females were observed in *dgt* encapsulated by the root. This strongly points at a malfunctioning feeding cell, which was characterized by a less extreme incompatible reaction: cell wall breakdown was severely hampered resulting in small feeding cells containing enlarged nuclei. This structure was lined by a thickened cell wall. Extensive callose deposit, metaxylem differentiation and cell division was observed in adjacent tissues (Fig. 2E). In the rare case a fully developed female was found on *dgt* roots, the length of the syncytium was halved as compared to Money Maker (data not shown). The occurrence of these abnormal feeding cells corresponds with the reduction in nematode development on roots of *dgt* shown in figure 1. This demonstrates that the *dgt* mutation obstructs early feeding cell development by cyst nematodes.

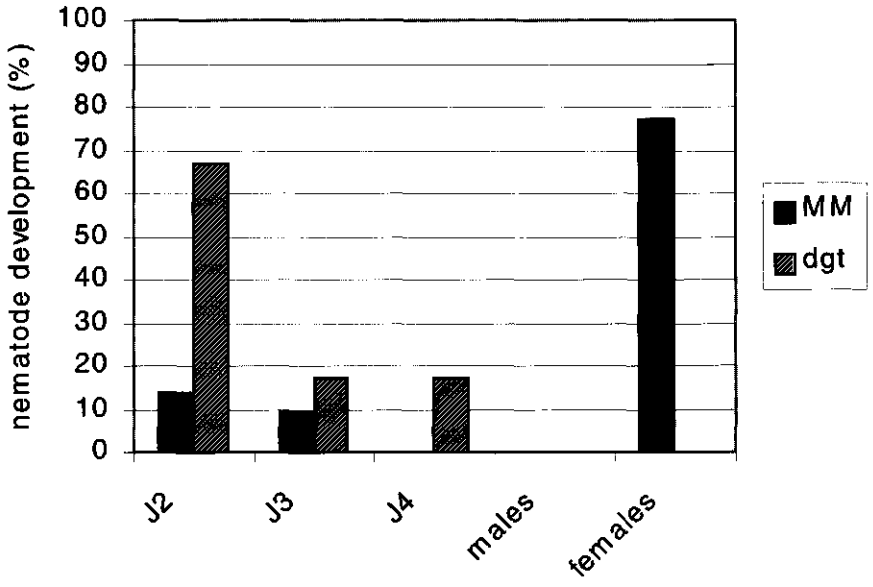

Fig. 1. *Potato cyst nematode development on roots of the auxin-insensitive tomato mutant* diageotropica (dgt) *and on the tomato cultivar Money Maker (MM) in vitro. Nematode development was expressed as the percentage of parasitic second stage juveniles (J2) that developed into a third juvenile (J3) or further (J4 or adult) at 49 dpi. The data presented are from one representative experiment out of three with comparable results.*

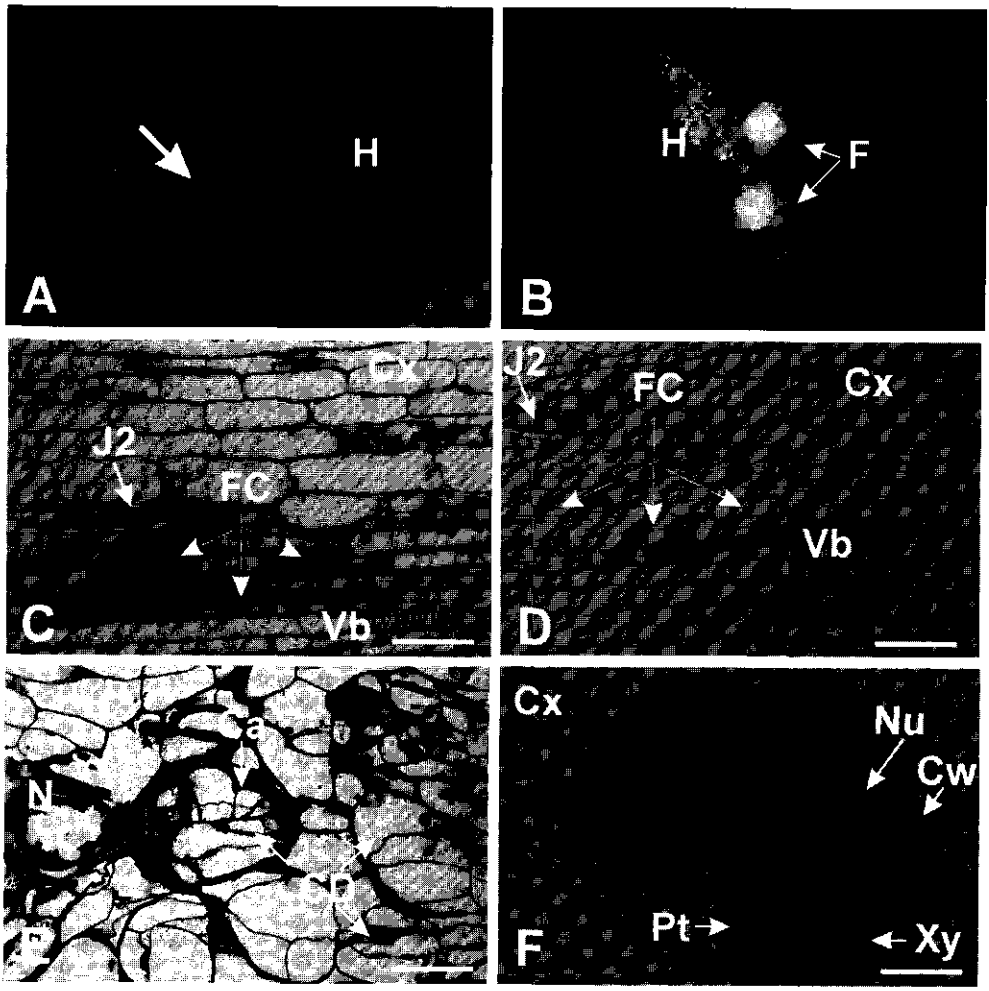

Fig. 2. *Feeding cell formation by the potato cyst nematode* Globodera rostochiensis *on roots of the auxin-insensitive tomato mutant* diageotropica (dgt) *and on the tomato cultivar Money Maker.* ***A.*** *Nematode-infected root of dgt 49 days post inoculation (dpi) (arrow = migratory track; H = hypertrophy).* ***B.*** *Adult females (F) on an infected control root (Money Maker) at dpi = 49 (H = hypertrophy).* ***C.*** *Cross section of an infected root of* dgt *at dpi = 3. The cytoplasmic content of the feeding cell (FC) is completely degraded (J2 = parasitic second stage juveniles; Cx = cortex; Vb = vascular bundle).* ***D.*** *Cross section of an infected control root (Money Maker).* ***E.*** *Cross section of an abnormal feeding cell in roots of* dgt *at dpi = 49 (N = poorly developed female; CD = disordered cell divisions; Ca = callose deposit).* ***F.*** *Normal syncytia formation in roots of Money Maker at dpi = 49 (Nu = nucleus; Pt = cell wall protuberances; Cw = cell wall dissolution; Cx = cortex; Xy = xylem). Bars = 50 μm.*

Nematode development on auxin-insensitive *Arabidopsis* mutants

To confirm the role of auxin in syncytium development, a range of auxin-insensitive *Arabidopsis* mutants (Table 1) was inoculated with the beet cyst nematode *H. schachtii* (*A. thaliana* is a non-host for *G. rostochiensis* (Sijmons *et al.*, 1991)). In contrast to *dgt*, lateral root formation was only slightly reduced in these mutants indicating a lower level of auxin-resistance. Only for *axr2*, nematode development was significantly reduced to 70% ± 0.4 (mean±SD) as compared to the wild type Columbia.

In *dgt* hypertrophy of the feeding cells was negligible as shown in Figure 2A. For the auxin resistant mutants, a similar - though less pronounced - reduction in the hypertrophy of the feeding cells was observed compared to syncytia induced on Columbia (Fig. 3A). For Columbia the hypertrophy of the feeding cells was 136±47 µm and 86±9 µm for the auxin insensitive mutants *axr1-3*, 43±23 µm for *axr1-12*, 101±53 µm for *axr2* (Fig. 3B), 101±28 µm for *axr4-2*, 99± 47 µm for *aux1-7/axr4-2* and 89±19 µm for the auxin influx carrier mutant *aux1-7*. Hypertrophy of the feeding cells was expressed as the mean±SD of the maximal increase in the diameter of the vascular bundle of infected roots compared with the diameter of the vascular bundle in uninfected roots.

Upon infection of *Arabidopsis* wild type roots (Columbia), lateral roots were formed at the infection site within 5 dpi (1.9±1.5 (mean±SD)). Since lateral root formation is stimulated by auxin, this phenomenon provides additional evidence for the role of auxin in feeding cell development. In the auxin insensitive mutants *axr4-2* and the double mutant *axr1-3/axr4-2* lateral root formation was completely absent at the infection site, whereas the number of lateral roots was halved in feeding sites induced in the mutants *axr1-3* (0.5±0.7), *axr1-12* (0.25±0.5), *axr2* (0.7±1.2), *axr4-1* (1.0±0.8), *aux1-7* (0.75± 0.7) and *aux1-7/axr4-2* (1.0±1.4).

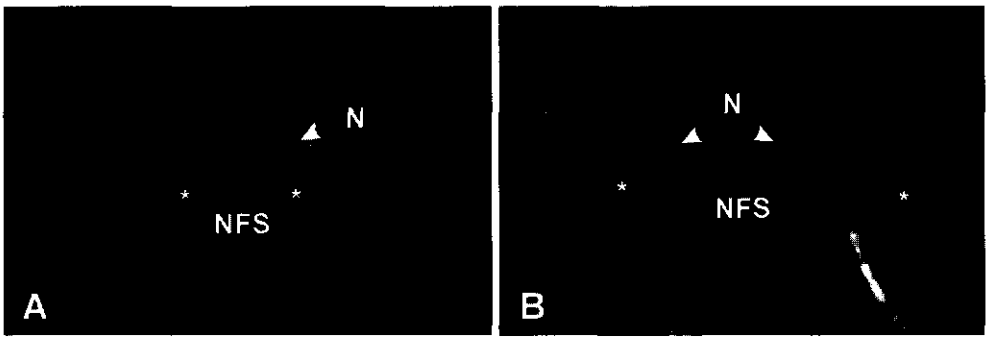

Fig. 3. In vitro *development of the beet cyst nematode* Heterodera schachtii *on* Arabidopsis thaliana *roots.* ***A.*** *Nematode development (N) on* A. thaliana *ecotype Columbia. The feeding sites are indicated with an asterisk.* ***B.*** *Nematode development on roots of the auxin-insensitive* Arabidopsis *mutant* axr2.

Transcriptional activation of an auxin-responsive promoter in early feeding cell development

To study local changes in the auxin balance upon beet cyst nematode infection the *Arabidopsis* promoter trap line 5-1E1 was used. This promoter responds specifically to auxin and is transcriptionally active in the root apex and in early stages of lateral root formation. No activation of this promoter is observed in response to other phytohormones like cytokinin, ethylene, ABA, methyl-jasmonic acid or GA3 (R. Offringa, unpublished results). In both infected and control roots, ß-glucuronidase (GUS) expression was observed in the root apex and lateral root primordia. Upon infection, transcriptional activation of the *gus* reporter gene was observed only during early feeding cell development (1 – 8 dpi) with strongest *gus* expression at 3.5 - 4.5 dpi (Fig. 4A). GUS staining

was specifically localized in the expanding syncytia and in cells surrounding the syncytia. The majority of these neighboring cells will be incorporated in the syncytium at a later stage of feeding cell development. Activation of the promoter was also observed in lateral root primordia formed upon infection (Fig. 4B and 4C). No GUS activity was detected in the cortex, epidermis or vascular bundle around this nematode infection site. Since *gus* expression was not induced by mechanical wounding of the roots (data not shown) or nematode migration, it is suggested that this early transient activation of the *gus* gene reflects a local increase of the auxin concentration, directly or indirectly induced by the infective juvenile.

Inhibition of polar auxin transport reduces feeding cell development

To investigate the role of polar auxin transport in feeding cell development, tomato seedlings were exposed to 1 μM of the phytotropin *N*-(1-naphthyl)phtalamic acid (NPA) and subsequently, roots were inoculated with the potato cyst nematode *G. rostochiensis*. In primary roots treated with NPA, feeding cell formation coincided with extreme galling and numerous swollen root airs (Fig. 5A). This phenomenon was not observed in uninfected NPA-treated roots (data not shown). A delay in nematode development was noticed and a reduction of about 60% in the number of adult females compared to untreated control roots (data not shown). Alternatively, the phytotropin triiodobenzoic acid (TIBA) was applied, but this inhibitor appeared to be toxic for potato cyst nematodes. The same holds true for 3,3',4',5,7-pentahydroxyflavone (quercetin), which belongs to a different class of auxin transport inhibitors, namely the flavonoids.

To investigate the effect of impaired polar auxin transport on feeding cell ontogeny, the abnormal feeding sites were sectioned. In the interaction tomato - *G. rostochiensis*, the initial syncytial cell is localized in the cortex and expansion involves the formation of a 'cortical bridge' towards the stele (Fig. 5B). NPA treatment hampers the formation of this cortex bridge. Especially when feeding cells are induced in the outer cortex, this radial expansion pattern of the syncytium is seriously restricted (Fig. 5C and 5D). Longitudinal expansion is apparently not affected by 1 μM NPA (data not shown). The gall structure around the feeding cells was the result of massive disordered cell divisions, loss of tissue structure, hypertrophy of the vascular bundle and metaxylem differentiation.

As an alternative to exogenous application of inhibitors, the *Arabidopsis* polar auxin transport mutants *aux1-7*, *pin1-1/ttg1* and *eir1-1* were tested. The first one shows a hampered influx of auxin into the cell, the latter two are involved in the efflux of auxin out of the cell. Only the auxin efflux carrier mutants *pin1-1/ttg1* and *eir1-1* resulted in a significant reduction of respectively 52% and 89% in nematode development (Fig. 6). The reduction in nematode development upon NPA treatment of tomato roots and upon infection of the *Arabidopsis* transport mutants *pin1-1/ttg1* and *eir1-1* indicate that polar auxin transport is involved in cyst nematode-induced feeding cell development. Unlike treatment with NPA, no abnormal feeding cell phenotypes were observed.

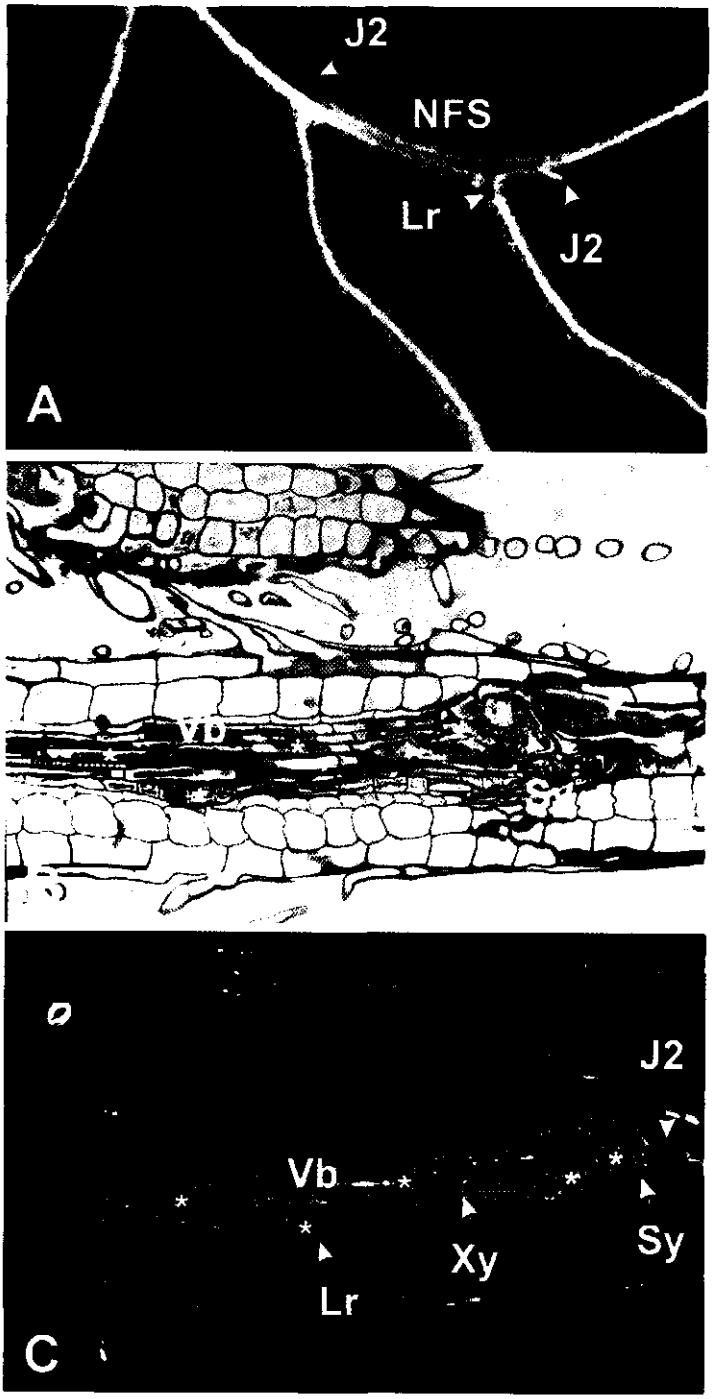
J2
NFS
Lr
J2
A
Vb
Sy
J2
Vb
Xy
Sy
Lr
C

Fig. 4. [←] *Transcriptional activation of the auxin-responsive promoter 5-1E1 in* Arabidopsis thaliana *roots upon infection with the beet cyst nematode* Heterodera schachtii. ***A.*** *The expression pattern of the* gus *reporter gene upon nematode infection at dpi = 4 (NFS = nematode feeding site; Lr = lateral root primordium).* ***B.*** *and* ***C.*** *Longitudinal sections showing GUS staining (asterisks) in the expanding syncytium (Sy) and in cells of the vascular bundle (Vb) that are about to be incorporated into the syncytium (Xy = xylem). In* ***B****, the section was stained with toluidine blue.* ***B*** *and* ***C.*** *were observed using respectively bright and dark field microscopy (320× magnification).*

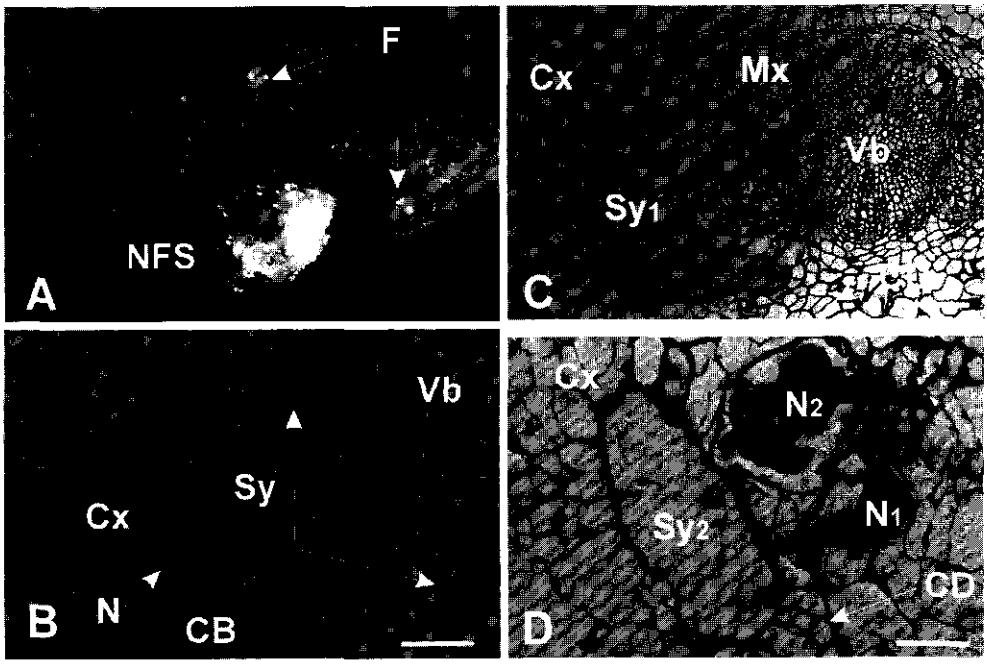

Fig. 5. [↑] *Effect of NPA treatment on potato cyst nematode* (G. rostochiensis) *development on tomato roots.* ***A.*** *Nematode feeding site (NFS) in NPA treated tomato roots (dpi = 8 weeks; F = young female).* ***B.*** *Cross section of a nematode-induced syncytium in untreated tomato roots (Sy; Cx = outer cortex; Vb = vascular bundle; CB = 'cortical bridge'; bar = 50 μm).* ***C.*** *and* ***D.*** *Cross sections of syncytia in NPA-treated tomato roots showing an aberrant morphology (N = nematode; Mx = metaxylem; CD = disordered cell division; bar C = 125 μm and bar D = 50 μm).*

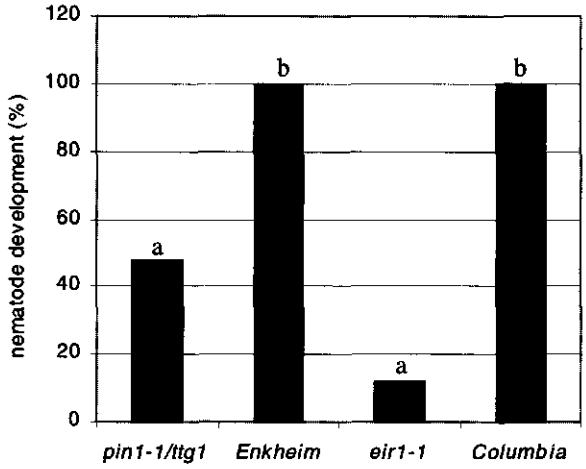

Fig. 6. *Development of the beet cyst nematode* Heterodera schachtii *on roots of the* Arabidopsis thaliana *transport mutants* pin1-1/ttg1 *and* eir1-1 *and the corresponding controls Enkheim and Columbia (dpi = 6 weeks). Infection was significantly reduced with respectively 52% (at $P < 0.05$) and 89% (at $P < 0.01$). Significant differences between the infection rates - indicated with a and b - were determined using a two-sample* t*-test ($\alpha = 0.05$).*

Table 1. *List of the* Arabidopsis thaliana *auxin and ethylene response mutants used for infection with the beet cyst nematode* Heterodera schachtii.

	Auxin	
Mutant	Ecotype	Reference
Axr1-3 *Axr1-12*	Col-0	Estelle and Sommerville, 1987
Axr2	Col-0	Wilson *et al.*, 1990
Axr4-1	Col-0	Hobbie and Estelle, 1995
Axr4-2	Col-0	Hobbie and Estelle, 1995
Axr1-3;axr4-2	Col-0	Hobbie and Estelle, 1995
Aux1-7	Col-0	Pickett *et al.*, 1990
Aux1-7;axr4-2	Col-0	Hobbie and Estelle, 1995
Pin1-1/ttg-1	Enkheim	Goto *et al.*, 1991

	ethylene	
mutant	Ecotype	Reference
Eir1-1	Col-0	Roman *et al.*, 1995
Eto1-1	Col-0	Guzman and Ecker, 1990
Eto2	Col-0	Kieber *et al.*, 1993
Eto3	Col-0	Kieber *et al.*, 1993

***Arabidopsis* ethylene-response mutants point at a role for ethylene in feeding cell development.**

The *Arabidopsis* mutant *eir1-1*, which encodes an auxin efflux carrier – is insensitive to ethylene (Roman *et al.*, 1995). In addition, auxin-inducible ethylene production is impaired in *dgt* (Kelly and Bradford, 1986). The same holds true for most of the auxin-insensitive *Arabidopsis* mutants (*e.g.* Wilson *et al.* 1990, Abel *et al.*, 1995). To investigate whether there could be a role for ethylene synthesis in feeding cell development, the dominant ethylene overproducing mutants *eto1*, *eto2* and *eto3* from *A. thaliana* were infected with the beet cyst nematode *H. schachtii* (Fig. 7A). The overproduction of ethylene by the host plant resulted in an acceleration of nematode development (data not shown) and hyperinfection of the roots; respectively 107± 32% for *eto1*, 174± 23% for *eto2* and 191± 55% for *eto3* compared to 100% infection on Columbia . Moreover, the adult females were larger in size as compared to the females grown on control roots (respectively a diameter of 422±71 µm for *eto1*, 442±87 µm for *eto2* and 452±60 µm for *eto3* versus 371±26 µm for Columbia). In cross-sections of infected *eto2* roots, the syncytia showed extensive cell wall dissolution and expansion. The remaining cell wall fragments were thickened (Fig. 7B and 7C). The enhanced cell wall breakdown in these ethylene mutants suggest that ethylene-induced cell wall degradation is involved in syncytium development.

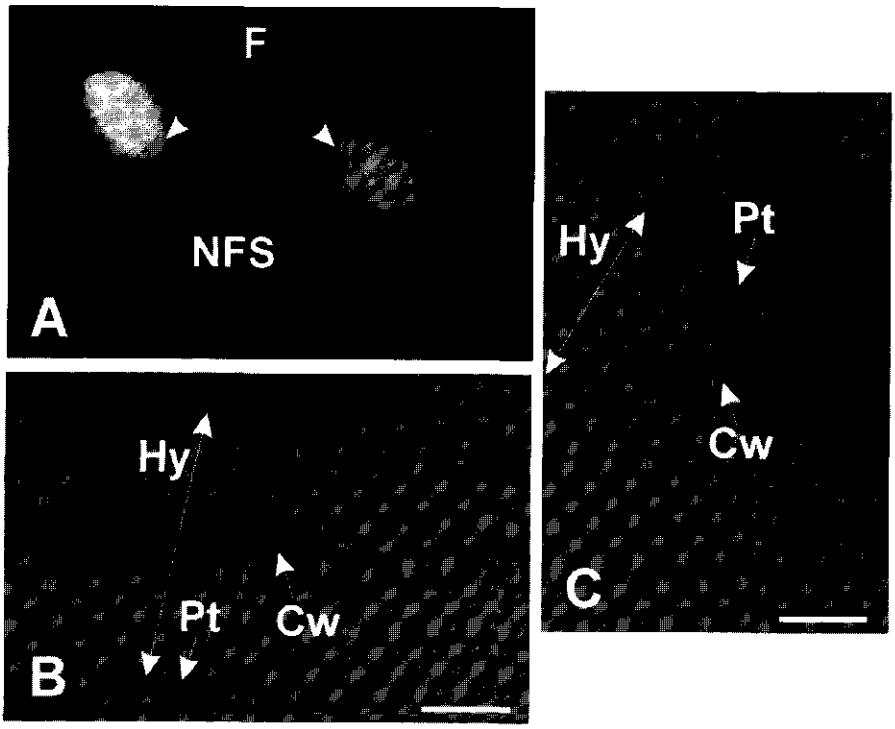

Fig. 7. *Development of the beet cyst nematode* Heterodera schachtii *on roots of the* Arabidopsis thaliana *ethylene response mutant* eto2. ***A.*** *Nematode development on roots of* eto2 *at dpi = 28 (NFS = nematode feeding site; F = females).* ***B.*** *Longitudinal section of a syncytium in* eto2 *showing increased cell wall degradation, cell wall deposition (CW) and hypertrophy of the vascular bundle (Hy; Pt = cell wall protuberances).* ***C.*** *Longitudinal section of a feeding cell induced in wild type roots of Columbia. Bars = 50 µm.*

DISCUSSION

Auxin-insensitive mutants from tomato and *Arabidopsis* were used to demonstrate the role of the phytohormone auxin in feeding cell development by two cyst nematode species. Whereas tomato is an excellent host for the potato cyst nematode *Globodera rostochiensis*, nematodes did not develop on roots of the extremely auxin-insensitive tomato mutant *diageotropica* (*dgt*). If lateral root formation is used as an indicator for auxin insensitivity, no equivalent for *dgt* could be found among the available *Arabidopsis* mutants. Nevertheless, a significant reduction in the number of developing beet cyst nematodes was observed in the highly auxin-insensitive mutant *axr2*. The auxin-responsive *Arabidopsis* promoter trap line 5-1E1 and polar auxin transport inhibitors were used to gather more information on the role of auxin in feeding cell development.

Auxin is involved in the early stages of feeding cell development

The failure of potato cyst nematodes to induce normal syncytia in the auxin-insensitive tomato mutant *dgt* points at a prominent role of auxin in early stages of feeding cell development. These results are supported by the experiments with a range of auxin-insensitive *Arabidopsis* mutants. The rapid and local accumulation of GUS in syncytia of the auxin-responsive promoter trap line 5-1E1 from *Arabidopsis* upon infection with *H. schachtii* suggests a local increase of the auxin concentration or an increased sensitivity towards this phytohormone. A local accumulation of auxin upon feeding cell induction is consistent with the substantial decrease in the mRNA levels of the auxin down-regulated genes *adr*-6, 11 and 12 after infection of soybean roots with *H. glycines* (Hermsmeier *et al.*, 1998). It is also consistent with the activation of the cell cycle genes *Arath;cycB1;1* and *cdc2a* in young feeding cells by *H. schachtii* (Niebel *et al.*, 1996), since both genes are known to be transcriptionally activated by auxin (Doerner *et al.*, 1996, John *et al.*, 1993).

The phytotropin *N*-(1-naphthyl)phtalamic acid (NPA) - a non-competitive auxin efflux inhibitor - severely hampered the directional expansion of the syncytium from the cortex to the stelar tissues and, as a consequence, nematodes developed poorly (60% reduction). It may be questioned why radial proliferation of the syncytium is more affected by NPA treatment than the longitudinal proliferation. Application of NPA disrupts existing auxin concentration gradients in the roots (Muday *et al.*, 1995; Ruegger *et al.*, 1997) and radial auxin gradients play a role in vascular pattern formation (Klee *et al.*, 1987, Uggla *et al.*, 1996) as well. Possibly, a radial auxin gradient is required to connect the initial syncytial cell to the vascular tissue. In addition, xylem differentiation was promoted only in the feeding sites and not in the uninfected tissues of tomato roots in the presence of a low NPA concentration (1 µM). A similar enhanced xylogenesis was observed in the inflorescence of the transport mutant *pin1* and in wild type *Arabidopsis* plants treated with a relatively high NPA concentration (15 µM) (Gälweiler *et al.*, 1998).

Nematode development was strongly reduced in the *Arabidopsis* polar auxin transport mutants *pin1-1/ttg1* (-52%) and *eir1-1* (-89%) supporting a role of polar auxin transport during feeding cell development. PIN1 was recently identified as a transmembrane component detected at the basal end of auxin transport-competent cells in vascular tissues both in shoots and roots. As such, PIN1 is likely to be part of the auxin efflux carrier complex (Gälweiler *et al.*, 1998). EIR1 (also known as PIN2) encodes a similar auxin carrier but this one is specifically expressed in the cortical and epidermal root cells (Luschnig *et al.*, 1998, Müller *et al.*, 1998).

A role for auxin-induced ethylene production

Intensive cross-talk between different hormones is a common phenomenon in plants. An example is the biosynthesis of ethylene, which is among others controlled by auxin (Yang and Hoffman, 1984). ACC synthase, a key enzyme of ethylene biosynthesis, is encoded by a multigene family which is subdivided into three groups (Oetiker *et al.* 1998). The expression of a subgroup - indicated as group III - is controlled by auxin. Hence, it is no surprise that the tomato mutant *dgt* shows a reduced expression of the ACC synthase genes ACS2, 3, 5, 6, and 7 (Abel and Theologis 1996; A. Theologis, pers. comm.). Similarly, the expression of ACS4 is reduced in the auxin-

insensitive *Arabidopsis* mutants *axr1-3, axr1-12, axr2* and *aux1-7* (Abel *et al.*, 1995). As such, a reduction in ethylene production could underlie to some degree the observed defects in syncytium development.

Remarkably, cyst nematodes flourish on the *Arabidopsis* ethylene overproducing mutants *eto1*, *eto2* and *eto3*. Hyperinfection of the roots and enhanced female development virtually correlated with the increasing levels of endogenous ethylene in the mutants *eto1*, *eto2* and *eto3*: respectively 40x (Guzmán and Ecker, 1990), 20x and 100x the wild type levels (Kieber *et al.*, 1993). Moreover, it was demonstrated that cell wall dissolution and deposit is enhanced in the presence of high endogenous ethylene concentrations. This apparently corresponds with the role of ethylene-mediated cell wall degradation during fruit ripening (Gray *et al.*, 1992, 1994, Metha and Mattoo, 1995). Hence, it is proposed that syncytium proliferation is mediated by ethylene which could activate cell wall degrading enzymes from the host plant itself.

Moreover, intensive cross-talk between auxin and ethylene is examplified by the *Arabidopsis* mutant *eir1-1*, which is insensitive to ethylene and to the phytotropins TIBA and NPA (Luschnig *et al.*, 1998). This ethylene responsive gene encodes for an auxin efflux carrier (EIR1/PIN2), which suggests that ethylene is involved in the regulation of polar auxin transport. This is supported by the observation that *eir1* acts downstream of *eto3* or in a pathway parallel to that in which *eto3* functions. In this paper, it is demonstrated that the efflux carrier EIR1/PIN2 is involved in feeding cell development and as such, it would be interesting to investigate the possible link between an increase in ethylene production and the regulation of polar auxin transport upon cyst nematode infection.

How do cyst nematodes increase the auxin concentration or auxin response?

The nematode produces and secretes IAA or IAA-like compounds. The infective juveniles themselves could produce and secrete auxin or a homologue of this phytohormone. The hypothesis that plant-parasitic nematodes disturb plant tissues by secreting auxins is relatively old. Goodey (1948) hypothesized that *'Anguillulina balsamophila'* (= *Anguina balsamophila*) caused galls in the leaves of its host by auxin or auxin-like secretions. Bird (1962) and Yu and Viglierchio (1964) used an auxin bio-assay to analyse extracts from root knot nematode-infected plant tissues. In addition, Johnson and Viglierchio (1969) used a similar approach to determine auxin activity in extracts of infective juveniles of the beet cyst nematode *H. schachtii*. In both cases the presence of auxin or auxin-like substances of nematode-origin was claimed. Using a monoclonal antibody directed against IAA and its precursor indole-3-acetamide, we could not confirm these findings. Despite the conjugation of free IAA to larger proteins using ethyl-3-(-3-dimethylaminopropyl)-carbodiimidehydrochloride (EDC) (Driss-Ecole and Perbal, 1987) and the detection of synthetic IAA on Western blot, no IAA could be detected in the homogenates of about 2000 pre-parasitic second stage juveniles of *G. rostochiensis* (data not shown). This suggests that auxin is present at an extremely low concentration or that auxin is not produced by infective juveniles.

The nematode induces the accumulation of auxin. A local increase in the auxin concentration could also be realized by stimulating the influx or inhibiting the efflux in a particular cell. Unlike *e.g.* cytokinins and abscisic acid which are transported mainly via the xylem and the phloem vessels, polar auxin transport occurs in cell files and this particularity makes auxin accumulation by local transport inhibition a practicable option.

Though auxin can be synthesized in the roots (Müller *et al.* 1998), the shoot apex is considered to be the main site of IAA production (*e.g.* Morris *et al.* 1969) and transport from the shoot towards the root tips occurs within the vascular bundle (acropetal transport). From the root tip auxin is transported upwards via cell files in the epidermis or the outer cortex (basipetal transport). Considering an individual plant cell, auxin can enter this cell freely in its protonated form. Alternatively, auxin can be taken up by an influx carrier. Efflux carriers present in the opposite plasma membrane mediate IAA export out of the cell (reviewed by Estelle, 1996 and Leyser, 1999).

Nodule formation by rhizobia in roots of legumes is accompanied by a local increase in the auxin concentration (Mathesius *et al.*, 1998a). Recently, it is determined that flavonoids, which are able to inhibit polar auxin transport in the roots by blocking the auxin efflux carriers, accumulate upon nodule formation. Hence, it is suggested that this is causing a local increase of the auxin concentration. In addition, it is proposed that flavonoids and flavonoid glycosides could modulate auxin levels by changing the turnover of auxin (Mathesius *et al.*, 1998b). In addition, it is observed that gall formation by root knot nematodes is associated with the activation of the flavonoid pathway (Hutangura *et al.*, 1999). It has to be investigated whether cyst nematodes use a similar strategy to manipulate the local auxin balance in order to activate auxin signaling pathways involved in feeding cell development.

The nematode stimulates the response to auxin. Recently, evidence is provided that infective juveniles of the potato cyst nematode secrete a mitogenic peptide(s) which co-stimulate(s) the proliferation of tobacco leaf protoplasts in the presence of auxin and cytokinin (Goverse *et al.*, 1999). Naturally-induced secretions did not affect the auxin or cytokinin concentration nor the sensitivity towards these phytohormones. Whether nematode secretions are involved in stimulating the auxin response in early feeding cell development needs further investigations.

Here, we demonstrate that a molecular genetic approach using phytohormone mutants is a powerful tool to identify factors that mediate the development of highly specialized nematode feeding cells. The most straightforward explanation of the results obtained in this study would be that syncytium development is accomplished by a local increase in the experienced auxin concentration directly or indirectly caused by the infective juvenile. Apart from other effects, this increase would result in an auxin-mediated increased ethylene production. In the interaction *Arabidopsis* - *H. schachtii* ethylene overproduction stimulated cell wall degradation and syncytium proliferation. Presumably, this observation holds true for other plant-nematode interactions as well.

MATERIALS AND METHODS

Plant materials

In vitro cultures of the homozygous tomato mutant *diageotropica* (*dgt/dgt*), which was kindly provided by M. Koornneef (Laboratory of Genetics, Wageningen University, the Netherlands), and its corresponding background *Lycopersicon esculentum* cv Money Maker were used. Seeds were surface-sterilized with 0.8% (w/v) commercial bleach for 25 min. Seeds were rinsed three times with an excess of sterile water and transferred to agar plates containing Murashige and Skoog culture medium supplemented with 2% sucrose (MS20). Plants were grown and maintained at 24°C with 16 hours of light. For nematode infection, stem cuttings were cultured on 1.5% agar plates (Difco Laboratories, Detroit, USA) prepared with Gamborg's B5 medium (including vitamins and minerals, pH 6.2) supplemented with 2% sucrose. For *in vivo* experiments, individual *dgt* plants were grown in 600 ml pots filled with sandy loam soil at 18°C with 16 hours of light.

the *Arabidopsis thaliana* mutants used in this study were kindly provided by the Arabidopsis Biological Resource Center (Columbus OH, USA). Ecotype *Lansberg erecta (Ler-1)*, *Columbia* (Col-0) and *Enkheim* (En-2) were included as corresponding genetic background for individual mutants. *Arabidopsis* seeds of the hemizygous auxin-responsive promoter trap line 5-1E1::GUS, with a single T-DNA insertion, was kindly provided by R. Offringa (Institute for Molecular Plant Sciences, Leiden University, The Netherlands). GUS expression was induced by auxin and not by cytokinin, ethylene, ABA, methyl-jasmonic acid or GA3 (R. Offringa, unpublished results). Seeds were surface-sterilized with 5% (w/v) hypochloride mixed 4:1 with absolute ethanol for 10 min, rinsed two times with absolute ethanol and dried overnight in the flow chamber. Seeds were transferred to plates with modified Knop medium as described by Sijmons *et al.* (1991). Plates were slightly lifted to allow roots to grow downwards. Growth conditions were 22°C with 16 hours of light.

Cyst nematode inoculation

In vitro cultures of tomato were inoculated with pre-parasitic second stage juveniles (J2) of the potato cyst nematode *Globodera rostochiensis* Rol Mierenbos. Surface-sterilized pre-parasitic J2 were obtained from dry cysts according to Heungens *et al.* (1996). Hatching was promoted by filter-sterile potato root diffusate (Clarke and Perry, 1977). After 3-5 days, J2 were collected on a 5 µm sieve and surface-sterilized using the following disinfectants: 0.5% (w/v) streptomycin sulphate-penicillin G (20 min), 0.1% (w/v) ampicillin-gentamycin (20 min), 5 min sterile tap-water and 0.1% (v/v) chlorhexidin-digluconate (3 min). After washing in sterile tap-water J2 were suspended and approximately 50 individuals were transferred to 8 - 10 days old root tips using a pipetman with siliconized tips (Costar Corporation, Cambridge MA, USA). When roots were grown under the agar, inoculation of the juveniles was facilitated by perforation of the agar with a sterile glass pipet. Inoculated plates were placed at 18°C in the dark. For nematode infections in pots, pre-parasitic J2 were obtained from dry cysts of *G. rostochiensis* Rol Mierenbos (De Boer *et al.*, 1992; Roosien *et al.*, 1993). Tomato plants of 39 days old were inoculated with 3500 pre-parasitic J2 and grown at 18°C with 16 hours of light.

In vitro cultures of 14 days old *Arabidopsis* plants were inoculated with sterile pre-parasitic J2 of the beet cyst nematode *Heterodera schachtii*. Cysts, obtained from monoxenic cultures on mustard (*Sinapsis alba*) kindly provided by F. Grundler (Institute for Phytopathology, Kiel University, Germany), were incubated on a 100 µm sieve in 3 mM $ZnCl_2$ for 5-7 days at 25°C in order to hatch J2 (Grundler, 1989). They were rinsed in sterile tap-water and 40 J2 were used for inoculation as described for *in vitro* cultures of potato cyst nematodes. Plates were sealed with Nesco film (Carl Roth GmbH & Co, Karlsruhe, Germany) and incubated in a slightly lifted position at 22°C with 16 hours of light.

Inhibition of polar auxin transport

Surface-sterilized seeds of the tomato cv Money Maker were germinated on plates with MS20 culture medium, which were positioned vertically to allow roots to grow onto the agar surface. After 10 days, 3 seedlings were transferred to a single plate with Gamborg's B5 medium supplemented with 2% sucrose and 1µM *N*-(1-naphthyl)phtalamic acid (NPA, Duchefa, Haarlem The Netherlands), which inhibits polar auxin transport. After one day, roots were inoculated with surface-sterilized pre-parasitic J2 of *G. rostochiensis* as described previously. Uninfected plants were used to observe the plant response to 1µM NPA, whereas a plate omitting 1µM NPA was used for inoculation to observe normal feeding cell development. This experiment was replicated two times. Feeding cell and nematode development were observed using a dissecting microscope (Leica, Wetzlar, Germany).

Histochemical GUS assay

Feeding cell development and the parasitic stage of the nematode were determined prior to tissue preparation using an inverted light microscope (Leica, Wetzlar, Germany). For each developmental stage (4 - 14 - 24 and 36 hours post inoculation and 3 - 5 - 8 and 15 days post inoculation) five to ten plants were examined. Histochemical GUS assay was performed according to Barthels *et al.* (1997) and GUS staining was observed using bright field and dark field microscopy (Leica, Wetzlar, Germany and Axioskop FS, Zeiss, Oberkochen, Germany).

Histology of nematode feeding cells

Infected roots of tomato and *Arabidopsis thaliana* were fixed in 4% freshly prepared formaldehyde supplemented with 0.25% glutaraldehyde in phosphate/citrate buffer pH 7.4 using a short vacuum infiltration step. Tissues were embedded in LR White resin according to the manufacturer (Aurion BV., Wageningen, The Netherlands) to prepare semi-thin sections on glass slides. Sections were stained with 1% toluidine blue and morphology of the feeding cells was observed using light microscopy (Leica, Wetzlar, Germany).

CHAPTER 6

General discussion

Cell cycle activation by plant-parasitic nematodes

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ABSTRACT

Sedentary nematodes are important pests of crop plants. They are biotrophic parasites that can induce the (re)differentiation of either differentiated or undifferentiated plant cells into specialised feeding cells. This (re)differentiation includes the reactivation of the cell cycle in specific plant cells finally resulting in a transfer cell-like feeding site. For growth and development the nematodes fully depend on these cells. The mechanisms underlying the intriguing ability of these nematodes to manipulate a plant for its own benefit are unknown. Nematode secretions are thought to play a key role both in plant penetration and feeding cell induction. Research on plant-nematode interactions is hampered by the minute size of cyst and root knot nematodes, their obligatory biotrophic nature and their relatively long life cycle. Recently, insights in cell cycle control in *Arabidopsis thaliana* in combination with reporter gene technologies showed the differential activation of cell cycle gene promoters upon infection with cyst or root knot nematodes. In this review, we integrate the current views of plant cell fate manipulation by these sedentary nematodes and made an inventory about possible links between cell cycle activation and local, nematode-induced changes in auxin levels.

INTRODUCTION

Virtually without exception sediment and soil ecosystems are inhabited by tremendous numbers of nematodes. The molecular diversity among the members of the phylum Nematoda is much larger as compared to their morphological diversity: most of them look alike. In general, nematodes are relatively small (< 2 mm in length), transparent and vermiform. Co-evolution between nematodes on the one hand and bacteria, fungi and plants on the other hand resulted in bacterial feeders such as the well-known *Caenorhabditis elegans*, in fungivorous nematode species and in obligatory plant parasites. Though representing a small minority within this huge phylum, the plant-parasitic nematodes receive ample attention, mainly because they are a major yield limiting factor in crops such as potato, beet, soybean and tomato.

When obligatory plant-parasitic nematodes are considered, a number of different strategies can be discriminated. One could be indicated as the hit-and-run strategy, this approach is employed by *e.g. Trichodorus* species. This nematode uses a stylet to penetrate the cell wall of the rhizodermis and to ingest the cell content. Subsequently another cell - not necessarily from the same plant - is visited. A more lasting strategy is employed by so-called endoparasites. These nematodes enter the plant and induce the formation of a feeding site. Once a feeding site is induced, the nematode fully depends on it for growth and development. If the feeding site becomes non-functional for whatever reason, the nematode by which the syncytium was induced will die. This durable strategy is successful: endoparasites invade a wide range of plant species and in agriculture they reside among the most persistent and harmful nematodes.

Cyst and root knot nematodes

Both cyst and root knot nematodes are endoparasites. The first group includes members of the genera *Globodera* and *Heterodera*. (In)famous are the two potato cyst nematode species *Globodera rostochiensis* and *G. pallida*, the beet cyst nematode *Heterodera schachtii* and the soybean cyst nematode *Heterodera glycines*. The second group consists of members of the genus *Meloidogyne*, probably the most successful plant-parasitic nematodes worldwide. Contrary to the cyst nematodes that have a narrow host range root knot nematodes such as *Meloidogyne incognita, M. javanica* and *M. arenaria* are highly polyphagous.Their relatively short generation time – less than 2 months - and their ability to multiply via parthenogenisis allows these species to rapidly build up high population levels.

The life cycles of cyst and root knot nematodes have a number of aspects in common. In both instances the second stage juvenile (J2) hatches from the egg, migrates through the soil in search for a suitable host plant. Root knot nematodes always penetrate the root just above the root

tip. Though cyst nematodes as well have a preference for this part of the root, they are more flexible in this respect. During this process the juveniles secrete cell wall-degrading enzymes such as β-1,4- endoglucanases which are produced in the oesophageal glands (Smant *et al.*, 1998; Rosso *et al.*, 1999). Hence, entering the root involves a combination of mechanic piercing by the stylet and enzymatic softening. After having entered a rhizodermis cell the root knot nematode migrates intercellularly whereas the J2 of a cyst nematode migrates intracellularly on its way to the vascular cylinder. Migration stops at the moment a cell is encountered that is suitable as a starting point for feeding site formation.

Regardless of the nematode - host plant combination, the mechanisms of feeding site induction are similar among cyst and among root knot nematodes. However, between the two groups the genesis of the feeding sites differs. Root-knot nematodes induce several giant cells embedded in a gall whereas cyst nematodes generate a syncytium, which can include up to 200 cells (Jones, 1981). The growing root knot nematode switches from giant cell to giant cell whereas the cyst nematodes feeds from one, single syncytium. Though the origins are different, both kinds of large, multinucleate feeding cells are functionally similar. Little is known about the mechanism underlying feeding site induction; secreted signaling molecules from the pre-parasitic juveniles are thought to mediate giant cell and syncytium development (Hussey, 1989; Williamson and Hussey, 1996).

The nematode-exploited plant cells are metabolically highly active and adapted to withdraw large quantities of nutrient solutions from the vascular system of the host plant. This function is reflected in the ultrastructure of the hypertrophied feeding cells: cell wall ingrowths adjacent to the xylem, breakdown of the large vacuole, dense granular cytoplasm with many organelles and numerous enlarged amoeboid nuclei (Bird, 1961; Jones and Northcote, 1972; Jones, 1981). Under optimal conditions, giant cell expansion can result in a final size of 600-800 µm long and 100-200 µm in diameter (Jones and Payne, 1978).

Once a J2 has successfully induced a feeding site the juvenile starts growing. After three moults the shape of the adult female has changed from vermiform to - depending on the species – saccate, lemon-shaped or spherical. Cyst nematodes are obligatory sexual and eggs will only be produced upon fertilization by the (again) vermiform and mobile males. Though sexual reproduction may occur in some *Meloidogyne* species present in the temperate regions, a number of important pathogens such as *M. incognita*, *M. javanica* and *M. arenaria* reproduce via mitotic parthenogenesis. Within the egg, the first stage juvenile moults and the resulting J2 will hatch under favorable conditions.

Positional cues are involved in feeding cell development

Both cyst and root knot nematodes select certain plant cells to initiate a feeding site. It is unknown what makes a given plant cell suitable to be an initial syncytial or giant cell. Nematodes could recognise cell types and differentiation status (Scheres *et al.*, 1997). Alternatively, nematodes could sense the response of cells to secreted signaling molecules. Only properly responding cells would be candidates as a starting point for feeding site induction. Though under natural conditions nematodes start feeding on roots only, feeding site induction is not bound to this organ. Under artificial conditions both root knot and cyst nematodes are able to induce feeding cells in hypocotyls and leaves as well (Linford, 1941; Powell and Moore, 1961; Bird, 1962; Sijmons *et al.*, 1991; A. Goverse, unpublished observations). Hence, feeding cell induction and development involve a rather general mechanism, which does not depend on root specific factors.

For giant cell induction root knot nematodes select 2-12 parenchymatic xylem cells located in the differentiation zone of the root (Christie, 1936; Guida *et al.*, 1991; Wyss, 1992). In contrast, cyst nematodes induce their syncytium in various tissues and the cell type is strictly depending on the plant-nematode relationship. The oat cyst nematode (*H. avenae*) and the beet cyst nematode (*H. schachtii)* usually select a pericycle or procambium cell as the starting point for feeding cell induction (Grymaszewska and Golinowski, 1991; Magnusson and Golinowski, 1991; Sijmons *et*

al., 1994a). On the other hand, a fully differentiated cortex cell is selected by the potato cyst nematode *G. rostochiensis* (Cole and Howard, 1958; Sembdner, 1963; Jones and Northcote, 1972; Rice *et al.*, 1985; A. Goverse, unpublished results). For the soybean cyst nematode *H. glycines*, the selection of the initial syncytial cell seems to be less critical and syncytia are induced in either cortex, endodermis, pericycle or phloem cells (Endo, 1964; 1991). Thus, both differentiated and undifferentiated cells are responsive to the infective juvenile resulting in the completion of the same morphogenetic program.

Irrespective of the cell type of the initial cell, syncytium induction starts close to one of the protoxylem poles (Endo, 1986; Golinowski *et al.*, 1996; A. Goverse, unpublished results). Remarkably, lateral root initiation in the pericycle as well as the formation of rhizobia nodule primordia occur at the same position (McCully, 1975; Peterson and Peterson, 1986 and Torrey, 1986; Libbenga and Harkes, 1973, respectively). This could imply that similar positional information or developmental cues are required in nematode syncytium, lateral root and nodule formation. From the stele of pea roots, a factor was isolated which enhances hormone-induced cell proliferation in the root cortex opposite the protoxylem ridges. This factor was identified as uridine (Smit *et al.*, 1995). Uridine could enhance the sensitivity of plant cells to auxin. Ethylene, in pea shown to be produced in the cell layers opposite to the phloem, is a potent inhibitor of cortical cell division and as such this phytohormone is a negative acting factor in the control of Nod factor-induced cell division (Heidstra *et al.*, 1997). It is not known whether uridine and ethylene play similar roles in the positioning of the feeding cell by cyst or root knot nematodes.

Nuclear changes accompanying feeding cell development

Genome multiplication

Nematode feeding cells share structural and functional similarities with rapidly growing nutritional organs of plants such as the tapetum and endosperm (Gheysen *et al.*, 1997). The multinucleate nature of these tissues is generated by incomplete cell cycles. Genome multiplication supports accelerated cell growth and an increase in cell size in these organs (Brodsky and Uryvaeva, 1977). Roughly, two mechanisms resulting in genome multiplication can be distinguished: polyploidizing mitosis and polytenization (D'Amato, 1984; Brodsky and Uryvaeva, 1985). Polyploidizing mitosis involves mitosis without cell division. Several mechanisms may underlie polyploidizing mitosis, a predominant one - acytokinetic mitosis - is characterized by the absence of cell plate formation or its incompleteness. A first acytokinetic mitosis will result in a binucleate cell. In a number of plant species, the multinucleate nature of tapetal cells is a result of one or more cycles of acytokinetic mitosis (Malallah *et al.*, 1996). Polytenization is established by endoreduplication *i.e.* the (repeated) doubling of the DNA strands in the interphase nucleus without their subsequent spiralization and division, leaving the chromosome number unchanged. In this way polytenization in the antipodal nuclei of wheat (*Triticum aestivum* L.) is realized (Wedzony, 1993). The number and size of the chromocenters are good measures to distinguish polyploid from polytene nuclei. In polyploid nuclei the number of chromosomes increases, whereas in polytene nuclei only the size of the chromocenters enlarges. However, chromocenters sometimes fuse resulting in fewer and larger chromocenters (Kabir and Singh, 1989).

Nematode-induced nuclear changes in plant cells

Plant parasitism by nematodes is often accompanied by nuclear changes. Though best studied in giant cell and syncytium formation by root knot and cyst nematodes, these changes are not restricted to these endoparasites. In several host plants, members of the ectoparasite family *Xiphinema* induce the formation of multinucleate cells by repeated mitosis without cell division. This is observed for *e.g. Ficus carica* roots infected by *X. index* (Wyss *et al.*, 1980) and for root-tip galls of strawberry and ryegrass parasitized by *X. diversicaudatum* (Griffiths and Robertson, 1988). Animal parasitic nematodes can manipulate their host in a similar way as the trichurid nematode

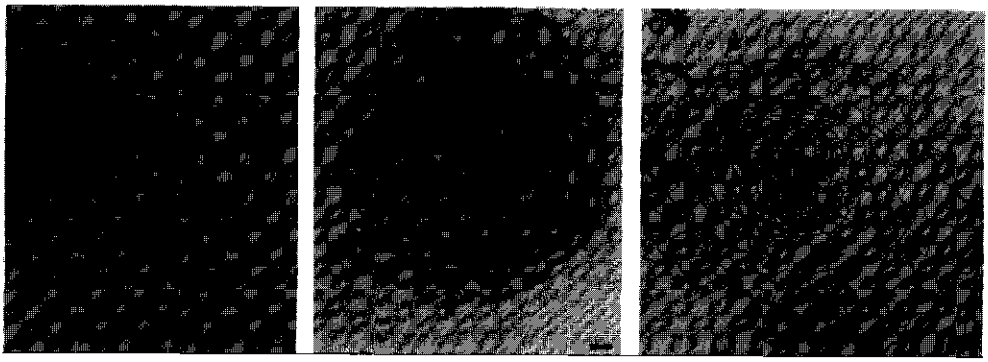

Fig. 1. *Cross sections of uninfected and nematode infected* Arabidopsis thaliana *roots. Root pieces were embedded in methacrylate medium, thin sectioned and stained with toluidine blue.* ***A.*** *Uninfected root.* ***B.*** *Syncytium induced by the cyst nematode* Heterodera schachtii *(3 days after infection).* ***C.*** *Gall induced by the root-knot nematode* Meloidogyne incognita *(3 days after infection). Abbreviations and symbols: N = nematode; S = syncytium; * = giant cells; bars = 12.5 μm.*

Capillaria hepatica induces multinucleate food cells in the liver of its mouse host (Wright, 1974). Hypertrophy of nuclei is a more commonly nematode-induced nuclear change in plant cells. It is a typical reaction to feeding in *e.g.* broad bean roots infected by *Pratylenchus penetrans* (Vovlas and Troccoli, 1990), roots of *Pinus clausa* parasitized by *Trophotylenchus floridensis* (Cohn and Kaplan, 1983) and parenchyma tissue of the vascular cylinder of redwood tree roots (*Sequoia sempervirens*) infected by *Gracilacus hamicaudata* (Cid del Prado and Maggenti, 1988). These data are merely based on histological data and only in case of giant cells and syncytia more details about the mechanisms underlying the nuclear changes are known.

Giant cell development is characterized by a rapid increase in the number of nuclei during the second parasitic life stage of the nematode (Starr, 1993; Paulson and Webster, 1970; Bird, 1973; Wiggers *et al.*, 1990; de Almeida Engler *et al.*, 1999). This observation was confirmed by the detection of ^{3}H-thymidine incorporation during DNA synthesis up to about 10 days post infection (Rubinstein and Owens, 1964; Rohde and Mc Clure, 1975; de Almeida Engler *et al.*, 1999). Acytokinetic mitosis results in 30-60 nuclei per individual giant cell, and in exceptional cases as many as 150 nuclei can be observed (*Meloidogyne incognita* on soybean (Dropkin and Nelson, 1960). In later stages of giant cell development polyploidizing mitosis is no longer observed. The continuation of DNA synthesis, which was demonstrated by Wiggers *et al.* (1990) in older infections in pea and enlargement of giant cell nuclei seen in older feeding cells of *Arabidopsis*, could be the result of DNA endoreduplication (de Almeida Engler *et al.*, 1999).

Though mitosis is observed in cells in the immediate vicinity of developing syncytia, extensive cytological observations indicated that it does not occur within syncytia themselves (Sembdner, 1963; Endo, 1964). The presence of enlarged nuclei both in the syncytium and in the neighbouring cells suggests DNA synthesis. This was confirmed by the incorporation of ^{3}H-thymidine in soybean roots infected by *Heterodera glycines* (Endo, 1971) and in *A. thaliana* roots infected by *Heterodera schachtii* (Niebel *et al.*, 1996; de Almeida Engler *et al.*, 1999). It is noted that - as compared to giant cells - the level of DNA synthesis is low in developing syncytia. In later stages ^{3}H-thymidine incorporation was mainly detected in the cells immediatelly surrounding the syncytium. This is confirmed by microscopic observations revealing enlarged nuclei and cell division activity in cells which are about to be incorporated in the syncytium

(Magnusson and Golinowski, 1991; Golinowski *et al.*, 1996; de Almeida Engler *et al.*, 1999; A. Goverse, unpublished results).

Interestingly, all interphase nuclei of giant cells and several nuclei of young syncytia induced in *Arabidopsis thaliana* contained more than the expected number of 10 chromocenters (de Almeida Engler *et al.*, 1999). This phenomenon is being investigated in more detail using confocal microscopy.

Cell cycle gene expression in nematode feeding sites

To compare nuclear changes in giant cells and syncytia at the molecular level, the transcriptional regulation of four cell cycle marker genes has been studied in early feeding cell development upon infection of the model host *Arabidopsis thaliana* with the beet cyst nematode *Heterodera schachtii* and with the root knot nematode *Meloidogyne incognita*.To monitor the manipulation of the cell cycle genes by these plant parasites, the expression patterns of two cyclin-dependent kinases (CDKs) (*cdc2aAt* and *cdc2bAt*) and two mitotic cyclins (*Arath;cycB1;1* and *Arath;cycA2;1*) were analysed.

In principle three reporter systems were available to monitor cell cycle gene expression upon nematode infection. Expression could be visualized by promoter-*gus* (coding region of β-glucuronidase), promoter-*gfp* (encoding the green fluorescent protein from *Aequorea victoria*) and promoter-*luc* (coding for firefly luciferase) fusions. Most results reviewed here are obtained by the detection of GUS activity. Attempts to monitor cell cycle gene expression by GFP failed as only very strong promoters can be followed *in planta* using the currently available *gfp* variants (M. Karimi, J. Verhees and A. Goverse, personal communication). The *luc* reporter gene is attractive because it encodes - contrary to *gfp* - an enzyme and it allows *in vivo* monitoring. Moreover, the instability of the firefly luciferase enzyme enables us to observe downregulation of gene expression as well. In this chapter, the suitability of this system will be illustrated.

The genes *cdc2aAt* and *cdc2bAt* encode two structurally and functionally distinct CDKs in *Arabidopsis* (Ferreira *et al.*, 1991; Imajuku *et al.*, 1992; Segers *et al.*, 1996; 1997). The *cdc2a* gene is constitutively expressed throughout the cell cycle, whereas the *cdc2b* gene is preferentially expressed during the S and G_2 phases. Expression of both genes is associated with actively dividing cells such as the root tip meristem and lateral root initials and with cells being competent to divide (Martinez *et al.*, 1992; Hemerly *et al.*, 1993; Segers *et al.*, 1996). The latter is illustrated by the fact that both *cdc2a* and *cdc2b* are expressed in the root pericycle (Hemerly *et al.*, 1993).

The expression of the cyclin genes *cycA2;1* and *cycB1;1* is associated with actively dividing cells (Ferreira *et al.*, 1994a). *cycA2;1* is also expressed in cells with competence to divide (Burssens *et al.*, 1998). Transcriptional activity of the *cycA2;1* gene starts in the S phase and reaches a maximum at the end of the G_2 phase. The expression of the *cycB1;1* gene is restricted to the late G_2 and M phase of the cell cycle with a maximum at the G_2-to-M transition (Shaul *et al.*, 1996; Mironov *et al.*, 1999). Normally, transcription of *cycB1;1* in the vegetative plant parts will be limited to the shoot and root meristem and the lateral root tips (Ferreira *et al.*, 1994b; Segers *et al.*, 1996).

Cell cycle activation by plant-parasitic nematodes

As mentioned above, in many plant-nematode interactions, parasitism is accompanied by enlargement of the plant nuclei. Based on these observations, one could hypothesize that cell cycle reactivation is a rather common strategy among obligatory plant-parasitic nematodes. Nucleus enlargement is not only induced by nematodes that start a relatively long lasting relationship with their host, but also by nematodes with a 'hit-and-run' strategy. The ectoparasite *Xiphinema diversicaudatum* was studied in more detail and both *cdc2a* and the *cycB1;1* were transcriptionally activated in *A. thaliana* roots (W. Robertson and L. Robertson, personal communication). In the following overview we will limit ourselves to endoparasites such as cyst and root knot nematodes.

Only for these families more information about cell cycle manipulation as an essential element of feeding cell development is available.

Both in cyst and root knot nematode-infected roots, strong expression of the four cell cycle genes was observed in the feeding cells within the first hours of parasitism (Niebel *et al.*, 1996; de Almeida Engler *et al.*, 1999). It is suggested that this rapid reactivation of the cell cycle is induced by an initial stimulus of the nematode. Remarkably, not only the proliferating giant cell or syncytium is affected by the presence of the pre-parasitic juvenile. Transcriptional activation of the *cdc2a*, *cdc2b* and *cycA2;1* promoter was also detected in non-dividing cells surrounding the proliferating giant cell or syncytium indicating their competence for mitotic stimulation.

In giant cells, the early and strong expression of the mitotic cyclin genes *cycA2;1* and *cycB1;1* is in accordance with the cytological observations of repeated mitosis caused by the root knot nematode. Interestingly, differential expression patterns in a gall demonstrate that distinct giant cells of the same gall are in different phases of the cell cycle (de Almeida Engler *et al.*, 1999). This phenomenon was previously suggested by Bird (1961) and Rubinstein and Owens (1964). Acytokinetic mitosis is restricted to the initial phases of giant cell formation and at 9 days post inoculation (dpi), expression of these cyclins could not be detected anymore in *A. thaliana* giant cells (de Almeida Engler *et al.*, 1999). From that point onwards mitotic figures were absent and no ^{3}H-thymidine incorporation was observed indicating that - as far as DNA duplication is concerned - giant cell development is completed. Consistently, acytokinetic mitosis rarely occurs in mature giant cells of pea, tomato, lettuce and broad bean (Starr, 1993).

Also in syncytia, *cycA2;1* and *cycB1;1* are strongly expressed during early syncytium development (up to dpi = 5). The expression of the mitotic cyclin gene *cycB1;1* indicates that the cell cycle progresses at least till late G_2. Considering that microscopical observations suggest that no mitosis occurs within the cyst nematode-induced feeding cell (Sembdner, 1963; Endo, 1964), it has been proposed that cyst nematodes induce cycles of DNA endoreduplication (G_1-S-G_2) shunting the M phase (Niebel *et al.*, 1996). Alternatively, it can not be excluded that an initial mitotic stimulation occurs upon cyst nematode infection, as proposed by Piegat and Wilski (1963).

In later stages of syncytium development, it is demonstrated that expression of *cycB1;1* and *cycA2;1* occurs primarily in cells surrounding the feeding cell. This is consistent with the cytological observations of mitotic activity in cells prior to syncytium incorporation (Magnusson and Golinowski, 1991; Golinowski *et al.*, 1996) and ^{3}H-thymidine incorporation in these cells (Endo, 1971; Niebel *et al.*, 1996; de Almeida Engler *et al.*, 1999). In contrast to giant cell development, expression of these cyclin genes at dpi = 9 indicates that the syncytium is still expanding and has not reached its maximum size.

Is cell cycle activation an essential element in feeding site induction?

Knowing that feeding site formation by cyst and root knot nematodes is accompanied by cell cycle activation, it may be questioned whether this is a coincidental side-effect or an essential part of plant parasitism by these nematodes. To answer this question de Almeida Engler *et al.* (1999) applied the cell cycle inhibitors oryzalin and hydroxyurea to *M. incognita* and *H. schachtii* - infected *A. thaliana* roots. The herbicide oryzalin inhibits plant microtubule polymerization and arrests cells at the early M phase (Morejohn *et al.*, 1987), whereas hydroxyurea is a cytostatic drug acting as a specific inhibitor of DNA synthesis (Young and Hodas, 1964). Control experiments showed that high concentrations of hydroxyurea or oryzalin were not harmfull for the nematodes themselves (Glazer and Orion, 1984; Orum *et al.*, 1979; de Almeida Engler *et al.*, 1999). In the past, several agrochemicals with cell cycle inhibiting properties have been tested for nematode control under field conditions (Davide and Triantaphyllou, 1968; Gershon, 1970; Orion and Minz, 1971; Romney *et al.*, 1974; Griffin and Anderson, 1979), but hardly any cytological data were given and consequently no firm statement can be made about their effect on feeding cell ontogeny.

Upon hydroxyurea treatment, early giant cell and syncytium development was blocked in *Arabidopsis* (de Almeida Engler *et al.*, 1999). This demonstrates that genome multiplication is essential for the formation of both types of feeding cells. Application of hydroxyurea at later stages

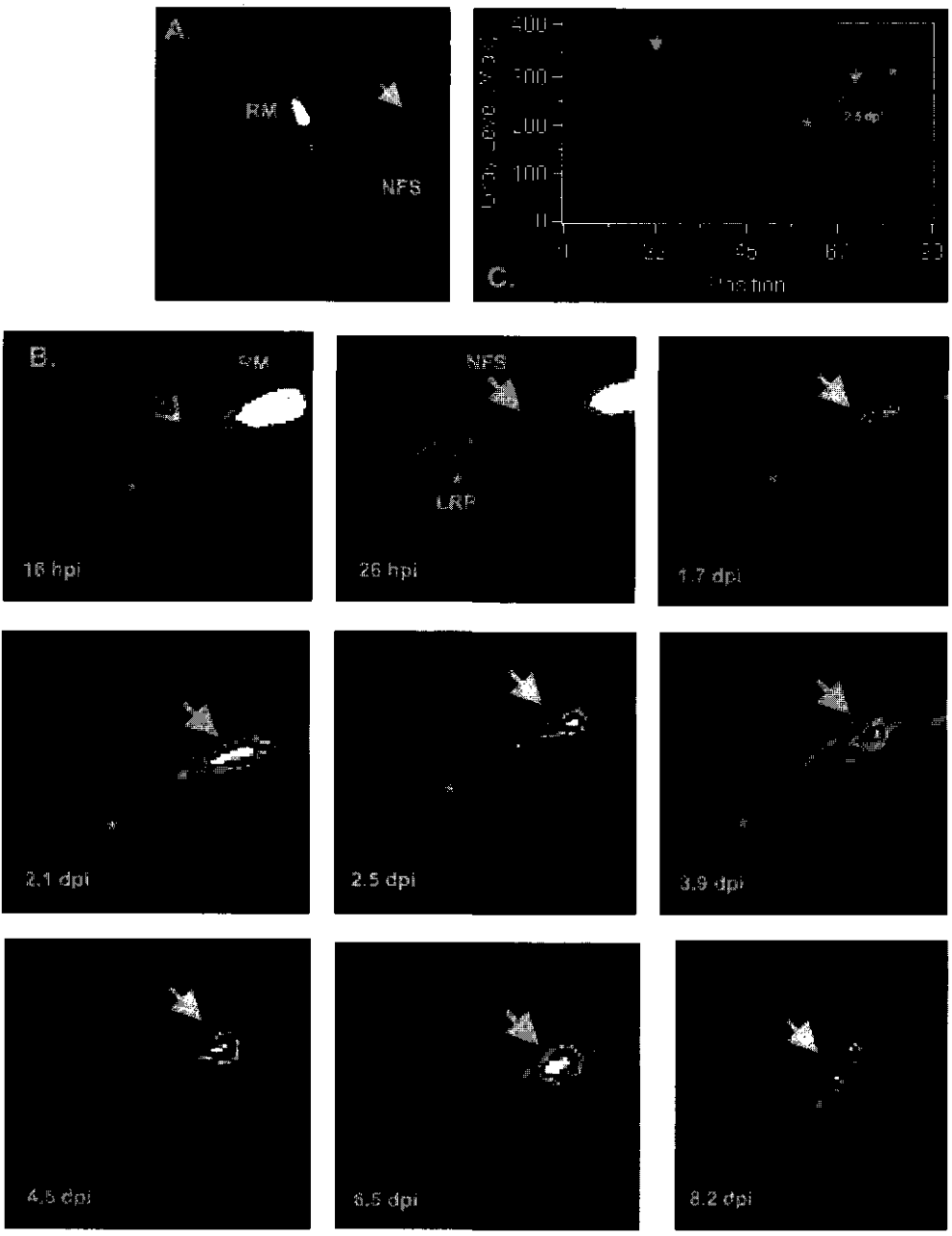
A.
RM
NFS
C.
B.
RM
16 hpi
NFS
LRP
26 hpi
1.7 dpi
2.1 dpi
2.5 dpi
3.9 dpi
4.5 dpi
6.5 dpi
8.2 dpi

Fig. 2. [←] ***A.*** *A pseudocolour image of luciferase activity in potato roots infected with the potato cyst nematode* Globodera rostochiensis. CycB1;1 *promoter activity was observed in the root meristems (RM) and in young nematode feeding sites (NFS). Strongest luciferase activity is indicated in white, whereas low activity is indicated in diminishing gray levels.* ***B.*** *Sequential images showing the transcriptional activity of the* cycB1;1 *promoter in a single nematode feeding cell (arrow). The feeding cell is induced just behind the root meristem (RM) and a similar transient expression pattern was obtained as depicted in Fig. 3. Strongest mitotic activity was detected in the center of the feeding cell where the head of the parasitizing nematode was located. Interestingly, low levels of mitotic activity were observed near the nematode feeding cell suggesting the formation of a lateral root primordium (asterisk). This mitotic induction completely disappeared after 4 dpi.* ***C.*** *This line drawing represents the spatial distribution of luciferase activity inside a single nematode feeding cell at 2.5 dpi (frame). This illustrates that highest mitotic activity is localized in the center of the feeding site, which gradually diminishes towards the edges.*

Luciferase activity as a means to monitor *Arath;cycB1;1* expression in *G. rostochiensis*-infected potato roots.

Firefly luciferase activity in combination with its substrate luciferin is an excellent tool to monitor *in vivo* gene expression. The enzymatic conversion of luciferin is accompanied by the emission of photons, which can be detected by a CCD camera. The rapid turnover of luciferase is advantageous as it allows for the detection of transient gene expression (Ow *et al.*, 1986). To monitor the spatial and temporal expression of a mitotic cyclin gene upon nematode infection, transgenic potato plants harboring a *cycB1;1* promoter-*luc* construct were inoculated with the potato cyst nematode *Globodera rostochiensis*. From about 16 hr post inoculation onwards LUC activity was observed. Subsequently, the activity increased and a maximum was reached at dpi = 6 (Fig. 2B and Fig. 3). Strongest activity was observed in the central region of the syncytium where the intial feeding cell was located and the expression was lower in the periphery of the growing syncytium (Fig. 2B and 2C). In feeding cells of young females (dpi = 15), no LUC activity was observed whereas *cycB1;1* promoter activity remained high in the root meristem. This temporal expression pattern is consistent with the transcriptional regulation of the *cycB1;1* gene in syncytia of *Arabidopsis* (de Almeida Engler *et al.*, 1999) and supports the hypothesis that reactivation of the cell cycle is a general phenomenon in cyst nematode-induced feeding cell development. For the analysis of gene expression at the cellular level, additional *in situ* hybridisation studies would be needed.

LUC activity as a means to follow gene expression in nematode-infected plants has - to the best of our knowledge - not been used before. In this pilot experiment we showed that exposure to luciferin does not affect the development of the potato cyst nematode *G. rostochiensis*. As such it could be an excellent and sensitive tool to study both spatially and temporally nematode-driven plant gene expression *in vivo*.

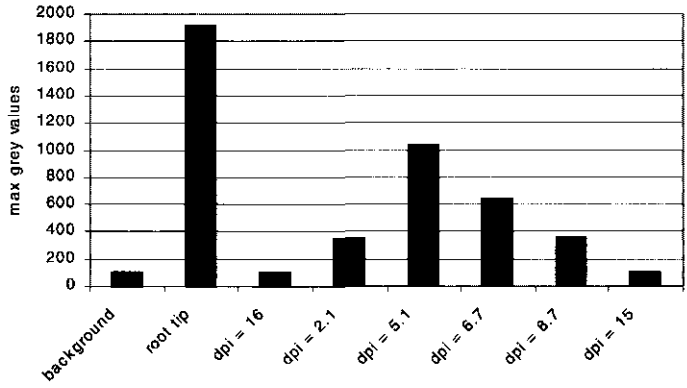

Fig. 3. *Transcriptional regulation of the mitotic cyclin promoter* Arath;cycB1;1 *in potato roots infected with the potato cyst nematode* Globodera rostochiensis *(16 hours - 15 days post inoculation). Promoter activity was monitored in infected plants using the firefly luciferase gene. Mitotic activity was observed in early feeding cell development reaching highest levels around five days post inoculation (dpi). The promoter activity in root tips is virtually twice as much as compared to nematode-induced syncytia.*

resulted in normal development of the nematodes. Similar results were obtained with tomato plants infected with root knot nematodes. Small highly vacuolated giant cells were induced resulting in an increased number of males (Stender *et al.*, 1986; Glazer and Orion, 1984).

Upon oryzalin application (dpi = 1 and 3), root knot nematode development in *Arabidopsis* was completely inhibited. The formation of giant cells was initiated but their development was severely hampered. Moreover, they contained a reduced number of nuclei as compared to untreated giant cells. When oryzalin was applied at later stages (dpi = 9), the majority of the nematodes was able to complete their life cycle. This is consistent with the fact that after 9 dpi no nuclear division occurs and that mitosis is required only for early giant cell differentiation. A similar drastic inhibition of gall formation was observed in oryzalin-treated cotton roots upon root knot nematode infection (Orum *et al.*, 1979). Microscopical analysis revealed that the nematodes entered the root but failed to initiate giant cells, and vascular tissue had differentiated around their heads.

If mitosis was not involved in syncytium formation, oryzalin should not affect cyst nematode development. Application of oryzalin at dpi = 1 resulted in the complete inhibition of syncytium development and no cysts were formed on these plants. When oryzalin was applied at later stages (dpi = 3 and 9) an increasing number of the infective juveniles developed into cysts. These data support the notion that mitotic activity is required for proper syncytium development. It was observed that oryzalin inhibits the mitotic activity in cells prior to syncytium incorporation and as a consequence, syncytium expansion is restricted.

Cell cycle regulation by phytohormones

Reactivation of the cell cycle requires (a) mitogenic stimulus(i). In animal cells, reentry of the cell cycle is accomplished by mitogens which stimulate the transcriptional regulation of D-type cyclins in quiescent cells (G_0) (Quelle *et al.*, 1993). Three D-type cyclin plant homologues have been isolated, which were transcriptionally activated in quiescent (G_0) *Arabidopsis* cells upon nutrient or cytokinin application (Soni *et al.*, 1995; Fuerst *et al.*, 1996; Rhiou-Khamlichi *et al.*, 1999). This

suggests an animal-like mechanism in plants that controls cell cycle activation in response to external stimuli.

The phytohormones auxin and cytokinin are considered to be key factors in controlling cell cycle progression in plants. This is achieved by regulating the expression and/or the activity of the cyclin dependent kinases (CDK) and the mitotic cyclins. Both auxin and cytokinin were able to induce gene transcription of the CDKs *cdc2aAt* and *cdc2Pet* in suspension-cultured cells (Hemerly *et al.*, 1993; Trehin *et al.*, 1998). A rapid increase in mRNA of the p34-cdc2-like protein was detected in tobacco pith upon auxin treatment and cytokinin was required for its activation and the induction of cell cycle activity (John *et al.*, 1993). Moreover, a putative auxin binding element was determined in the *cdc2aAt* promoter (Chung and Parish, 1995) indicating a more direct role for auxin in regulating cell cycle gene expression. Evidence has been provided that cytokinin regulates the cell cycle by tyrosine phosphorylation of the cyclin dependent kinase *Cdc2* (Zhang *et al.*, 1996). Alternatively, it is proposed that cytokinins might interact with the ATP-binding sites of kinases (Redig *et al.*, 1996).

In addition, the expression of the mitotic cyclin gene *cycB1;1* was rapidly induced in roots of *Arabidopsis* by exogenous auxin (Ferreira *et al.*, 1994a; Doerner *et al.*, 1996). The lack of auxin in *Arabidopsis* cell suspension cultures resulted in a strong decrease in mRNA levels of the cyclin genes *cycA2;1, cycA2;2, cycB1;1* and *cycB2;2* (Ferreira *et al.*, 1994b) indicating that transcription of these cyclins is also regulated by auxin. In the shoot apical meristem, expression of *cycA2;1* could be induced by cytokinin treatment as well, whereas the expression in roots was reduced by cytokinin (Burssens *et al.*, 1998).

The role of auxin in feeding cell development

A number of observations point at a role for auxin in feeding cell induction by root knot and cyst nematodes. To test whether there is a direct relation between this phytohormone and nematode development in a host plant, mutants having a defect in their auxin household can be a powerful tool. In principle tomato is a good host for root knot as well as potato cyst nematodes. However, both species could hardly develop on the strongly auxin-insensitive tomato mutant *diageotropica* (*dgt/dgt*) (Richardson and Price, 1984; Goverse *et al.*, 1998a; Helder *et al.*, 1998). The majority of the second stage juveniles penetrated the root of *dgt* and subsequently failed to induce the formation of a feeding site. These observations suggest that auxin signaling is essential in both syncytium and giant cell formation and implies a change in the local auxin balance upon nematode infection. In *Arabidopsis*, Sijmons *et al.* (1994b) tested a range of auxin mutants including both auxin insensitivity mutants as well as plants mutated in the auxin biosynthesis pathway(s). The authors did not detect a significant difference in infection efficiency by root knot and cyst nematodes between the mutants and the controls. For the mutants *aux1-7* and axr2, only a reduction in the number of lateral roots was observed at the feeding site (Sijmons *et al.*, 1994b and Vercauteren *et al.*, 1995, respectively). The discrepancy between their results and the more recent outcomes using tomato could be explained by the leakiness of the *Arabidopsis* mutants used.

The notion that a local accumulation of auxin could be part of the series of events finally resulting in feeding site induction is supported by a number of independent observations. A number of biochemical studies reported on the accumulation of indole compounds in galls formed by the root knot nematodes *M. incognita*, *M. hapla* and *M. javanica* (Setty and Wheeler, 1968; Balasubramanian and Rangarwami, 1962; Yu and Viglierchio, 1964; Viglierchio and Yu, 1968). If there is indeed a local accumulation of auxin or a local increase in the sensitivity towards this phytohormone upon nematode infection, this should be reflected in the transcriptional regulation of auxin-responsive genes. Hermsmeier *et al.* (1998) reported a decreased expression of *adr*-6, 11 and 12 in soybean roots infected with *H. glycines*. These genes are known to be auxin downregulated. A local and relatively strong upregulation of the reporter gene *gfp* preceeded by either the CaMV 35S promoter or the TR2' promoter from *Agrobacterium tumefaciens* was observed in young syncytia upon infection with *G. rostochiensis* (Goverse *et al.*, 1998b). Both promoters harbor auxin responsive elements. Furthermore, a local increase in auxin is consistent with the activation of the

cell cycle genes *cycB1;1* and *cdc2a* in young feeding cells induced by *H. schachtii* and *M. incognita* (Niebel *et al.*, 1996), since both genes are transcriptionally regulated by auxin as mentioned above (Doerner *et al.*, 1996; John *et al.*, 1993).

There is considerable evidence that auxin is involved in lateral root initiation (for recent review Malamy and Benfey, 1997). If nematodes were locally manipulating the auxin household, this could be somehow reflected in lateral root formation. Already in 1936, Christie wrote about infection of tomato by *'Heterodera marioni'* (= root knot nematode): 'division of the pericycle, stimulated by the presence of the parasite, results in a layer of small-cell parenchyma, outgrowths of which form the lateral roots that so frequently occur'. A number of host plant - cyst nematode interactions were studied in more detail and from this limited data set the picture arises that lateral root formation is promoted only if the initial syncytial cell is located in or in the immediate vicinity of the pericycle. Lateral roots are formed upon infection of wheat with the oat cyst nematode (*H. avenae*) (Grymaszewska and Golinowski, 1991) and of rape and *A. thaliana* with the beet cyst nematode *Heterodera schachtii* (Magnusson and Golinowski, 1991; Sijmons *et al.*, 1994b). In tomato the syncytium formation by the potato cyst nematode *Globodera rostochiensis* starts in the cortex and only in a few instances concomitant lateral root initiation was observed (Goverse *et al.*, unpublished data). Illustrative in this respect is Fig. 2 which shows a temporary (16 hpi - 4 dpi) expression of *cycB1;1* in *G. rostochiensis*-infected potato roots just outside the syncytium. The most plausible explanation for this LUC activity would be a transient activation of this cell cycle gene in the pericycle cells. In this particular case the activation did not result in lateral root formation.

Options for mechanisms underlying local auxin manipulation by root knot and cyst nematodes

If local changes in the auxin household are involved in feeding site induction by root knot and cyst nematodes, a range of options are open as far as the underlying mechanism is involved. First of all the concentration of auxin could be increased locally or, alternatively, the sensitivity of the plant tissue towards this phytohormone could be raised. Assuming that auxin accumulates locally, this could in principle originate from either the nematode or the plant.

Goodey (1948) hypothesized that auxins are present in nematode saliva. Auxin determination by paper chromatography indicates that the type and level of auxins in the gall are characteristic for the parasitizing nematode (Yu and Viglierchio, 1964; Viglierchio and Yu, 1968). There are several reports which describe the presence of auxin-like substances in adult females of *M. javanica* (Bird, 1962) and in exudates of hatched J2 from *M. incognita* (Setty and Wheeler, 1968) and *M. hapla* (Yu and Vigliergio, 1964). In contrast, the release of an auxin inactivating compound was reported for *Ditylenchus dipsaci, M. hapla* (Viglierchio and Yu, 1965) and *M. javanica* (Bird, 1966). For cyst nematodes, auxin activity was determined in homogenate of hatched second stage juveniles of *H. schachtii* (Johnson and Viglierchio, 1969). It is difficult to draw any conclusion from these - partially contradictory - papers. Keeping in mind that many plant pathogens produce auxin (see *e.g.* Glickman *et al.*, 1998), there is no reason to rule out the option that nematodes produce and secrete auxins or homologues of this phytohormone.

Alternatively, nematodes could manipulate the auxin household of the plant. Polar auxin transport from cell to cell is carrier mediated. Auxin enters a cell through the action of a saturable auxin uptake carrier or directly (in its protonated form) and it can leave the cell only via an efflux carrier (for recent review about auxin transport see Leyser, 1999). If nematodes would secrete substances inhibiting the efflux of auxin - *e.g.* flavonoids - this could result in an auxin accumulation. Recently, it is observed that auxin induction triggers gall formation by root knot nematodes, which is associated with the activation of the flavonoid pathway (Hutangura *et al.*, 1999). A similar mechanism to locally raise the auxin concentration by perturbing its flow is thought to be employed by *Rhizobium leguminosarum* in white clover roots: endogenous flavonoids probably act as inhibitors of auxin transport in early stages of nodule initiation (Mathesius *et al.*, 1998a,b). Alternatively, auxin accumulation could be brought about by the

release of auxin from auxin conjugates. Conjugated IAA constitutes about 90% of the total IAA in vegetatively growing tissues, so if a nematode could free a part of this inactivated auxin this as well could result in a local accumulation of IAA.

Another option would be that nematodes locally increase the sensitivity towards IAA. Sensitivity towards IAA varies dramatically within plants, roots being one of the most auxin sensitive organs. Little is known about the background of the highly different (tissue specific) sensitivities towards IAA within plants, but is certainly conceivable that nematodes can manipulate this variable.

Perspectives

Cell cycle activation is an essential element in host plant exploitation by endoparasitic nematodes. We begin to understand what cell cycle elements are affected by nematodes but this does not alter the fact that it is largely unclear how the nematode - an organism fully unrelated to plants - realizes this masterpiece of parasitism. In this review, a few pieces of the puzzle are highlighted and more pieces are about to be identified. Currently, we are investigating the biological relevance of naturally-induced cyst nematode secretions in feeding cell induction and their ability to change the developmental program of (un)differentiated plant cells. Stylet secretions from infective juveniles are thought to include pathogenicity factors and - as such - collection of these secretions is highly relevant in this research area. In particular, the use of potato cyst nematodes is advantageous because for this particular cyst nematode a natural trigger - potato root diffusate - can be used to make nematode secrete. In this way, stylet secretions from millions of pre-parasitic second stage juveniles can be collected. Recently, it was shown that (a) small peptide(s) present in nematode secretions are responsible for a stimulation of tobacco protoplast division. This peptide(s) was functionally dissimilar to the phytohormones auxin and cytokinin (Goverse *et al.*, 1999). Apart from being a tool in research, this peptide could be a target for artificial resistance approaches.

It is unclear whether the components in stylet secretions responsible for feeding cell induction act extra- or intracellularly. In case they act extracellularly, D-type cyclins could be relevant. It is assumed that the expression of corresponding genes results in reactivation of the cell cycle in the presence of extracellular signals (Soni *et al.*, 1995; Fuerst *et al.*, 1996; De Veylder *et al.*, 1999). As such, plant D-type cyclins will be good marker genes to study the induction of the cell cycle in response to nematode signaling molecules.

Cell cycle inhibition by chemical treatment results in the disruption of feeding cell formation and as such, in the restriction of both cyst and root knot nematode development, and most likely also of other nematode types that activate the plant cell cycle as part of their infection strategy. Hence, biotechnological approaches which affect the cell cycle could be promising in engineering resistance to a broad range of nematode species. Besides blocking the signals that come from the nematode, several strategies can be envisaged to prevent the activation of the cell cycle. Dominant negative mutations of the *cdc2* gene have been shown to be effective in inhibiting cell division in yeast and tobacco (Hemerly *et al.*, 1995). Expressed behind a promoter that is activated in nematode feeding cells these dominant mutant genes would block the infection process and have no effect on the rest of the plant. Similarly, the feeding-site specific expression of inhibitors of the cell cycle could be equally effective.

Another possibility lies in the better understanding of how exactly the nematode modifies the plant cell cycle into a process of mere genome multiplication and cell enlargement without cell division. This process exists in healthy plants but is poorly understood at the molecular level, except for the maïs endosperm where an S-phase specific kinase and an M-phase inhibitor have been identified as mediators of endoreduplication (Grafi and Larkins, 1995). Regularisation of the nematode-induced shortened cell cycles into normal ones would prevent the infected cells from expanding into giant cells and this might be another way of depriving the nematode from its food source. These different examples illustrate the tools that could be adapted to engineer the cell cycle and it will be interesting to see how their specific application will affect nematode-induced feeding cell development.

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SAMENVATTING

Het cystenaaltje

Nematoden of aaltjes zijn in het algemeen wormvormige organismen met veelal microscopische afmetingen. Ze vormen het fylum der Nematoda. De leden van dit fylum komen op de meest uiteenlopende plaatsen ter wereld voor waar zij soms onder extreme omstandigheden leven. Zo zijn er nematoden waargenomen op Antarctica en op grote diepten in de bodem van oceanen. Veel nematoden leven vrij in de bodem en voeden zich met dood organisch materiaal, schimmels, bacteriën en andere nematoden. Slechts een klein gedeelte van dit fylum leeft parasitair op volstrekt ongerelateerde organismen zoals planten en dieren. Dit vereist speciale aanpassingen die kenmerkend zijn voor parasitaire nematoden en die hen in staat stellen om zich succesvol te kunnen ontwikkelen in de aanwezigheid van de juiste gastheer of waardplant.

De plantenparasitaire nematoden, ook wel aaltjes genoemd, behoren tot de orden der Tylenchida en Dorylaimida en worden gekenmerkt door een stevige, meestal holle stekel in de kop van de dieren. Het infectieuze aaltje gebruikt de stylet als een priem om de plant binnen te dringen en als een soort rietje om zich te voeden. Door de stekel heen en weer te bewegen, vaak in samenhang met celwandafbrekende enzymen, wordt de harde celwand doorboord en krijgt het aaltje toegang tot de inhoud van de cel of kan het zich een weg banen in de plant op zoek naar voedsel. Er bestaan diverse manieren waarop aaltjes de plant aantasten. Zo zijn er bijvoorbeeld de ectoparasieten zoals *Tylenchorhynchus dubius* die leven in de rhizosfeer en zich voeden met de inhoud van de buitenste cellaag van plantenwortels en de migrerende endoparasieten zoals *Pratylenchus penetrans* die de wortel binnendringen om voedsel op te nemen en vervolgens de plant weer verlaten. Dit in tegenstelling tot de sedentaire endoparasieten die na binnendringing in de wortel een permanente voedingsrelatie aangaan met de plant. Het aaltje wordt vervolgens immobiel en is volledig afhankelijk van de waardplant voor zijn verdere ontwikkeling en voortplanting.

Tot deze laatste groep behoren de cystenaaltjes (*Globodera* spp. en *Heterodera* spp.) en de wortelknobbelaaltjes (*Meloidogyne* spp.).In tegenstelling tot wortelknobbelaaltjes, parasiteren cystenaaltjes een klein aantal plantensoorten. De aardappelcystenaaltjes *Globodera rostochiensis* en *G. pallida* kunnen bijvoorbeeld alleen aardappel, tomaat en aubergine infecteren. Aardappelcystenaaltjes zijn de veroorzakers van aardappelmoeheid, een ziekte die veel schade toebrengt aan de aardappelteelt in Nederland. Cysten- en wortelknobbelaaltjes vormen zowel in de tropen als in de gematigde streken een bedreiging voor diverse landbouwgewassen.

In aanwezigheid van een waardplant worden cystenaaltjes geactiveerd en kruipen infectieuze larven uit het ei op zoek naar wortels. De wortels worden bij voorkeur net achter de wortelpunt geïnfecteerd waarna het aaltje dwars door de cortex cellen heen op zoek gaat naar een geschikte voedingscel. Wanneer deze geselecteerd is, wordt die voorzichtig aangeprikt en ontwikkelt deze zich vervolgens tot een meerkernige voedingscel. Doordat aangrenzende celwanden oplossen ontstaat een conglomeraat van gefuseerde cellen, dat wel uit 200 cellen kan bestaan. Tevens neemt de metabolische activiteit van dit syncytium toe en vergroten zich de kernen. De voedingscel breidt zich uit naar de vaatbundel waaraan voedingsstoffen worden onttrokken. Wanneer het voedselaanbod optimaal is ontwikkelt het aaltje zich tot een volwassen vrouwtje dat opzwelt en uit de wortel barst. Na bevruchting ontwikkelen de eieren zich in het lichaam van het vrouwtje dat afsterft. Haar huid blijft de eieren omhullen en beschermt deze in de bodem in afwachting van een nieuwe waardplant. Afhankelijk van de soort zijn er meestal één of slechts enkele generaties per groeiseizoen.

Dit proefschrift beschrijft de resultaten van het onderzoek naar de mechanismen die ten grondslag liggen aan het tot stand komen van een succesvolle interactie tussen het cystenaaltje en de waardplant. Hierbij stond de vraag centraal hoe het cystenaaltje in staat is om de ontwikkeling van de waardplant ten gunste van de eigen ontwikkeling te beïnvloeden.

De biologische activiteit van secreties afkomstig uit de speekselklieren

Plantenparasitaire nematoden worden niet alleen gekenmerkt door de aanwezigheid van een stekel waarmee ze plantencellen aanprikken, maar ook door twee typen speekselklieren: een dorsale klier en twee subventrale klieren. Om de rol van deze klieren bij de inductie van een voedingscel vast te stellen is gezocht naar een methode om de inhoud van deze klieren te analyseren (Hoofdstuk 2). Door de microscopische afmeting en de biotrofe levenswijze van het aaltje is het zeer lastig om aan voldoende biologisch materiaal te komen. Door het aardappelcystenaaltje bloot te stellen aan wortelexudaat was het mogelijk om op een zo natuurlijk mogelijke wijze de infectieuze larven te stimuleren tot het uitscheiden van speekseleiwitten (*svps*). Dit effect werd versterkt door het toedienen van de serotonine analoog DMT (5-methoxy-N,N-dimethyltryptamine-hydrogen-oxalaat). Met behulp van monoclonale antilichamen die zijn gericht tegen de subventrale speekselklieren van het aardappelcystenaaltje kon worden aangetoond dat deze klieren basische eiwitten produceren, die via de stekel van het aaltje worden uitgescheiden. De productie van *svps* door vroeg-parasitaire stadia en de secretie van *svps* in aanwezigheid van wortelexudaat ondersteunt de hypothese dat de subventrale klieren een belangrijke rol spelen in de plant-nematode interactie.

Het cystenaaltje is in staat om volledig gedifferentiëerde wortelcellen te redifferentiëren tot een voedingscel. Dit gaat gepaard met de inductie van celcyclusactiviteit. Om de biologische relevantie van secreties bij dit proces aan te tonen is een gevoelige bioassay onontbeerlijk. In hoofdstuk 3 is de opzet van een dergelijke assay beschreven, waarbij gebruik gemaakt is van tabaksprotoplasten. Deze cellen delen zich in aanwezigheid van de plantenhormonen auxine en cytokinine. Het toedienen van secreties afkomstig van infectieuze larven bleek te resulteren in een significante toename in celdelingsactiviteit. Een klein eiwit (< 3 kDa) bleek verantwoordelijk te zijn voor deze toename. De stimulerende werking van secreties kwam alleen tot stand in aanwezigheid van auxine en cytokinine, maar bleek onafhankelijk van de hormoonconcentratie te zijn. Een vergelijkbaar co-stimulerend effect werd waargenomen na toediening van secreties aan humane bloedcellen (PBMC). Er werd een significante toename in T-cel proliferatie waargenomen in de aanwezigheid van optimale concentraties phytohaemagglutinin (PHA). Dit is een mitogen dat T-cellen aanzet tot celdeling. Hiermee is aangetoond dat secreties - mits de juiste mitogenen aanwezig zijn - in staat zijn celdeling te stimuleren in zowel dierlijke als plantaardige cellen. In hoeverre dit peptide betrokken is bij de inductie van voedingscellen vergt verder onderzoek.

Analyse van genexpressie tijdens de vroege ontwikkeling van voedingscellen

De inductie en ontwikkeling van voedingscellen toont aan hoe intiem de relatie tussen het cystenaaltje en de waardplant is. Vermoedelijk zetten signaalmoleculen van het aaltje deze veranderingen in de initiële voedingscel in gang. Hierbij treden grote veranderingen in genexpressie op. Tot voor kort kon alleen met behulp van destructieve methoden de regulatie van genen in geïnfecteerde wortels worden bestudeerd. Daarbij wordt gebruik gemaakt van bijvoorbeeld het reporter-gen *gus* dat codeert voor het enzym ß-glucuronidase. In 1996 werd ontdekt dat een fluorescerend eiwit, het van de kwal *Aequorea victoria* afkomstige Green Fluorescent Protein (GFP), gebruikt kon worden voor het monitoren van genexpressie in levende organismen. In hoofdstuk 4 beschrijven we het gebruik van dit reporter-gen om de activiteit van twee krachtige wortelpromoters, te weten de 35S promoter afkomstig van het Bloemkool Mozaïek Virus en de TR2' promoter afkomstig van de bacterie *Agrobacterium tumefaciens*, te bepalen na infectie met het aardappelcystenaaltje. Door gebruik te maken van confocale laser scanning microscopie kon GFP fluorescentie worden aangetoond in vroege ontwikkelingsstadia van de voedingscel. Bovendien werd GFP waargenomen in het spijsverteringssysteem van het voedende aaltje, waaruit blijkt dat dit eiwit niet toxisch is voor de nematode. Blijkbaar kunnen eiwitten met een molecuulmassa van circa 32 kDa de 'feeding tube' van het aardappelcystenaaltje passeren.

Aan het gebruik van GFP als *in vivo* reporter-gen bleken ook een aantal nadelen te kleven, waaronder de stabiliteit van het eiwit en de hoge expressieniveaus die nodig zijn voor de detectie van GFP fluorescentie in geïnfecteerde wortels. Deze problemen werden ondervangen

door gebruik te maken van het luciferase-gen dat afkomstig is van het vuurvliegje. Bij de omzetting van het substraat luciferine worden fotonen uitgezonden die kunnen worden gedetecteerd met behulp van een gevoelige camera. Mede door de snelle *turnover* van het enzym wordt een betrouwbaar beeld verkregen van genexpressiepatronen tijdens de inductie en ontwikkeling van voedingscellen. Met behulp van de cycline promoter *Arath;cycB1;1* – een marker voor celcyclusactiviteit – en het luciferase-gen werd vastgesteld dat de celcyclus wordt geactiveerd in de voedingscel vrijwel direct nadat het aardappelcystenaaltje de wortels heeft geïnfecteerd (Hoofdstuk 6). Tijdens de eerste dagen nam de expressie sterk toe, maar na 8 dagen was er vrijwel geen celcyclusactiviteit meer waarneembaar in de voedingscel. Hiermee is aangetoond dat luciferase een krachtig reporter-gen is voor het analyseren van subtiele genexpressiepatronen tijdens de ontwikkeling van voedingscellen.

De rol van auxine tijdens de ontwikkeling van voedingscellen

Plantenhormonen spelen een belangrijke rol in tal van ontwikkelingsprocessen waaronder de inductie van zijwortels, reactivatie van de celcyclus en de differentiatie van cellen. De rol van plantenhormonen bij de inductie en ontwikkeling van voedingscellen is grotendeels onbekend. Om inzicht te krijgen in de functie van auxine werd de tomatenmutant *diageotropica* geïnoculeerd met het aardappelcystenaaltje (Hoofdstuk 5). Door een verstoorde ontwikkeling van de voedingscellen werden nauwlijks vrouwtjes gevormd in de wortels van deze auxine-ongevoelige plant. Soms werd een vrouwtje waargenomen veelal in de aanwezigheid van een abnormale voedingscel. Blijkbaar is auxine betrokken bij de ontwikkeling van voedingscellen die geïnduceerd worden door cystenaaltjes. Dit werd bevestigd door de infectie van auxine-ongevoelige *Arabidopsis* mutanten met het bietencystenaaltje *Heterodera schachtii*. Hierbij was zowel de vorming van zijwortels op de plaats van infectie en de hypertrofie van de voedingscel sterk gereduceerd.

Bovendien werd binnen 24 uur na inoculatie van de auxine-induceerbare *Arabidopsis* promoter trap lijn 5-1E1 met *H. schachtii gus* expressie gedetecteerd in de voedingscel. De expressie nam sterk toe tot een maximum (dpi = 4) en was niet meer waarneembaar vanaf dpi = 8. De expressie was gelocaliseerd in de syncytia, de cellen van de vaatbundel rondom het syncytium en de zijwortelprimordia die vaak worden gevormd in geïnfecteerde *Arabidopsis* wortels. Dit is een indicatie dat de vroege ontwikkeling van voedingscellen gepaard gaat met een plaatselijke en tijdelijke verstoring van de auxine balans.

Een derde aanwijzing dat auxine een belangrijke rol speelt bij de vorming van voedingscellen werd verkregen door experimenten met de auxine transportremmer NPA. Blootstelling aan NPA van geïnoculeerde tomatenzaailingen resulteerde in de vorming van afwijkende voedingscellen en een sterke afname in de ontwikkeling van de nematoden. Uit histologisch onderzoek bleek dat de morfologie van de voedingscel sterk was verstoord in de NPA-behandelde tomatenplantjes. Met name de radiale expansie van de voedingscel richting de vaatbundel was verstoord. Verder nam de ontwikkeling van metaxylem en ongeordende celdelingen in het omliggende weefsel sterk toe. De rol van polair auxine transport bij de ontwikkeling van voedingscellen werd bevestigd door inoculatie van *Arabidopsis* auxine transport mutanten met het bietencystenaaltje.

Diverse plantenhormonen beïnvloeden elkaar wederzijds. Dit wordt geïllustreerd door onder andere de *Arabidopsis* mutant *eir1-1*, die betrokken is bij het polair auxine transport in de wortel en tevens ongevoelig is voor ethyleen. Een ander voorbeeld is het defect in de tomatenmutant *dgt* en de meeste auxine-ongevoelige *Arabidopsis* mutanten. Deze mutanten zijn vaak ook ongevoelig voor ethyleen. Vandaar dat de rol van ethyleen is bestudeerd met behulp van de ethyleen-overproducerende *Arabidopsis* mutanten *eto1, eto2* en *eto3*. Bietencystenaaltjes bleken zich beter en sneller te ontwikkelen op deze ethyleen mutanten. Een mogelijke verklaring zou kunnen zijn dat de ethyleen-geïnduceerde celwandafbraak, welke essentiëel is voor de fusie van cellen tot een voedingscel, sneller verloopt in deze ethyleen-overproducerende mutanten.

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NAWOORD

Ik herinner me nog als de dag van gisteren dat Jaap Bakker me tijdens mijn doctoraalonderzoek op de vakgroep Nematologie de mogelijkheid aanbood om het onderzoek voort te zetten als promotieassistent. Het antwoord was een volmondig 'ja' en ik heb geen moment spijt gehad van die beslissing. In tegendeel, het plant-nematode onderzoek is mijn inziens nog nooit zo boeiend geweest. Dit proefschrift is het resultaat van vijf intensieve jaren, waarin we getracht hebben om een tipje van de sluier op te lichten. Dit alles is tot stand gekomen dankzij de inzet en het enthousiasme van diverse mensen, die ik vanaf deze plaats met name wil noemen.

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CURRICULUM VITAE

Aska Goverse, geboren op 18 oktober 1969 te Amsterdam, behaalde in 1988 het VWO diploma aan de Chr. Scholengemeenschap Assen. De belangstelling voor de biologie bracht haar naar Wageningen, alwaar ze de studierichting Plantenziektekunde ging volgen aan de Landbouwuniversiteit (LUW). Tijdens de doctoraalfase heeft zij onderzoek verricht in het Laboratorium voor Nematologie (LUW) onder begeleiding van dr. J. Roosien. In deze periode werd een methode ontwikkeld om de twee nauwverwante aardappelcystenaaltjes soorten *Globodera rostochiensis* en *G. pallida* te onderscheiden. Hierbij werd gebruik gemaakt van individuele juvenilen in combinatie met randomly amplified polymorphic DNA (RAPD) markers. Vervolgens deed zij onderzoek aan de schimmel *Botrytis cinerea* onder begeleiding van dr. J. van Kan (Laboratorium voor Fytopathologie, LUW), waarbij verschillende karyotypen werden onderscheiden met behulp van chromosoom-specifieke markers. In 1993 vertrok ze naar het lab van prof. R.S. Hussey (Department of Plant Pathology, University of Georgia, Athens, USA) alwaar het onderzoek in de nematologie werd hervat. In samenwerking met E.L. Davis (PhD) werden diverse monoclonale antilichamen geproduceerd die specifiek met secreties uit de speekselklieren van het sojabooncystenaaltje *Heterodera glycines* reageerden. In 1994 werd de studie in de fysiologische en moleculair-biologische aspecten van de gewasbescherming afgerond. Aansluitend kon zij zich als toegevoegd onderzoeker in het Laboratorium voor Nematologie (LUW) verder verdiepen in het onderzoek naar de secretieproducten van cystenaaltjes. Vervolgens is zij als Onderzoeker in Opleiding aangesteld op het NWO-SLW project "Gene expression analysis in feeding cells induced by sedentary plant-parasitic nematodes" (805.45.010 Dwarsverband Gewasbescherming; 1 september 1994 –1998). Dit onderzoek richtte zich op de rol van secreties in de plant-nematode interactie, en de manier waarop cystenaaltjes de ontwikkeling van de plant beïnvloeden. Het voor u liggende proefschrift beschrijft de resultaten en inzichten die zijn voortgekomen uit dit onderzoek. Sinds januari 1999 is de auteur werkzaam als post-doc op het Laboratorium voor Monoklonale Antistoffen (LUW).

NOTES