Endogenous cellulases in stylet secretions
of
cyst nematodes

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Endogenous cellulases in stylet secretions
of
cyst nematodes

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Proefschrift

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in het openbaar te verdedigen

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des namiddags te vier uur in de Aula.

Stellingen

1. In de loop van de evolutie hebben cystenaaltjes genen die coderen voor cellulasen middels 'horizontal gene transfer' verkregen van plant-pathogene bacteriën.

   Dit proefschrift

2. De uitgebreide theoretische redenering over de vermeende éénrichtingsklep in de pompkamer van de metacorpus van Tylenchide nematoden wordt verworpen door experimentele data.

   Wyss, U. and Zunke, U, 1986. Observations in the behaviour of second stage juveniles of Heterodera schachtii *inside host roots. Rev. Nematol. 9, 153-165.*
   Dit proefschrift

3. Het was geen exceptionele vorm van zelfverheerlijking dat B.Y. Endo in 1971 als eerste endoreduplicatie in syncytia beschreef.

   Endo, B.Y., 1971. Synthesis of nucleic acids at infection sites of soybean roots parasitized with the soybean cyst nematode, Heterodera glycines. *Phytopathology 61, 395-399*

4. Als men de vloed aan informatie uit *Caenorhabditis* sequencing projecten wil gebruiken voor de identificatie van genen die van belang zijn voor de pathogeniciteit van plant-parasitaire nematoden, kan men zich beter richten op de verschillen dan op de overeenkomsten tussen beide typen van nematoden.

5. De biologische relevantie van de uitkomsten van experimenten met secreties van nematoden is afhankelijk van de stress die de nematoden ondervinden bij die experimenten.

   Dit proefschrift

6. De immuunrespons op molekuulgewichtfracties verkregen met 'continuous flow electrophoresis' wordt voornamelijk bepaald door enkele immunodominante eiwitten in deze fracties, en is niet representatief voor alle eiwitten die in de fracties aanwezig zijn.

   Dit proefschrift

7. De naam Freund's adjuvans is een pertinente misleiding.

8. De gewijzigde verhoudingen op de arbeidsmarkt voor hooggeschoolden luiden het einde in van het AIO/OIO-stelsel in zijn huidige vorm.

9. Tumoren zijn net zo uniek, en net zo onvoorspelbaar, als mensen.

10. Bij veel discussies wint de kracht van de persoonlijkheid het ten onrechte van de kracht van het argument.

11. Gelukkig hebben niet alle primaten, gekenmerkt door een opponerende duim, toegang tot de oppositie bij een promotieceremonie.

Stellingen behorende bij het proefschrift getiteld: "Endogenous cellulases in stylet secretions of cyst nematodes" van Geert Smant.

Wageningen, 6 oktober 1998

Bibliographic abstract
This thesis describes the identification of β-1,4-endoglucanases (cellulases) in stylet secretions of the two cyst nematodes species, *Globodera rostochiensis* and *Heterodera glycines*. A novel method was developed to raise monoclonal antibodies that were directed to subventral oesophageal gland secretions. These monoclonal antibodies were used to characterise and to immunopurify two secretory proteins. Partial sequence data from these proteins enabled the cloning of two homologous genes from each of the two cyst nematode species. The predicted amino acid sequences revealed a high similarity with bacterial cellulases, whereas no homology was found with eukaryotic cellulases. Evidence is provided for the endogenous origin the nematode cellulases that may have been acquired from bacteria by horizontal gene transfer.

Contents

Chapter 1

General Introduction

The annual losses world-wide caused by plant-parasitic nematodes are estimated to be approximately 100 billion US dollar [77]. This represents one third of the total losses caused by pest and diseases in commercially grown crops [94]. A substantial part of the losses is due to infestations by root-knot nematodes (*Meloidogyne* spp.), and cyst nematodes (*Heterodera* spp., and *Globodera* spp.). In the past the control of these nematodes relied on a combination of crop rotation, the use of nematode resistant cultivars, and the application of nematicides. Because of the undesirable side effects of many nematicides, farming has become more dependent on the availability of resistance against plant-parasitic nematodes. Such a resistance can either be introduced into commercial cultivars from natural sources by conventional plant breeding, or via biotechnological approaches.

Resistance against cyst nematodes acquired via biotechnological approaches requires a detailed understanding of the molecular interaction between cyst nematodes and host plants [79]. Important in these interactions are those pathogenicity factors released by infective cyst-nematode that determine host-nematode compatibility. The objective of this thesis is the identification and characterisation of pathogenicity factors in oesophageal gland secretions of cyst nematodes.

Life history of cyst nematodes

Cyst nematodes are parasitic on a relatively small host-plant range, *e.g.* potato cyst nematodes can only reproduce on Solanaceous species, such as potato, egg plant, and tomato [34]. The infective second-stage juveniles (J_2) of potato cyst nematodes reside dormantly in eggs in the soil where they stay viable for many years [19,68]. The remnant of the body of the parent female, which transforms into a thick-walled cyst after death [18], en closes the eggs as a protective container. In some cyst nematode species dormant juveniles only hatch from eggs when they are activated by root exudates from host-plants [69,70]. Once hatched the juveniles leave the cyst in search for a nearby root. The roots of host plants that are encountered by juveniles are subsequently invaded at particular sites, and inside the root the parasitic juveniles migrate through the cortex to the stele (Fig. 1.1).

At this stage they select a cell within or close to vascular cylinder and induce a multicellular feeding site, a so-called syncytium [23,54,55]. The syncytium is the only means by which the obligatory biotrophic cyst nematodes obtain the necessary nourishment to develop into the reproductive life stage. While feeding, the body wall muscles in the juveniles disintegrate, and as a consequence they loose their migratory abilities [21]. The sex of the juveniles is determined epigenetically early in the development. Sex determination is related to protein content and amino acid composition of the syncytial cytoplasm [41]. During development females undergo extensive swelling of the body, which causes them to burst out of the root epidermis. It is estimated that sedentary adult females are able to retrieve 4 times their body volume in fluid per day from the syncytial cytoplasm [80]. Adult males regain their mobility and migrate out of the root to fertilise the adult females. Migratory males are attracted to the females that expose themselves on the roots [40]. Soon after fertilisation female nematodes die while still containing their offspring within the rigid cuticle.
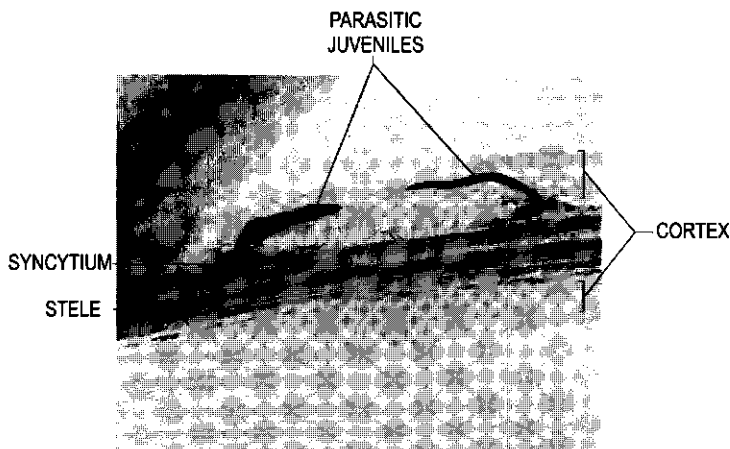

Figure 1.1 *Soybean root infected with juveniles of the soybean cyst nematode* Heterodera glycines. *The roots are incubated in acid fuchsin, which results in a differential staining of the juveniles. The juveniles are feeding from syncytia located in the stele of the root.*

Parasitic behaviour

In the parasitic behaviour of cyst nematodes three distinct phases can be discerned: i) penetration of the root, ii) intracellular migration, and selection of the initial syncytial cell, and iii) induction and maintenance of a syncytium. These three phases have been studied in detail for several nematode-plant interactions, *e.g. Globodera rostochiensis* on tomato (*Lycopersicon esculentum*) seedlings [84], *Heterodera cruciferae* on Brussels sprouts (*Brassica oleracea* var. gemmifera) [22], *Heterodera schachtii* on the crucifers oil rape (*Brassica napus*) [98], rapeseed (*Brassica rapa*) [96], and *Arabidopsis thaliana* [39]. Despite the differences between individual plant-nematode combinations the basal *modus operandi* is the same for all cyst nematodes species. In the following paragraphs the three phases will be reviewed with an emphasis on the role of nematode secretions.

Phase I: Penetration of roots

In 1972 Steinbach [84] published a detailed time-course study on the penetration of *G. rostochiensis* (at that time classified as *Heterodera rostochiensis*) in young tomato seedlings. He described how J_2 preferentially invaded the epidermis cell layer of the root at the differentiation zone. Others have observed that *H. schachtii* [98], *H. cruciferae* [22], and *H. glycines* [1] preferentially invade the root either at the elongation zone or at the base of emerging lateral roots.

In the proximity of roots the *G. rostochiensis* juveniles show elevated stylet activity presumably under the influence of root exudates [84]. After initial uncoordinated and undirected stylet thrusts the nematodes continued with careful touching of a small selected area of the epidermal cell wall. Steinbach suggested that nematodes release secretions containing cellulolytic enzymes while touching the cell wall [84]. After 19 min. of localised and enforced stylet thrusting, at a rate of 120 thrusts per min., the stylet completely perforated the cell wall for the first time. Cyst nematodes repeat this action a number of times thereby creating a slit in the epidermal cell wall. The juveniles enter the epidermis by forcing their head skeleton through the slit. The complete penetration of the epidermis of *G. rostochiensis* lasted approximately one hour from the first contact with cell wall onwards [84].

Phase II: Intracellular migration and selection of initial syncytial cell

Following penetration cyst nematodes move intracellularly through several cortical cells towards the vascular cylinder [75,84,98]. Each time the migrating juvenile encounters a cell wall it cuts a slit in it by highly co-ordinated stylet thrusts (up to 150 thrusts per min.). The destructive force used by cyst nematodes during their migration through the root cortex results in a necrotic reaction of the cells aligning the migration track [73]. In potato roots infected with *G. rostochiensis* and *Globodera pallida* the necrotic browning in cells on either side of the migration track results in extensive

autofluorescence [75].

At the molecular level, Hansen *et al.* [45] reported the up-regulation of *wun1*, a wound inducible-promoter, in potato tissue infected with *G. pallida.* Previously, the transcriptional activation of *wun1* was correlated with callose deposition [58]. Root-knot nematodes, *Meloidogyne* spp., which migrate intercellularly, did not induce this promoter. Hence the migration strategy used by root-knot nematodes has less devastating effects in the cortex than the one adopted by cyst nematodes. In addition, the expression of the cell wall protein extensin is up-regulated at the necrotic penetration sites and migration tracks in tobacco infected with *Globodera tabacum* [63]. A similar transcriptional activation pattern with a fragment of the *gst1* promoter was observed in cortex cells of potato roots infected with *G. pallida* [87]. The *gst1* gene was previously characterised as a plant hormone-responsive defence gene encoding glutathion-S-transferase [44]. Moreover, in a less localised mode the infection of potato with *G. pallida* is accompanied by the transcriptional activation of a potato catalase gene [64].

During their migration through the cortex the oesophageal gland cells in *H. schachtii* [98] and *H. glycines* [1] are packed with secretory granules which is interpreted as a measure for the activity of the gland cells (Fig. 1.2). However, Wyss concluded that in the case of *H. schachtii* secretions from these gland cells are not involved in the migration of these juveniles because the pump chamber in the oesophageal metacorpus was not active, and no flow of secretions was observed during intracellular migration [98]. Migration of the J_2 in the cortex was therefore thought to occur by means of mechanical force only.

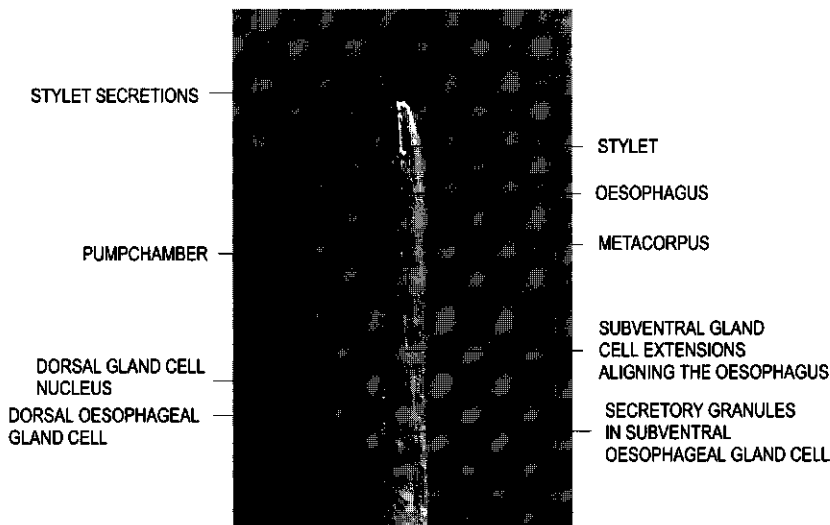*Figure 1.2* *Image of the anterior part of a* H. glycines J_2 *acquired with Nomarski optics, which is illustrating the oesophagus and oesophageal gland cells. Precipitated stylet secretions are visible as particulates at the tip of the stylet.*

As soon as the juveniles reach the vascular cylinder their somewhat blunt behaviour changes into a more refined probing of neighbouring cell walls. This exploratory behaviour may take several hours during which the juveniles apparently determine whether a cell is suitable for syncytium induction. At present those factors decisive for the selection of the initial syncytial cell are not well understood. Golinowski *et al.* [39] observed in *A. thaliana* roots that *H. schachtii* juveniles show a marked preference for (pro)cambial cells as their initial syncytial cell. Juveniles of *H. schachtii* infecting plants under conditions favouring male development only induce functional syncytia in pericycle cells [83]. By contrast, juveniles of *G. rostochiensis* usually select a cortical cell in potato roots to initiate a syncytium [20,56,73]. Juveniles of *H. glycines* infecting soybean roots are less stringent in their selection of the initial syncytial cell: syncytia were initiated in cortex, endodermis, pericycle, and phloem parenchyma cells [25,30]. Nevertheless, when taking into consideration the time used by the nematode to select an initial syncytial cell, and the specificity of the cell type it selects, it seems plausible that the juvenile is perceptive for signals indicating the ability of a cell to differentiate into a syncytium. These signals can either originate from the cell under scrutiny by the juvenile. Alternatively, the nematode may be able to interpret positional information defining cell differentiation [78,92].

Phase III: Initiation, and maintenance of the syncytium

Juveniles can only initiate a syncytium in a selected cell when the protoplast does not collapse after the first complete perforation of the cell wall. At the following steps of the syncytium initiation the stylet ceases to move and remains protruded in the cell lumen for several hours [80,85,96,98]. During this preparation period no ingestion is observed, but Wyss [98] noted significant changes in the activity of the oesophageal glands cell in *H. schachtii*. Secretory granules accumulated in the ampulla of the dorsal gland, whereas the number of secretory granules in the subventral gland cells decreased. The end of the preparation period is demarcated by continuous pulsation of the pump chamber in the median bulb.

It has proven to be difficult to unravel the molecular mechanisms underlying syncytium formation due to a poor accessibility and recognition of the initial syncytial cell. Syncytia are thought to arise from root cells by intervention of the normal developmental program of the cell. The strongest line of evidence to support this hypothesis comes from the transcriptional activity of two promoters, *cdc2a* and *cyc1At*, that are indicative for cell cycle activity in *A. thaliana*. *Cdc*2a is a protein kinase that may reflect the competence of a cell to divide [47], whereas *cyc1At* is only expressed early in the transition from G2 to M phase in the mitotic cell cycle [35]. Both promoters are activated during syncytium initiation by *H. schachtii* in *A. thaliana*. In addition, moderate levels of ^{3}H-thymidine incorporation were observed in syncytial nuclei induced by *H. glycines* [26] and *H. schachtii* [62]. Cells at the border

of syncytia induced by *H. schachtii* showed ^{3}H-thymidine incorporation, too [38]. ^{3}H-thymidine incorporation indicates that a cell proceeds through the S-phase of the cell cycle. Gheysen *et al.* [38] suggested that cyst nematodes could force the syncytial nuclei into repeated cycles of endoreduplication. As no complete mitosis is observed in syncytia, endoreduplication may be the consequence of shunting the cell cycle at the G2/M to the G1 phase [62]. Alternatively, syncytial cells may also arise from initial mitotic stimulation of cells at the borderline of the syncytium [38].

G. rostochiensis [85] and *H. schachtii* [96,98] juveniles feed from the syncytium in a consistent pattern of repeated cycles of nutrient ingestion (I), retraction and re-insertion of the stylet (II), and salivation (III). This feeding pattern is only interrupted during moulting when the juveniles refrain from feeding. While the intervals of stylet retraction and re-insertion, and salivation remain fairly constant in time, the developing juveniles use increasingly more time for nutrient withdrawal.

During each salivation-period a new intracellular feeding tube is formed in the cytoplasm of the syncytial cells (Fig. 1.3). In the case of cyst nematodes the feeding tubes are amorphous structures embedded in endoplasmic reticulum. The basal part of the feeding tubes is connected to the plasmamembrane just opposite to the stylet orifice. The origin and the nature of the feeding tube is not clarified yet, but there are several observations indicating that feeding tubes are at least in part formed from oesophageal gland secretions [50]. In the initial syncytial cell the inserted stylet does not penetrate the plasmamembrane, but is invaginated by it. It is not clear how gland cells secretions pass the plasmamembrane to form a feeding tube in the plant cytoplasm without disturbing the integrity of the plasma membrane. It is difficult to imagine an active transport mechanism that facilitates the transport of secretions across the membrane. Therefore, nematodes may have evolved a sophisticated method to make a minute opening in the plasma membrane. Considering the high pressure (9,000-10,000 hPa) in the syncytial cells [14] the connection between the stylet orifice and the plasmamembrane should be able to resist extreme forces.

The prevailing view on the function of a feeding tube envisages that it filters ingested cytosolic fluid from syncytial cells thereby preventing the stylet from choking. This view originates from the experiments done by Böckenhoff and Grundler [14] who determined a size exclusion limit (Stokes radius of 3.2 to 4.4 nm) for the uptake of dextrans from syncytia by *H. schachtii*. The stylet orifice of cyst nematodes is approximately 100 nm [27,97] wide thus can not explain the exclusion limit found. It is not clear to what extent the size exclusion limit is also relevant for proteins in the cytosolic fluid. Small proteins such as the oryzastatin protein inhibitor (17 kDa) [90] and the green fluorescent protein (27 kDa) [91] are not excluded from ingestion by the feeding juveniles.

The syncytium is the sole nutrient source for cyst nematodes during their development. The scientific interest in cyst nematodes can be ascribed to cellular and molecular structure of the syncytia. There is ample descriptive data in the literature on nematode-induced syncytia [13,30,39,54-56,60,73,83,97]. In summary, syncytia develop from cells that are fused to the initial syncytial cell by cell wall degradation at the pit fields. The process of cell incorporation continues till the fourth developmental

stage of the juvenile. Characteristically, cells incorporated in the syncytium have dense cytoplasm that is filled with ribosomes, endoplasmic reticulum, lipid bodies, mitochondria and plastids. Syncytial nuclei are hypertrophied, and have an irregular shape with prominent nucleoli. The central vacuole is replaced by numerous smaller vacuoles, sometimes associated with paramural bodies including remnants of degraded cell walls. In addition, the syncytial cells become increasingly hypertrophied during the course of feeding by the nematode, whereas cells at the perimeter of the syncytium show hyperplasy. Cell walls in a syncytium abutting xylem elements have extensive finger-like cell wall ingrowths.
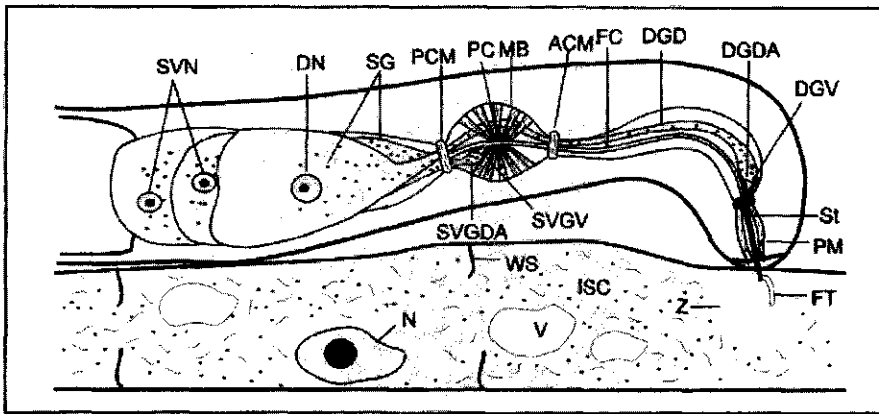

Figure 1.3 *Drawing of a* H. schachtii J_2, *feeding from an initial syncytial cell, approximately 36 h after induction (ACM = anterior sphincter muscle of the metacorpal bulb; DGV = dorsal gland valve; DN = dorsal oesophageal gland nucleus; FC = oesophageal lumen; FT = feeding tube; ISC = initial syncytial cell; MB = metacorpal bulb; N = hypertrophied nucleus; PC = pump chamber; PCM = posterior sphincter muscles of the metacorpal bulb; PM = stylet protractor muscles; SG = secretory granules; St = stylet; SVGDA = subventral gland ampulla; SVGV = subventral gland valve; SVN = subventral oesophageal nucleus; V = vacuole; WS = remnants of degraded cell walls; Z = zone of modified cytoplasm; Courtesy of Dr. U. Wyss).*

For weeks the biotrophic cyst nematodes continuously retrieve enormous amounts of water and solutes from syncytia induced in host plants [80]. The question is how plant metabolites, ions, and water in syncytial are replenished from surrounding plant tissue to prevent a metabolic crash in the syncytial cells. Böckenhoff *et al.* [15] have shown that syncytia induced in *A. thaliana* roots by *H. schachtii* function as metabolic sinks for phloem derived ^{14}C-labeled sucrose. Phloem unloading of plant assimilates into syncytia was shown to be strictly unidirectional, and could therefore not follow the regular symplastic pathway. This observation led the authors to the conclusion that some yet unknown anomalous pathway of solute transport mediates the transport of assimilates from phloem into syncytia [42].

Structures similar to the cell wall ingrowths in the syncytial cell walls abutting xylem elements are found in transfer cells [56,66]. Transfer cells are associated with intensive apoplastic transport over short distances in various plant organs, *e.g.* reproductive tissues. The cell wall ingrowths in syncytia result in a significant increase in surface area of the aligning plasma membrane, thus facilitating a strong influx of water and ions from xylem vessels into the syncytial cells. Since nematodes can only retrieve nutrients in a solubilised form there is an obvious high demand for water in syncytia, that needs to be supplied from xylem elements.

The key question is how cyst nematodes induce the aforementioned changes in plant development. It has been shown for root-knot nematodes that a continuous stimulus from the nematode is required for feeding cell maintenance, though such a signal molecule has not been identified in cyst and in root-knot nematodes. Williamson and Hussey [95] have postulated a model that could explain how secretions released by the nematode trigger the formation of a syncytium. Pathogenicity factors in secretions may interact with receptors in the extracellular matrix, or in the initial syncytial cell membrane. Alternatively, signal molecules may initiate a response by binding to intracellular receptors, or by interacting directly with the host-plant's transcription/translation machinery.

Nematode secretions

Secretions or excretions from cyst nematodes that are putatively involved in the interaction between plant and nematodes may be produced at four sources in the nematode's body —the secretory-excretory pore, the cuticle, the amphids, and the stylet.

Many animals produce an excretory fluid to balance internal ion levels, and to eliminate their metabolic waste products. Osmoregulatory functions in nematodes are thought to depend on a secretory-excretory system [10,12]. The secretory-excretory system is a tubular structure with lateral extensions that opens in the cuticle via a so-called secretory-excretory pore [9]. Others have suggested that a function for the secretory-excretory system may also be the secretion of a glycoprotein surface coat [10]. Several lectin species react with secretions from the secretory-excretory pore, and the amphids in *H. schachtii* males [2,3]. However, none of these secretions have been identified so far.

Cuticular exudates accumulate on many cyst-nematode species, e.g. *G. rostochiensis* [37,52], *H. schachtii* [2,32], and *H. glycines* [33]. For instance, the cuticle of the infective *H. glycines* J_2 is covered with a thin layer of fibrillar exudates that originates in the cuticle hypodermis during the establishment of the syncytium [33]. Despite their common presence on cyst nematodes the biochemical nature of the cuticular exudates has not been elucidated.

As a third source, secretions may originate in the sheath cells at the basal region of the amphids. The amphids are chemosensory organs located adjacent to the stylet shaft in the cephalic framework of the nematode's head [27,36,53]. Amphidial secretions may contain material that is used in the formation of a feeding plug, between the

perforated cell wall of the initial syncytial cell and the protruded stylet [27]. Possibly, the feeding plug seals the perforated cell wall during the retraction period in the feeding cycle, and during moulting. In more recent studies Jones [53] could not confirm that the material of the feeding plug is part of the amphidial secretions, but observed a compositional resemblance between material found in the feeding plug and in cuticular striae at the utmost anterior region of the nematode's head [52].

Historically, secretions from the oesophageal glands have received much attention because they are considered to be important factors in the pathogenicity of sedentary plant-parasitic nematodes [6-8]. Oesophageal gland secretions are released to the immediate environment of the nematode via the hollow stylet [48,49]. Cyst nematodes have three single-celled oesophageal glands located directly anterior to the intestine [28]. Based on their positions in the pseudocoelom two gland cells are indicated as subventral glands whereas the third most anterior gland cell is named the dorsal oesophageal gland. In the following paragraphs the structure and function of the oesophageal glands are treated in more detail.

Oesophageal gland cells
Oesophageal gland cells in cyst nematodes have a typical bottle-like shape, with at the basal region a cell lobe and at the apical region a narrow cell extension leading into an ampulla (Fig. 1.4 [28]). The subventral oesophageal gland cells are connected to the lumen of the oesophagus in the median bulb just behind the pump chamber, whereas the dorsal gland cell opens in the oesophagus just behind the stylet base. The positions where the gland cells are attached to the oesophagus have been used as an argument in the debate about the function of the two types of oesophageal glands (see chapter 3).

The morphology of the three oesophageal gland cells in cyst nematodes resembles the structure of other polarised secretory cells found in many animals [5,24], e.g. pituitary [43], parotid and pancreatic acinar cells [86]. A large part of the cytoplasm in the gland cells is filled with subcellular compartments involved in the secretory pathway of proteins [4,28,46,57,65]. This is illustrated in Figure 1.4 where close to the basal membrane of a gland cell a large nucleus with prominent nucleolus is depicted, surrounded by an extensive network of rough endoplasmic reticulum. At the perimeter of the endoplasmic network numerous Golgi-complexes are observed including small vesicles that bud from the *trans*-Golgi network. Larger, membrane delineated secretory granules, completely fill the cell cytoplasm at the more apical region of the gland cell lobe [29,31]. The secretory granules are frequently observed to move anteriorly through the gland cell extension towards the distal part of the oesophageal gland cell, the ampulla [98]. The extensive arrays of microtubuli that are present in the gland cell extensions [29] may mediate the selective transport of the secretory granules to the ampulla. In the ampulla the secretory granules accumulate awaiting a trigger for secretion. Once secretion is initiated in the gland cell the secretory granules in the ampulla dock and fuse to the plasma membrane in the ampulla thereby releasing the contained proteins by exocytosis. In sedentary nematodes the apical plasma membrane in the ampulla has evolved into a membranous valve that covers a small duct to the oesophageal lumen [28].
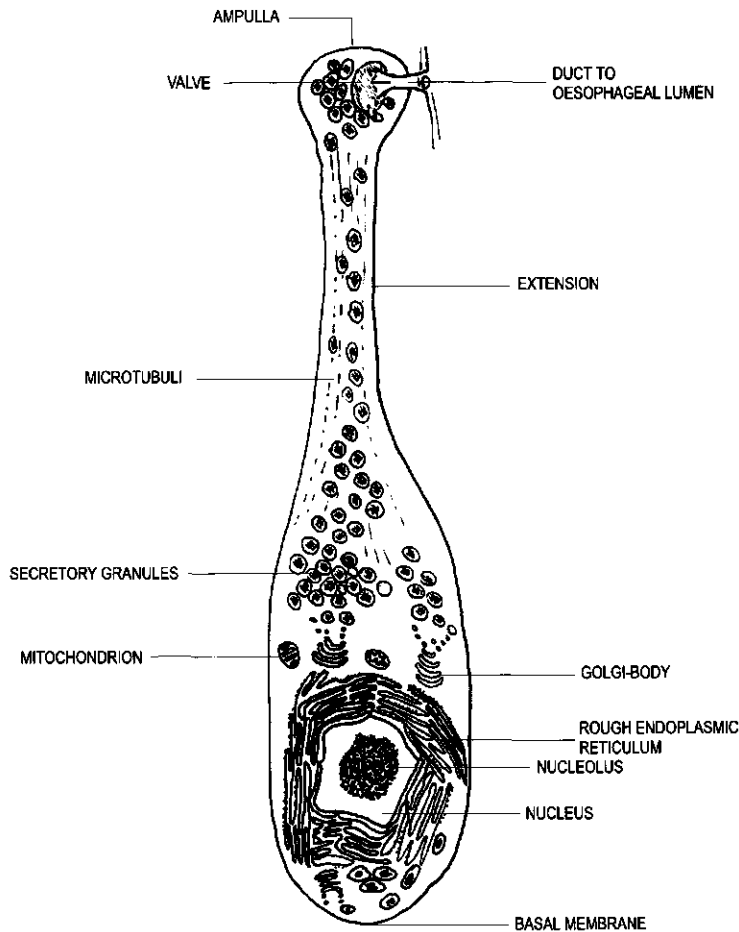
Figure 1.4 Schematic representation of a oesophageal gland cell dissected from a cyst nematode.

Recent studies have revealed that the fundamental mechanism of granule docking and membrane fusion is conserved between different pathways in eukaryotes [4,46,59,67,76,88]. Homologues of integral membrane proteins involved in the initial steps of exocytosis of secretory granules have been identified in *Caenorhabditis elegans*, too [61]. In summary, eukaryotic cells can have two types of exocytotic pathways for Golgi-derived vesicles [16]. The constitutive secretory pathway that is present in all living cells maintains the internal recycling of membrane lipids, membrane proteins, and proteins required in the cell's extracellular matrix. The small vesicles (approx. 100 nm) of the constitutive pathway mediate a continuous bi-directional trafficking between the intracellular compartments of the cell, and the

plasma membrane. The larger secretory granules (approx. 1 μm) form a distinct subcellular compartment in the regulated secretory pathway that is only observed in cells specialised in secretion. The secretory granules bud from the *trans*-Golgi network as smaller condensing vesicles, which fuse to form the larger secretory granules. In several cell types secretory granules undergo maturation during which the concentration of the granule contents increases finally resulting in a higher electron density. The function of the secretory granules is to store secretory products until the hosting organism/cell is opposed by a condition in which large quantities of the secretory products are required. Exocytosis of the secretory granules in the regulated pathway is triggered by a molecular signal, such as elevated cytoplasmic Ca^{2+}-concentrations [76]. Typically, the products contained in secretory granules are functionally not restricted to the immediate environment of the secreting cell.

Oesophageal gland secretions

Despite the many efforts to elucidate the nature of oesophageal gland secretions no secretory products have been identified from sedentary plant-parasitic nematodes. In older work the partial identification of oesophageal gland secretions relied on histochemical methods [6,11,17,71,89]. These techniques were applied either to gland cells or collected stylet secretions. The sometimes-contrasting results make it difficult to draw conclusions from the histochemical data, but three aspects of the oesophageal gland secretions and stylet secretions are noticed consistently in several independent studies. It seems evident that multiple proteins are secreted by sedentary plant-parasitic nematodes, of which at least some proteins are glycosylated and show a relative high abundance of basic amino acids (see chapter 3).

From a study with the root-knot nematode *Meloidogyne incognita* the isolation was reported of the secretory granules from the oesophageal gland using isopycnic centrifugation [72]. However, no compelling evidence was provided for the origin of the isolated fraction in nematodes. Moreover, at least nine different proteins were detected on SDS/PAGE in collected stylet secretions from adult females of *M. incognita*, among which three proteins were shown to be glycosylated [93]. Hussey *et al.* [51] used a monoclonal antibody to immunopurify a 212 kDa glycoprotein from the secretory granules of the dorsal gland cell in *M. incognita* J$_2$ (see Chapter 2). This large protein seems to be refractory to N-terminal amino acid sequencing, and has not yet revealed its identity.

Outline of this thesis

The objective of this thesis was to identify and characterise secretory proteins expressed in the subventral oesophageal gland cells of cyst nematodes. Two relatively unrelated cyst nematodes species, *Globodera rostochiensis* (Woll.) Skarbilovich [34,81] and *Heterodera glycines* Inchinohe [74,82], were selected for this purpose.

When dealing with the single celled oesophageal glands in such small animals as cyst nematodes there is an obvious need for a powerful experimental tool for the isolation of proteins. In chapter 2 we describe a novel method using continuous flow gel electrophoresis to raise monoclonal antibodies directed to denatured secretory

proteins from the oesophageal gland cells. One monoclonal antibody was selected as suitable for large-scale purification of secretory proteins from the subventral oesophageal gland cells.

The third chapter describes the biochemical characterisation of subventral oesophageal secretions recognised by the subventral gland specific antibodies. The primary goal was to find clues for the biological significance of the subventral gland secretions, and to determine whether it would be worthwhile to pursue the isolation of these secretory proteins.

Chapter 4 describes the cloning and characterisation of two genes from both cyst-nematode species encoding β-1,4-endoglucanases (cellulases). Several lines of evidence are provided in this chapter converging to the conclusion that the cellulases are expressed from endogenous nematode genes, independently from symbiotic micro-organisms.

The predicted amino acid sequences of the nematode cellulases showed a remarkable homology with bacterial cellulases, while no homology was evident with eukaryotic cellulases. This led us to speculate upon an ancient acquisition of the cellulase genes from a bacterial source by horizontal gene transfer. Chapter 5 describes how we studied the genomic structure of the cellulase genes including their flanking regions in search for additional indicators that could point to the origin of the genes. In chapter 6 the structure and function of the nematode cellulases is discussed in relation to their importance for pathogenicity, and in relation to their evolutionary origin.

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Chapter 2

Production and selection of monoclonal antibodies directed to subventral oesophageal gland secretions of the potato cyst nematode, *Globodera rostochiensis*[1]

Abstract

Sodium dodecyl sulfate-extracted proteins from second stage juveniles (J_2) of the potato cyst nematode *Globodera rostochiensis* were fractionated by preparative continuous flow electrophoresis, and monoclonal antibodies (mAbs) were raised against the 38-40.5 kDa protein fraction. Screening of the hybridoma culture fluids by immunofluorescence microscopy of J_2 resulted in the identification of 12 mAbs that bound specifically to the subventral oesophageal glands. These mAbs have been tested for immunolabelling of the secretory granules in the oesophageal gland cells, and for binding collected stylet secretions. On western blots of J_2 these mAbs identified four protein bands with apparent molecular weights of 30, 31, 39 and 49 kDa. It is concluded that one or more of these proteins are present within the secretory granules, and in DMT-induced stylet secretions. Immunofluorescence microscopy of J_2 from other plant parasitic nematode species showed that some of these mAbs also bind to the subventral glands of *G. pallida, G. tabacum, H. glycines,* but not of *Heterodera schachtii, Meloidogyne incognita* or *M. hapla.*

INTRODUCTION

The identification and characterisation of oesophageal gland secretions from plant-parasitic cyst-nematodes is seriously hindered by the minute size of these tiny animals. The thread shaped body of the second-stage juvenile of the potato cyst nematode *Globodera rostochiensis* measures only 20 µm in width and 450 µm in length. It is therefore practically not feasible to dissect an amount of oesophageal gland cells from the nematode's pseudocoelom, which is sufficient for protein analysis. In the face of such a challenge, that merely resembles the search for a needle in a haystack, the hybridoma technology can be a powerful tool.

To date, monoclonal antibodies (mAbs) have many applications in the identification of proteins [13,14,20]. The versatility of mAbs is mainly due to their high binding affinity combined with epitope specificity. These two features are the reason why many attempts have been made to raise mAbs against secretory proteins in the oesophageal gland cells of several plant-parasitic nematode species. Such mAbs are particularly useful for the purification of minute amounts of a protein from a complex protein pool.

So far, mAbs have been used in attempts to identify the oesophageal gland secretory proteins of the sedentary plant-parasitic nematode *Heterodera glycines* [1,12] and *Meloidogyne incognita* [4,15]. For both species, screening procedures using immunofluorescence microscopy have identified mAbs that react with secretory granules of the dorsal and subventral gland cells. Various antigens have been used to produce these monoclonals: with *H. glycines* mice were immunised with homogenates from hatched J_2, unhatched J_2, stylet secretions of J_2, and adult females or anterior and

posterior parts thereof [1,12]; with *M. incognita* the immunogens were a total protein homogenate of J_2, a subcellular granule fraction from J_2, anterior and posterior parts of females, and stylet secretions of females [4,15]. In both species the immunosuppressive drug cyclophosphamide was used to enhance the effect of the immunisations [1,4], and with *M. incognita* intrasplenic immunisations were employed to administer minute quantities of immunogen [4]. However, the success of these mAbs in identifying oesophageal gland secretory proteins, either by immunostaining of gel electrophoresis patterns or by purification with chromatography techniques, has remained limited. Hussey *et al.* [16] used a mAb reactive with both the dorsal and subventral glands in *M. incognita* to isolate a secretory component from homogenised J_2. This glycoprotein had an apparent molecular weight of more than 212 kDa. A mAb binding to the subventral oesophageal glands of adult females of *M. incognita* was used to isolate a gene from a cDNA expression library [19]. Because this mAb also bound to the body-wall muscles in J_2 of *M. incognita* and because the sequence of the isolated gene showed homology with the rod portions of myosin heavy chains, it was suggested that this antigen may be involved in the movement of secretory granules rather than being itself secreted [19].

In this study, we report the production and selection of mAbs that bind to secretory proteins in the subventral oesophageal glands of the potato cyst nematode, *Globodera rostochiensis*. In advance we defined four criteria for a mAb to be suitable for the purification and identification of subventral oesophageal gland secretory proteins. These four criteria include that a mAb should

- i). be able to bind to the oesophageal gland cells in immunofluorescence microscopy,
- ii). label the secretory granules in these gland cells using immunogold labelling,
- iii). recognise its antigen in collected stylet secretions in immunofluorescence microscopy, and
- iv). detect its antigen on western blots.

As immunogen we used a protein fraction from homogenised J_2, which was collected by preparative continuous flow polyacrylamide gel electrophoresis. Finally, the mAbs selected for *G. rostochiensis* were tested with fluorescence microscopy for cross-reactivity with other endoparasitic nematode species.

MATERIALS AND METHODS

Nematodes

Second stage juveniles (J_2) of *Globodera rostochiensis* pathotype Ro1, and of *G. pallida* pathotype Pa$_2$ were hatched by soaking cysts on a 100 μm sieve in potato root diffusate [2]. J_2 of *Meloidogyne hapla* were a gift from Mrs. E. Jansen, DLO Research Institute for Plant Protection, Wageningen, The Netherlands. The J_2 suspensions were mixed with an equal volume of 70% (w/v) sucrose in a centrifuge tube, covered with a layer of tap water, and centrifuged briefly at 1000 *g*. Purified

juveniles were collected from the sucrose-water interface with a Pasteur pipette, washed with tap water and used for experiments. J_2 of *G. tabacum*, *Heterodera glycines*, *H. schachtii* and *M. incognita* were obtained as described by Goverse *et al.* [12].

Stylet secretions

Stylet secretions were induced using DMT (5-methoxy-N,N-dimethyltryptamine-hydrogen-oxalate; Research Biochemicals, Natick, USA), and collected according to Goverse *et al.* [12]. For each replicate an estimated 20,000 preparasitic J_2 were collected and exposed overnight to 4 ml of a test solution at 20°C. When appropriate, 0.01% Coomassie Brilliant Blue G-250 (CBB G-250) was added to enhance the visibility and collection of stylet precipitates.

Preparative electrophoresis

In total 2.75 million J_2 of *G. rostochiensis* were homogenised in 208 mM Tris-HCl pH 6.8 supplemented with 8.33% (v/v) 2-mercaptoethanol at 5°C using a small glass mortar and pestle. The J_2 were homogenised in aliquots of approximately 300,000 individuals. Following homogenisation the samples were pooled and mixed in a ratio of 3:2 (v:v) with a solution of 5% SDS / 25% glycerol / 0.1% Bromophenol Blue, thus producing standard SDS-sample buffer [18]. The homogenate was heated for 5 min in boiling water, centrifuged for 5 min at 10,000 *g*, and the supernatant was stored at -80°C until used. The nematode sample (2 ml, approximately 13 mg of protein) was fractionated by preparative SDS-PAGE using a Model 491 Prep Cell apparatus (Bio-Rad, Richmond, California, USA), essentially according to the instruction manual, with the correction that 0.1% SDS was added to the buffers of the acrylamide gels. The cylindrical separating gel was 50 x 28 mm (height x diameter) and contained 10% acrylamide. The stacking gel contained 4% acrylamide and was 15 mm in height. The proteins were separated with a current of 40 mA, and 1.5 ml fractions were collected using an elution buffer flow rate of 1 ml/min. The fractions were concentrated by freeze drying, washed three times in phosphate buffered saline pH 7.4 (PBS) using 1.5 ml microcentrifuge filter concentration units with molecular weight cut-off of 5 kDa (Ultrafree-MC, Millipore Corp., Bedford, USA), taken up in 50 μl PBS and stored at -20°C. Groups of 2 to 5 fractions were pooled, and each pooled sample was diluted in PBS to obtain three 200 μl aliquots for successive immunisations.

Immunisations and cell fusions

Sixteen mice were immunised intraperitoneally with the isolated protein fractions, which were mixed 1:1 with Freund's incomplete adjuvant. After 4 weeks a second immunisation was given, also with incomplete adjuvant. Two weeks later antiserum samples were collected for immunofluorescence microscopy. One mouse that was selected for monoclonal antibody production received a final booster injection 12 weeks after the first immunisation. Three days later the mouse was sacrificed, and mAb-producing hybridoma cell lines were obtained by fusing spleen cells with SP 2/0 myeloma cells [11,21].

Immunofluorescence microscopy

Mouse antisera and hybridoma cell line culture fluids were screened by indirect immunofluorescence microscopy for reaction with J_2 of *G. rostochiensis* as described in detail elsewhere [6]. Briefly, J_2 were fixed in 2% paraformaldehyde for 3 days, dried, and cut into sections with a razor blade. Following treatment with proteinase-K, methanol, and acetone, the nematode sections were incubated with the primary antibody and stained with rat-anti-mouse FITC-conjugated immunoglobulins. The mouse antisera were tested in a 1:400 dilution. Hybridoma cell lines producing antibodies to the subventral oesophageal glands of *G. rostochiensis* were subcloned to stability and stored in liquid nitrogen. Isotyping of the light and heavy chains of the mAbs was done as described by Schots *et al.* [21]. Immunofluorescence testing of J_2 from *G. pallida* and *M. hapla* followed the same procedure as for *G. rostochiensis*, with the exception that the initial fixations in paraformaldehyde were different: two days for *G. pallida*, and one day for *M. hapla*. Immunofluorescence testing of J_2 from *G. tabacum*, *M. incognita*, *H. glycines* and *H. schachtii* was as described by Goverse *et al.* [12].
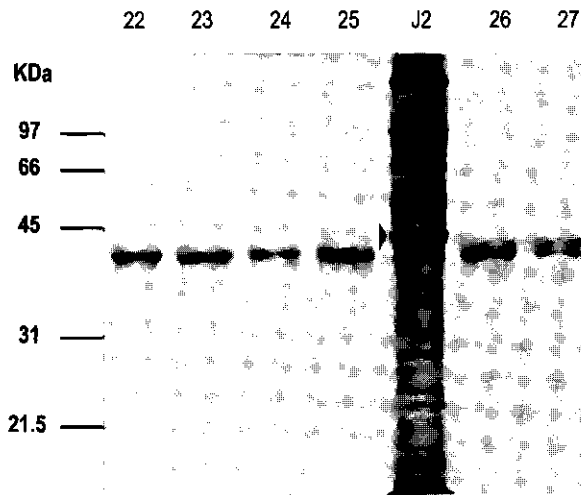
Figure 2.1   SDS-polyacrylamide gel electrophoresis pattern of selected protein fractions purified from homogenised second stage juveniles (J_2) of Globodera *rostochiensis by preparative continuous flow electrophoresis. The lanes numbered 22 to 27 show successive fractions of increasing molecular weight. The mouse that was immunised with the pooled fractions 22 to 25 (molecular weight range 38-40.5 kDa) produced antiserum to the subventral oesophageal glands, and was used for monoclonal antibody production. The lane labelled "J2" shows a total protein homogenate of J_2. The arrow indicates a characteristic major protein band (presumably actin) which can be used to compare Fig. 2.1 with Fig .2. 4.*

Coomassie brilliant blue-stained stylet secretions were collected by micropipet and used for indirect immunofluorescence microscopy as described [3,12] 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) bovine serum albumin in PBS for 10 min before antibody treatments.

SDS-PAGE and Western blotting

Analytical mini SDS-PAGE was performed essentially as described by De Boer *et al.* [5]. For the examination of the protein fractions that were obtained with preparative electrophoresis, 4 µl of SDS-sample buffer was added to 2 µl from the concentrated fractions in PBS, and these samples were applied to 20 µl slots in the stacking gel. Following electrophoresis, the gels were stained with colloidal Coomassie Blue G-250 [17]. For Western blot testing of mAbs, J_2 of *G. rostochiensis* were homogenised as described above, and per minigel approximately 10,000 J_2 were added to a single 73 mm wide slot in the stacking gel. An adjacent reference well (3 mm wide) was filled with prestained molecular weight markers (Bio-Rad, Richmond, USA). Following electrophoresis (13% separating gel) the proteins were transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, USA) using a semi-dry blotting apparatus. A continuous transfer buffer system was used containing 39 mM glycine, 48 mM Tris and 20% (v/v) methanol. Transfer was carried out with 0.8 mA/cm^2 for 1 hour. The blots were cut into strips, which were blocked overnight in PBS pH 7.4 / 0.1% (v/v) Tween-20 (PBST) supplemented with 5% (w/v) skimmed milk powder. Following a wash in PBST / 1% milk powder, the strips were incubated for 2 hours in hybridoma culture fluid, diluted 1:6 in PBST/1% milk powder. For mAb MGR 48 affinity-purified antibody (1 µg/ml) was used instead of culture fluid. After washing three times in PBST / 1% milk powder, the strips were next incubated

Table 2.1. Immunoreactivity of monoclonal antibodies (mAbs) directed to subventral oesophageal glands of Globodera rostochiensis. mAbs were used in immunogold labeling (IEM) of ultrathin sections of preparasitic second-stage juveniles (J_2). Stylet secretions were collected after incubating J_2 24 hours in 6.5 mM DMT and subsequently tested in an indirect immunofluorescence (IF) assay.

Antibody (MGR)		46	47	48	49	50	53	54	55	56	57	59	60
IEM in J_2	location	SG	SvG	SG	SG	SvG	SG	SvG	SvG	SG	SG	SG	SvG
	intensity	+++	+	++++	+++	+/-	++	+	++	+++	++	++	+
IF stylet-secretions	intensity	++	-	++	++	-	++	+	-	++	+	+	+

SG = labelling in secretory granules of subventral oesophageal glands; SvG = labelling of subventral oesophageal glands, no clear localisation in secretory granules;
+/++/+++/++++ = increasing labelling intensity; - = no specific labelling

individually in alkaline phosphatase conjugated rat-anti-mouse IgG (H+L) (Jackson Immuno Research Laboratories Inc, West Grove, USA) diluted 1:5,000 in PBST / 1% milk powder for 1 hour. After washing in PBST / 0.1% milk powder (1x) and PBST (3x) the strips were stained individually in 0.1 M ethanolamine-HCl pH 9.6, supplemented with 4 mM $MgCl_2$, 0.06 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.1 mg/ml nitro blue tetrazolium.

Electron microscopy

For ultrastructural examination, J_2 of *G. rostochiensis* were fixed at 4°C with 4% paraformaldehyde in 0.1 M HEPES-buffer pH 7.5 for 2 days. The suspension of fixed J_2 was spread on a microscope slide and the nematodes were chopped into pieces with a razor blade. The nematode sections were then sequentially fixed with 2% glutaraldehyde and 1% osmium tetra-oxide (both in 0.1 M HEPES pH 7.5), and stained with 1% aqueous uranyl acetate. Following stepwise dehydration in ethanol, the J_2 were infiltrated for 1 day with Spurr epoxy resin [22]. The nematode fragments were then transferred to fresh epoxy resin in a BEEM capsule, centrifuged to the bottom, and polymerised at 60°C. Thin sections were cut with an LKB ultra-microtome, collected on formvar-coated 100 mesh copper grids, and post-stained with 2% uranyl acetate in 50% methanol.

For immunogold labelling, J_2 of *G. rostochiensis* were fixed for 2 days at 4°C in 2% paraformaldehyde in PBS pH 7.4. After chopping them into pieces, the J_2 were washed 2 times in distilled water, dehydrated in 30%-50%-70%-96% ethanol, infiltrated at room temperature for 1 hour in a 1:1 mixture of 96% ethanol and LR-White acrylic resin (London Resin Co. Ltd., Basingstoke, England), and subsequently in pure LR-White resin for 4 hours. The nematode fragments were transferred to fresh resin in a gelatin capsule, centrifuged to the bottom, and polymerised at 60°C. Following ultramicrotomy, thin sections were collected on formvar-coated copper grids and immunolabelled with colloidal gold according to the following protocol (Aurion Immuno Gold Reagents, Wageningen, The Netherlands): 10 min PBS pH 7.6 / 0.05 M glycine, 2 hours affinity purified mAb MGR 48 diluted to 1 µg/ml in incubation buffer (PBS pH 7.6 / 0.2% BSA-C / 20 mM NaN_3), 6x 5 min wash with incubation buffer, 2 hours colloidal gold solution (10 nm particle size, conjugated with goat-anti-mouse-IgG immunoglobulins; Aurion Immuno Gold Reagents, Wageningen, The Netherlands) diluted 1:10 in incubation buffer, 6x 5 min wash with incubation buffer, 3x wash with PBS, and 3x wash with distilled water. The sections were stained with 1% aqueous uranyl acetate. Control labelling experiments were performed with a mouse monoclonal antibody specific to the body wall musculature of J_2 of *G. rostochiensis.*
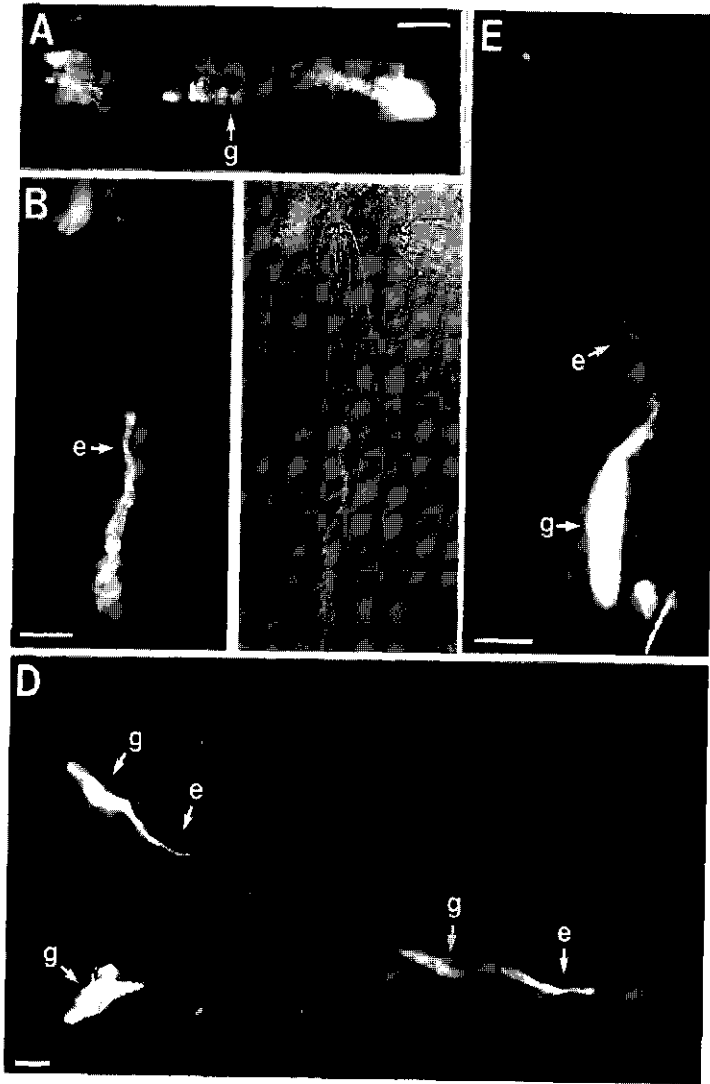
Figure 2.2 *Immunofluorescence labelling of the subventral oesophageal glands (G) and their extensions (E) in second stage juveniles of potato cyst-nematodes. (Because both glands lie in close apposition, their cell bodies and their extensions appear as single structures in the micrographs).* **A.** Globodera rostochiensis *stained with mouse antiserum raised to a 38.5-40 kDa protein fraction derived from J$_2$ of G. rostochiensis. Individual secretory granules can be distinguished within the gland cells.* **B, C, and D,** G. rostochiensis *labelled with monoclonal MGR 48. Additional bright field illumination (C) shows that the gland extensions terminate in the metacorpus (M) of the oesophagus.* **E,** G. pallida *labelled with monoclonal MGR 48. (Due to the cutting of the J$_2$ prior to immunolabelling, the subventral glands have come to lie outside this juvenile, with only their extensions still in place). Bars: 20 μm.*

RESULTS

Antibody production

Homogenised J_2 of *G. rostochiensis* were separated by preparative continuous flow electrophoresis in the presence of sodium dodecyl sulfate (SDS) into 50 protein fractions. The average molecular weight of these protein fractions ranged from 30 to 52 kDa. Examination of successive fractions by analytical SDS polyacrylamide gel electrophoresis (SDS-PAGE) revealed narrow protein zones, with a very gradual increase in molecular weight (Fig. 2.1). Therefore the original fractions were pooled to form a representative set of 16 protein samples of increasing molecular weight, which were used for immunising 16 mice. Antisera collected after the second immunisation with these samples were screened with immunofluorescence microscopy for reaction with the oesophageal glands of J_2 of *G. rostochiensis*. It was found that the eight mice that were immunised with the successive protein fractions from 36.5 kDa to 52 kDa had produced antisera that showed a strong reaction with the subventral oesophageal glands (Fig. 2.2A). In addition, a very weak reaction with the subventral glands was observed with the antiserum to the 31-33 kDa fraction. The mouse that was immunised with the 38-40.5 kDa protein sample (Fig. 2.1) was chosen for monoclonal antibody production.

Immunofluorescence microscopy

Screening of the hybridoma culture supernatants by immunofluorescence microscopy of nematode sections identified 12 mAbs that bound specifically to the subventral glands of J_2 of *G. rostochiensis* (Fig. 2.2B, C, D). The staining of the glands by the mAbs was found to be variable, both between individual J_2 within a single experiment and between repeated experiments with the same antibody. Nevertheless, 10 mAbs (MGR 46, 47, 48, 49, 50, 53, 54, 55, 56, 59) were able to stain the entire contents of subventral gland cells, including the gland extensions (Fig. 2.2D). Two mAbs (MGR 57 and 60) persistently failed to penetrate the gland cells sufficiently to obtain an even staining reaction.

Substantial quantities of precipitated stylet secretion from preparasitic J_2 were obtained upon exposure of the J_2 to 6.5 mM DMT. In an indirect immunofluorescence assay the 12 mAbs (MGR 46 to 48) were tested for labelling of DMT-induced stylet secretions (Table 2.1, and Fig. 2.3). MGR 46, 48, 49, 53, 54, 56, 57, 59, and 60 stained only parts of the precipitates. 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 oesophageal glands nor metacorpal activity was observed, the use of DMT was strongly correlated with stylet thrusting. Immunofluorescence microscopy was also used to test the cross reactivity of the mAbs with J_2 of other species of plant parasitic nematodes (Table 2.2). It was found that nearly all mAbs also bound to the subventral glands of *G. pallida* (Fig. 2.2E) and *G. tabacum*. However, except for MGR 48, which reacted with *H. glycines*, none of the mAbs showed binding to the oesophageal glands of the other species examined.
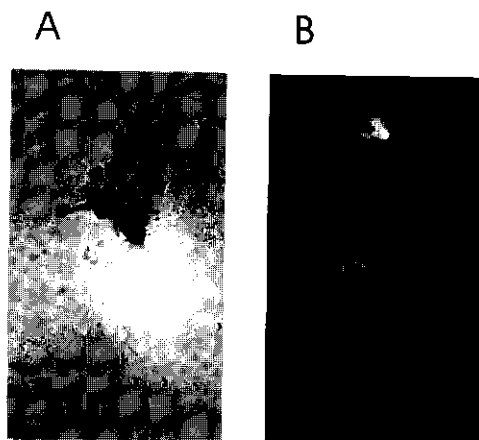
Figure 2.3 *Light (A) and indirect immunofluorescence (B) microscopy of MGR48 binding collected stylet secretions of* Globodera rostochiensis *J_2 hatched in potato root diffusate and incubated for 24 hours in DMT (bar = 10 μm).*
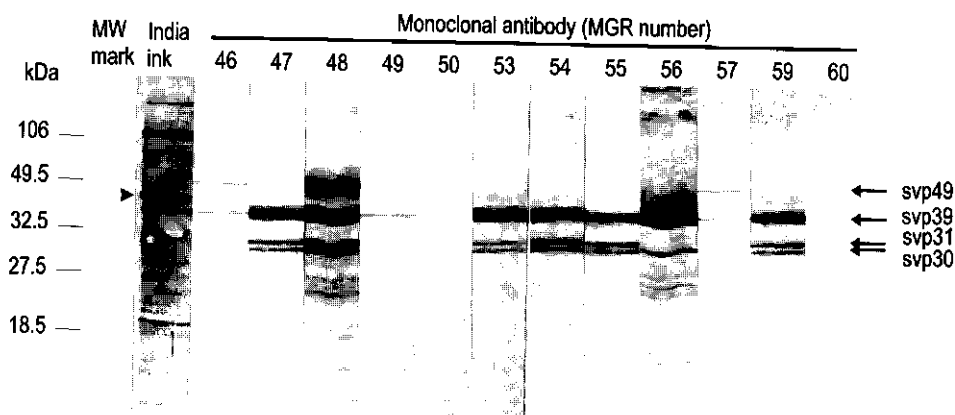
Figure 2.4 *Western blot of second stage juveniles (J_2) of* Globodera rostochiensis *stained with a panel of monoclonal antibodies (MGR 46 to 60) specific to the subventral oesophageal glands. Four major protein bands are identified, labelled svp30, svp31, svp39, and svp49. MW Mark = pre-stained molecular weigh markers. India ink staining shows the complete protein pattern of J_2. The arrow indicates a characteristic major protein band (presumably actin) which can be used to compare Fig. 2.4 with Fig. 2.1.*

Western blotting

The mAbs were tested for reactivity with protein homogenates of J$_2$ which were separated by SDS-PAGE and electroblotted onto PVDF membrane. Eleven of the 12 mAbs stained protein bands in the electrophoresis pattern (Fig. 2.4). Together these mAbs identified four different polypeptides with apparent molecular weights of 30 kDa (*svp*30), 31 kDa (*svp*31), 39 kDa (*svp*39) and 49 kDa (*svp*49) respectively. All reactive mAbs bound to the *svp*39 band, with 10 showing additional binding to one or more of the other bands. MGR 47, 53, 54, 55, and 59 all reacted strongly with the *svp*30, *svp*31 and *svp*39 bands, while MGR 46, 57 and 60 showed moderate binding to *svp*39 and *svp*49. Reactivity with all four protein bands was found only with MGR 48. In addition, MGR 48 stained several minor bands in the gel region below *svp*30. Staining of minor bands was also observed with MGR 56, both below *svp*30 and above *svp*39.

Electron microscopy

Because ultrastructural information on the subventral glands in potato cyst nematodes was completely lacking and could only be inferred from studies with related plant-parasitic nematode species [7-10], we have first examined subventral gland morphology in hatched J$_2$ of *G. rostochiensis*. Thin sections showed that both the cell body (Fig. 2.5) and the cell extension are packed with secretory granules. It was only in the immediate vicinity of the nucleus that parts of the cytoplasm were free of granules. The secretory granules invariably possessed an electron translucent halo, which surrounded an electron dense core. This morphology made it easy to identify the subventral glands in the low-contrast formaldehyde fixed specimens that were used for immunolabelling. Immunogold labelling of formaldehyde-fixed J$_2$ with 12 subventral gland specific mAbs was found to be variable in intensity and location (Table 2.1). MRG 46, 48, 49, 53, 56, 57, and 59 clearly labelled in the electron dense granule matrix, and to a lesser extent to the patches of cytoplasm surrounding the granules (Fig. 2.6). A positive correlation was found between the labelling intensity of the antibody and the location of the antigens in the granule matrix.

DISCUSSION

In this paper we used a novel method of immunogen preparation to produce monoclonal antibodies specific to oesophageal gland antigens of a sedentary plant parasitic nematode. Mice were immunised with protein fractions, which were produced by preparative continuous flow SDS-electrophoresis of homogenised J$_2$. Following immunofluorescence screening of hybridomas we identified a panel of 12 mAbs specific to the subventral glands of J$_2$ of *G. rostochiensis*. Most of these antibodies differed from mAbs, which we obtained previously to the subventral glands of *G. rostochiensis* [6] in that they bound to SDS-denatured epitopes. This enabled the identification of subventral gland specific proteins on Western blots of J$_2$ of *G. rostochiensis*.
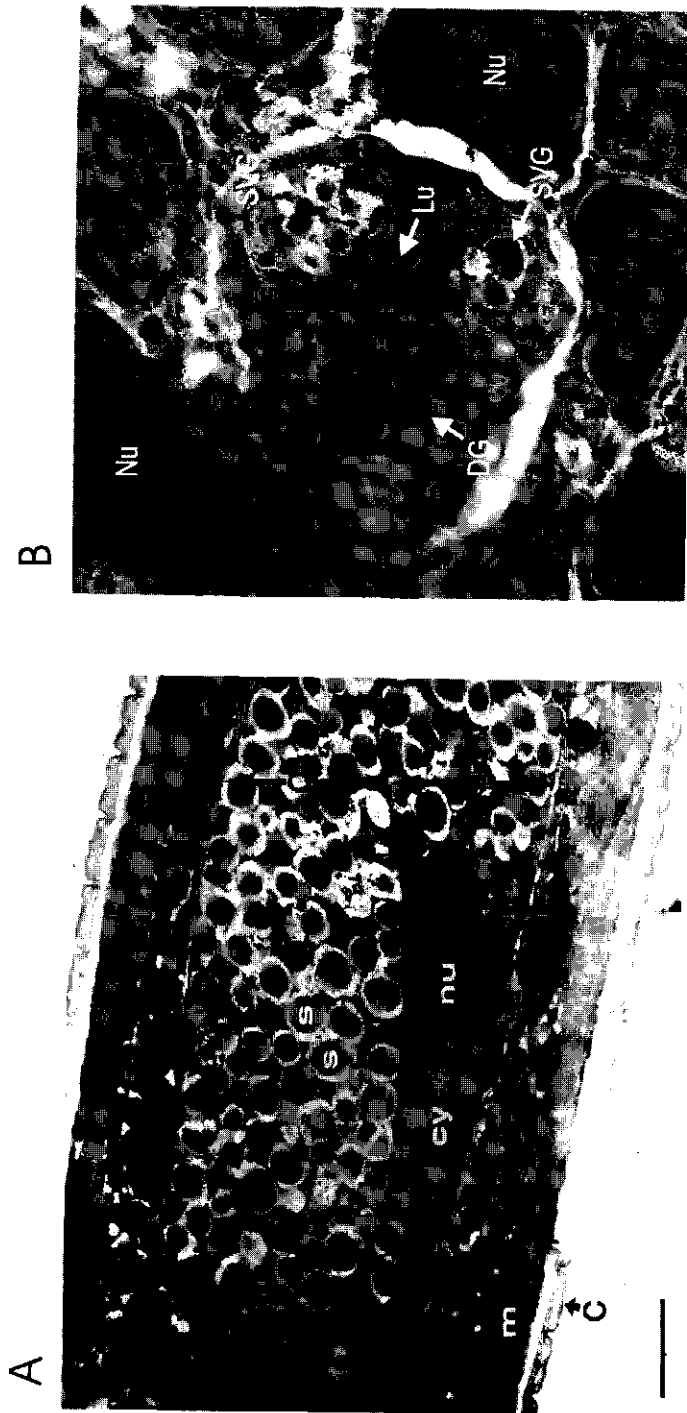
Figure 2.5 A. Longitudinal section through the subventral gland region of a second stage juvenile of Globodera rostochiensis. The two subventral gland cells (g1 and g2) lie adjacent to each other and they are both packed with secretory granules (s). Cytoplasm free of secretory granules (cy) could only be found near the subventral gland nucleus (nu). m = body wall muscle filaments. C = cuticle. Bar = 2 μm. B. Cross section through the oesophageal gland region where the extensions of the three gland cells align forward. The extensions of both types of oesophageal glands are packed with secretory granules. Lu = triradiate lumen of the oesophagus; DG = dorsal gland extension; SVG = subventral gland extensions.

The panel of mAbs presented here identified four major protein bands in the electrophoresis pattern of J_2 of *G. rostochiensis*. All the mAbs that gave a positive reaction on Western blot identified a protein band of 39 kDa (*svp39*). This molecular weight corresponds with the protein fraction that was used for immunisation. In addition three other proteins with smaller and larger molecular weights (*svp30, svp31,* and *svp49*) were identified. It is likely that these additional polypeptides are structurally related to the *svp39* band. Possibly these four proteins represent variants of a single gene product, which differ in post-translational modification or in the length of the polypeptide chain. The different reactivities with our panel of mAbs would then give information about the epitopes that are available on these related polypeptides. Thus MGR 47, 53, 54, 55, and 59 may be directed to an epitope that is present only on the *svp30, svp31* and *svp39* bands, whereas MGR 46, 57 and 60 may bind to an epitope specific for *svp39* and *svp49*.
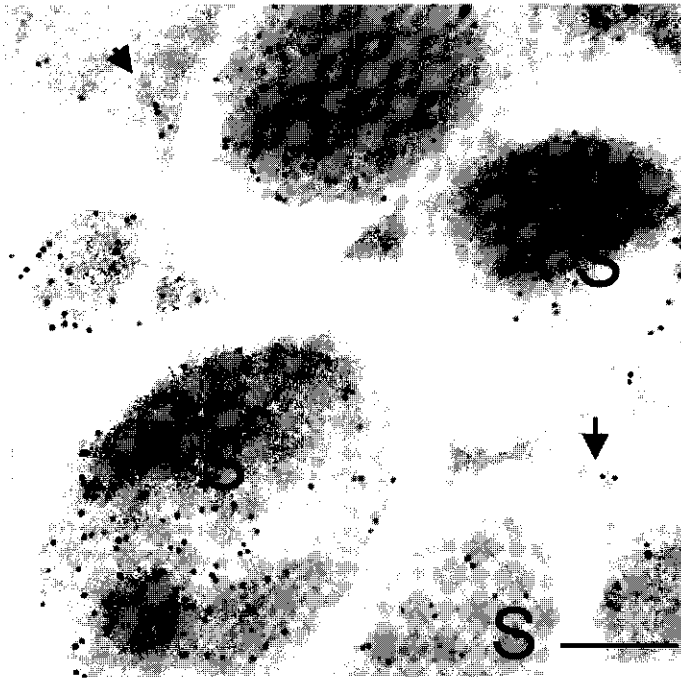
Figure 2.6 *Immunogold labelling of a subventral oesophageal gland of a J_2 of* Globodera rostochiensis *with monoclonal antibody MGR 48. The nematodes were fixed with formaldehyde, and embedded in LR-White resin. The gold particles localise to the relatively electron dense core of the secretory granules (S). Labelling is also found outside the granules (e.g. arrows). Bar = 0.25 μm.*

MAbs, which identified four protein bands on western blots of J_2, were used to determine the ultrastructural location of these polypeptides within the subventral gland cells. Immunogold labelling showed an intense binding of MGR 46, 48, 49, 53, 56, 57,

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and 59 to the electron dense core of the secretory granules. It is therefore concluded that one or more of the proteins identified by these mAbs are localised within the secretory granules of the subventral gland. Some labelling was also found in patches of cytoplasm between the granules. This could be the result of a release of antigen from the secretory granules due to the combined effects of mild tissue fixation and ethanol dehydration during specimen preparation. Alternatively, this may indicate that one or more of the polypeptides (also) reside outside the granules, for instance in the endoplasmic reticulum.

Table 2.2 List of monoclonal antibodies to the subventral oesophageal glands of Globodera rostochiensis and their immunofluorescence cross-reactivity with other species of sedentary plant-parasitic nematodes (Globodera pallida [G. pall.], G. tabacum [G. taba], Heterodera schachtii [H. schac], H. glycines [H. glyc], Meloidogyne incognita [M. inco], and M. hapla) . "+" -reaction with subventral glands, "-" no reaction with subventral glands

Antibody[a]	Isotype		G.pall	G. taba	H. schac	H. glyc	M. inco	M. hapla
	Heavy	Light						
MGR46	IgG2a	λ	+	+	·	-	-	-
MGR 47,50, 53, 55,56,59	IgG1	κ	+	+	-	-	-	-
MGR 48	IgG1	κ	+	+[b]	-	+	-	-[b]
MGR 49	IgG3	κ	+	-	-	-	-	-
MGR 54	IgG2a	κ	+	+	-	-	-	-
MGR 57, 60	IgG1	λ	+	+	-	-	-	-

[a] Affinity-purified antibody (MGR 48) or hybridoma culture fluid (all other mAbs).
[b] Background staining.

The neurotransmitter analogue DMT was used to investigate the presence of subventral oesophageal gland antigen in stylet secretions of *H. glycines* [12]. We have used DMT to determine whether the 12 mAbs reactive for the subventral glands in *G. rostochiensis* recognise antigens in stylet secretions, too. It was found that labelling of collected stylet secretions is positively correlated with binding to the core of the secretory granules in the gland cells for five mAbs (MGR 46, 48, 49, 53, and 56).

From the panel of antibodies mAb MGR48 seems to be a good candidate antibody for further biochemical and molecular studies because this antibody exhibits the highest binding affinity in immunolabelling experiments. The binding affinity of MGR48 is illustrated by the observation that the antibody allows the detection of proteins extracted from less than ten individual J_2 on a dot blot (data not shown). In addition, mAb MGR48 seems to be particularly useful because it recognises all four *svp*s described in this study.

In conclusion, we have identified subventral gland specific proteins of J_2 of *G. rostochiensis* using monoclonal antibodies that were raised against a highly purified

SDS-denatured immunogen. These antibodies will be valuable tools in future studies relating to the nature of these antigens and their possible function in the host-parasite interaction. We have shown that immunisation with electrofractionated total protein homogenates can be a useful method for obtaining mAbs against oesophageal gland antigens of plant parasitic nematodes. It is expected that by using fractionated proteins from other molecular weight ranges or from other developmental stages, a systematic search for additional oesophageal gland antigens of *G. rostochiensis* will be possible.

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Chapter 3

Potato root diffusate-induced secretion of soluble, basic proteins originating from the subventral oesophageal glands of potato cyst nematodes[1]

Abstract

In preparasitic second stage juveniles (J_2) of *Globodera rostochiensis*, six proteins with molecular weights of 30, 31a/b, 32, 39, and 49 kDa were recognised on western blots by a monoclonal antibody (MGR48) specific for the subventral oesophageal glands. All these subventral gland proteins (*svps*) focused in the basic range (pI 6.8-8.6) of an immobilised pH gradient. The *svps* were present in preparasitic and in parasitic J_2, and not in following juvenile stages and adult females. Minor *svp* quantities were also observed in adult males. Immunogold labelling of preparasitic (J_2) showed that the *svps* were localised in the rough endoplasmic reticulum and secretory granules of the subventral oesophageal glands. Potato root diffusate triggered the secretion of the *svps* through the stylet and 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 oesophagus (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 oesophageal glands play an important role in the early events of this nematode-plant interaction.

INTRODUCTION

Nematode oesophageal gland secretions are thought to play an important role in the host-parasite interaction [17,18]. Cyst nematodes have three large oesophageal gland cells, that is one dorsal and two subventral glands. The dorsal gland has a long cytoplasmic extension that terminates in an ampulla, a reservoir for secretory granules. The ampulla is connected, via a sclerotised duct with an elaborate valve, with the oesophageal lumen just behind the stylet knobs [13,14]. 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 oesophageal glands terminate in ampullae within the oesophagus located posterior to the metacorporal pump chamber [13]. It has been argued that the metacorporal pump chamber of cyst nematodes, and also of root-knot nematodes, acts as a unidirectional valve which precludes forward flow [12,30,32]. It has therefore been suggested that subventral gland secretions are solely transported posteriorly and involved in intracorporal digestion [12] or the mobilisation of lipid reserves [31].

Various artificial agents are used to obtain stylet secretions from plant-parasitic nematodes *in vitro* [6,7,16,19]. For example, the serotonin analogue, 5-methoxy-N,N-

dimethyltryptamine-hydrogen-oxalate (DMT), induces stylet secretions, which are precipitated at the lip region [6,16]. Using monoclonal antibodies it was shown that antigens from the subventral glands of preparasitic J_2 of *Heterodera glycines* are present in DMT-induced stylet secretions [16]. Comparable results for subventral oesophageal gland proteins were obtained with *Meloidogyne incognita* using resorcinol as the inducing agent of stylet secretions [7].

Detailed information on the nature of stylet secretions of plant-parasitic nematodes is essential for understanding the complex feeding behaviour 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 characterisation [15]. In the last decade research on stylet secretions focused on generating monoclonal antibodies against the secretory granules within the oesophageal glands. Various panels of monoclonal antibodies have been raised against stylet secretions of *H. glycines* [16], *Heterodera avenae* [6], *Globodera rostochiensis* ([10] see also chapter 2), and *M. incognita* [7,8]. However, biochemical information on the corresponding antigens is scarce.

To study the secretory pathway of proteins from the subventral oesophageal gland cells to the stylet secretions of *G. rostochiensis*; we used monoclonal antibody (MGR48) recognising six subventral gland proteins (*svps*). One could argue that chemically induced stylet secretions are a consequence of the stress that is imposed upon the nematode by the chemical compounds used for this purpose. Therefore, we have investigated the biological relevance of the DMT-induced stylet secretions, and the concomitant release of subventral oesophageal gland antigens. 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.

MATERIALS AND METHODS

Nematodes
Globodera rostochiensis (Wollenweber) Skarbilovich pathotype Ro_1 was used in all experiments. Nematodes were reared and hatched as described previously [9,23]. When diapause is completed, a minority of J_2 will spontaneously hatch upon hydration in tap water. The resulting J_2 are indicated in this paper as tap-water-hatched J_2. When eggs are incubated in potato root diffusate (PRD) [5] the majority of the J_2 will hatch. These J_2 are named PRD-hatched J_2.

Samples of parasitic J_2 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 (J_3) were collected 16 days post-inoculation, whereas mixed 3rd and 4th (J_4) 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.* [9]. Eggs with young J_2 were collected from females 2 months post-inoculation. Eggs containing J_2 in diapause were collected from dry cysts.

Protein extraction and SDS-PAGE

SDS-extracted proteins from PRD-hatched J_2 of *G rostochiensis* were obtained by homogenisation in 10 mM sodium phosphate buffer pH 7.2 using a 2-ml Potter-Elveh-jem homogeniser 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.* [10]. Pre-stained SDS-PAGE standards (Biorad, Richmond, USA) were used in each run.

Two-dimensional gel electrophoresis

Freshly PRD-hatched J_2 of *G. rostochiensis* were homogenised in 1% (v/v) Nonidet P-40 and 2% (v/v) 2-mercaptoethanol. Protein concentration was estimated according to Bradford [4] 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, Germany) 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 immobilised pH 3 to 10 gradient (Immobiline Dry Strips 3-10L, Pharmacia LKB Biotechnology, Upsala, Sweden). The immobilised 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 separated by second-dimension electrophoresis in an 8-18% (w/v) SDS-PAGE gradient gel (ExcelGel 110x245x0.5 mm, Pharmacia). The SDS-PAGE gel was either used for semi-dry western blotting onto PVDF-membrane at 0.8 mA/cm^2 for 1 h, or for silver staining [20].

Induction and collection of stylet secretions

Unless stated otherwise PRD-hatched preparasitic J_2 were used for the induction of

stylet secretions. For each replicate an estimated 20,000 preparasitic J_2 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 [7,16].

To test the effect of hatching conditions on the production of stylet secretions, either tap-water-hatched or PRD-hatched J_2 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 [24]. 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 J_2 suspension vigorously on a whirl mix followed by centrifugation at 10,000 x g for 5 min. The supernatant contained the solubilised stylet secretions and was checked for absence of J_2 using a dissecting microscope. Secretions were either processed for denaturing western blotting or for native dot blotting.

Incubation fluids with solubilised 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.* [11]. For dot blot assays, 2 ml native solubilised stylet secretion was concentrated onto a nitrocellulose blotting membrane under mild vacuum (Schleicher and Schuell, Dassel, Germany). Subsequently, the dot blot was left to dry and processed for immunodetection as described [11]. 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.* [29]. 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 preparasitic J_2 was performed similar to De Boer *et al.* [11]. Coomassie brilliant blue-stained stylet secretions were collected by micropipet and used for indirect immunofluorescence microscopy as described [7,16] 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) bovine serum albumin in PBS for 10 min before antibody treatments.
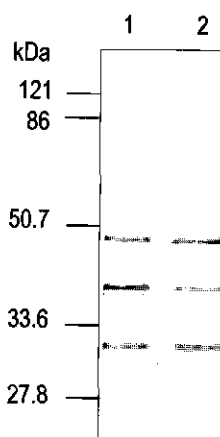
Figure 3.1 *Western blot of SDS-extracted proteins from preparasitic J_2 of* Globodera rostochiensis *after incubation in periodic acid. The experimental lane (2) was treated with 50 mM periodic acid in sodium acetate buffer (pH 4.5) to allow oxidation of carbohydrate side-chains. As compared to the control lane (1), no reduction in labelling intensity was observed using MGR48.*

Immunoelectron microscopy.

G. rostochiensis preparasitic J_2 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) [11]. Thin roots of potato (*S. tuberosum* spp. *tuberosum* cv. Bintje) were inoculated with *G. rostochiensis* [27]. Parasitic J_2 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 [28].

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) bovine serum albumin-C (Aurion, Wageningen, NL) in PBS, 2 h on monoclonal antibody hybridoma supernatant (diluted 1:20 in 0.2% (v/v) bovine serum albumin-C in PBS), six washes of 5 min each with 0.2% (v/v) bovine 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) bovine serum albumin-C in PBS, four washes of 5 min each with 0.2% (v/v) bovine 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 [22].

RESULTS

Electrophoretic characterisation of subventral gland proteins

SDS-extracted proteins from homogenised preparasitic J_2 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. 3.1, lane 1) and non-reducing conditions (data not shown). Presumably due to a different homogenisation procedure as compared to De Boer *et al.* [11], *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 an effect on the binding pattern of MGR48 on western blot (Fig. 3.1; lane 2).

Six protein spots were detected with MGR48, when protein extracts of preparasitic J_2 were separated with two dimensional electrophoresis (IPG-Dalt; Fig. 3.2a) and electroblotted on PVDF-membrane (Fig. 3.2b). *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 blot before (see chapter 2), 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.

Secretion of subventral gland proteins

Immunogold labelling experiments on thin sections of aldehyde-fixed preparasitic PRD-hatched J_2 with MGR48 revealed clearly a specific concentration of gold particles within the matrix of secretory granules of the subventral oesophageal gland cells (Fig. 3.3A). MGR48 also bound within the lumen of rough endoplasmic reticulum surrounding the nucleus of the subventral gland cells (Fig. 3.3B). Localisation in smaller subcellular organelles such as Golgi body was not possible due to
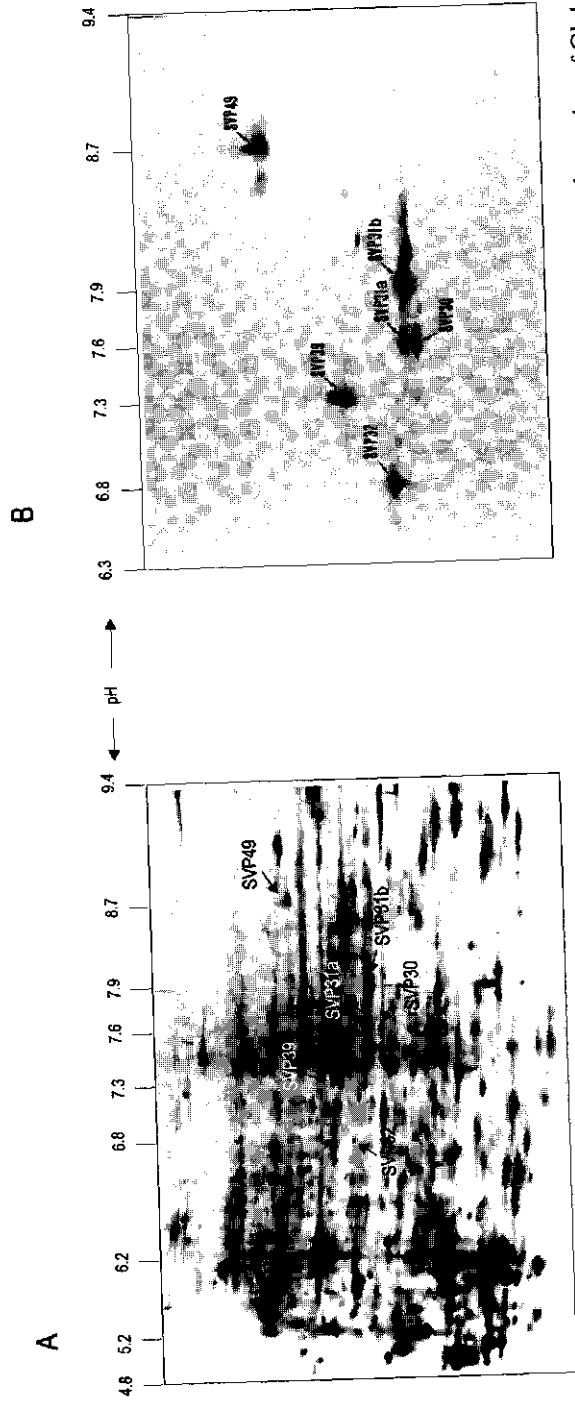
Figure 3.2 *Two dimensional gel electrophoresis (IPG-Dalt) and western blotting of urea/Nonidet P-40- extracted proteins of Globodera rostochiensis. Isoelectric focusing was done in a immobilised pH gradient (pH range is 3 to 10). In the second dimension, a linear SDS-polyacrylamide gradient (8-18%) was used. (A) Silver stained gel including arrows that indicate the proteins species recognised by MGR48 on western blot (B). Only the part of the western blot that showed labelling with MGR48 is depicted.*

poor preservation of the ultrastructure.

Indirect immunofluorescence with MGR48 was used to compare DMT-treated and non-treated preparasitic PRD-hatched J_2. A more granular and less intense labelling of the subventral oesophageal glands was observed in DMT-treated J_2 suggesting a decrease in gland content (data not shown). Immunogold electron microscopy of DMT-exposed J_2 confirmed these observations. Both the dorsal and subventral oesophageal 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 translucent structures was observed among the typical homogeneous electron-dense granules in the cell body of the dorsal oesophageal gland (Fig. 3.4).

The precipitation of stylet secretions that is 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 J_2 in various acidic buffers (pH<5.5) without DMT. All acidic buffers used resulted in the precipitation of secretions at the stylet tip.
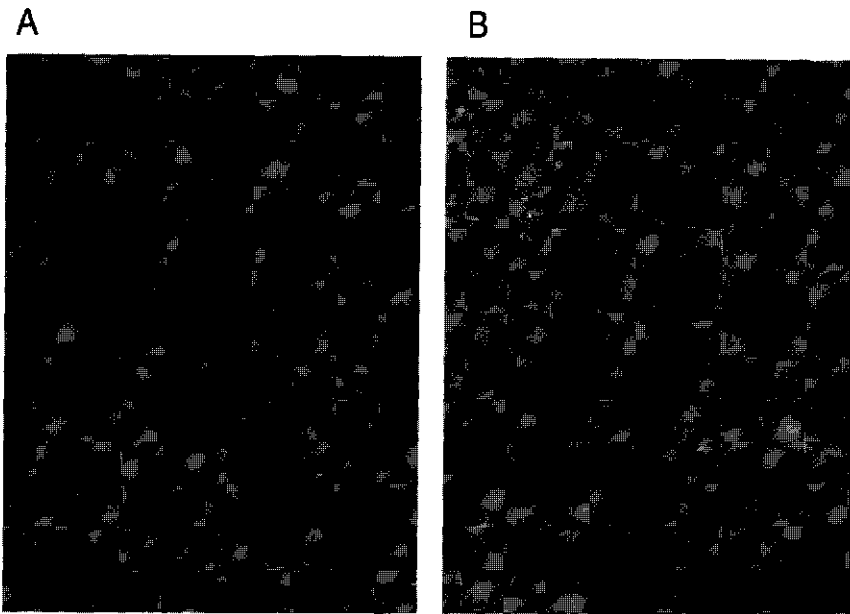

A **B**

Figure 3.3A and B Immunogold labelling of the subventral oesophageal gland cell of a J_2 of Globodera rostochiensis *with mAb MGR48. The gold particles localise to the rough endoplasmic reticulum (ER) surrounding the nucleus (N), and the dense core of the secretory granules (SG) (bars = 400 nm).*

Testing the effect of different hatching conditions showed that, apart from stimulation of hatching, potato root diffusate also triggers the secretion of *svps*. Incubation of tap-water-hatched J_2 in standard pore water did not result in the secretion of *svps* at a detectable level on dot blots using MGR48 (Fig. 3.5A). In contrast, substantial quantities of *svps* were detected on dot blot when PRD-hatched J_2 were used.

DMT supplemented to the incubation fluid (standard pore water) resulted in an additional effect on secretion of *svps*. DMT stimulated secretion of *svps* by tap-water-hatched J_2. Secretion of *svps* was further increased if PRD-hatched J_2 were used instead of tap-water-hatched J_2. Exposure of J_2 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 *svps* (31, 39 and 49 kDa) were present in standard pore water when PRD-hatched J_2 were used. Among these proteins *svp*31 was predominant (Fig. 3.5B).
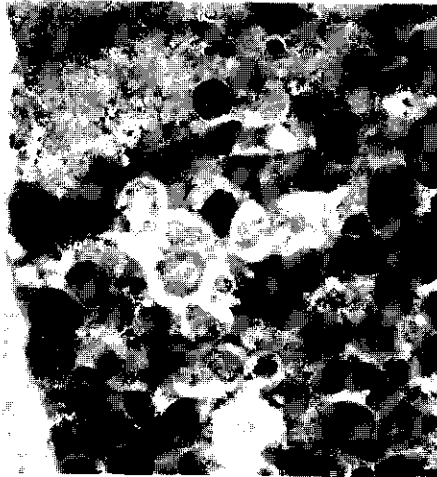
Figure 3.4 *Electron micrograph of the dorsal oesophageal gland cell in* Globodera rostochiensis *after treatment with 6.5 mM 5-methoxy-N,N-dimethyltryptamine-hydrogen-oxalate (DMT). Translucent structures (arrows) appear among the typical electron-dense secretory granules (SG) of the dorsal oesophageal gland (bar = 400 nm).*

Developmental regulation of *svp*

The developmental regulation of *svps* throughout the nematode's life cycle was determined by western blot analysis (Fig. 3.6). The protein content of each nematode extract was estimated and equal protein quantities of each developmental stage were analysed. The *svps* were neither detectable in fresh eggs containing young J_2 nor in dry eggs containing J_2 in diapause. Maximum immunodetection of *svps* occurred in homogenates from preparasitic J_2, PRD-hatched from dried cysts which had passed the diapause. The staining intensity of *svp*31/32 decreased in parasitic J_2, while in this
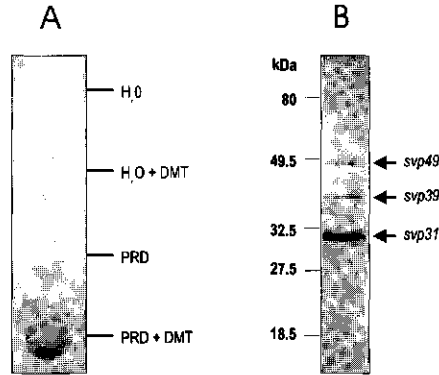
Figure 3.5 *Concentrated solubilised stylet secretions of* Globodera rostochiensis *tested with MGR48 on western blot and dot blot. A. A dot blot of concentrated incubation fluids of J$_2$ either hatched in tap-water (H2O) or potato root diffusate (PRD) after 24 hr in buffered standard pore water. B. Western blot of concentrated incubation fluid of potato root diffusate-hatched J$_2$ after an incubation period of 24 hr in standard pore water. The 31 kDa protein band is predominating in stylet secretions. The addition of 5-methoxy-N,N-dimethyltryptamine-hydrogen-oxalate to a final concentration of 4 mM is indicated as +DMT.*

stage no substantial decrease was observed for *svp*39 and *svp*49 (Fig. 3.6). None of the three *svps* 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 oesophageal 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 preparasitic J$_2$. To exclude the possibility that the apparent absence of *svp*s in other stages than J$_2$ 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 3.6.

Immunogold labelling of parasitic J$_2$ *in planta*

Potato roots infected with J$_2$ 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 J$_2$ inside plant tissue. As a consequence the nematodes shrink after polymerisation of the resin and soft tissue in the nematode shows morphological distortion. However, in some cases the cuticularised lumen of the oesophagus remained well preserved in thin sections. Serial sections of the oesophagus showed immunogold labelling of *svp* antigens within the circular lumen of the oesophagus anterior to the metacorpal pumpchamber (procorpus) (Fig. 3.7). Using MGR48 the labelling intensity was not strong, but very specific to oesophageal lumen both in cross (Fig. 3.7A) and longitudinal (Fig. 3.7B) sections.
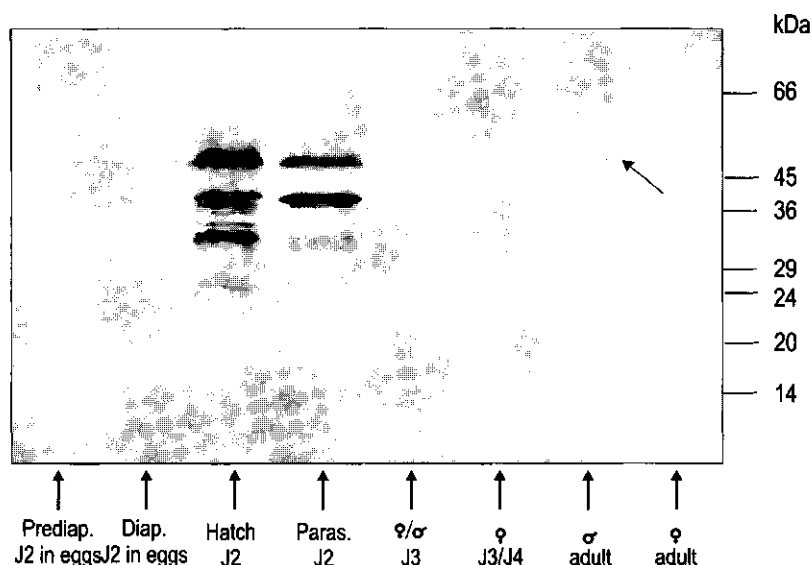
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Figure 3.6 *Western blot of SDS-extracted proteins from various life stages of* Globodera rostochiensis. *Immunodetection was done with MGR48. Prediap.= eggs containing young J₂; diap. J2 = eggs containing J₂ in diapause; hatch. J2 = freshly in potato 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.*

DISCUSSION

The subventral oesophageal glands of the potato cyst nematode *G. rostochiensis* contain basic proteins (*svp*). On two-dimensional electrophoresis five out of six *svp*s focused in the basic part of the immobilised pH gradient. The silver-stained 2-DGE gel illustrates that *svp*-proteins are not expressed at high levels. It is therefore unlikely that the *svp*-proteins are the sole secretory proteins in the subventral gland cells. By analogy to the exocrine acinar cells in mammalian pancreas *svp*-proteins may co-segregate in the secretory granules with several other distinct protein species [2,3].

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 [29]. 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 recognises successive stages of post-translational protein processing.

It has been argued [32] that the metacorporal pump chamber of beet cyst nematode *Heterodera schachtii* forms an anatomical barrier that precludes the secretion of
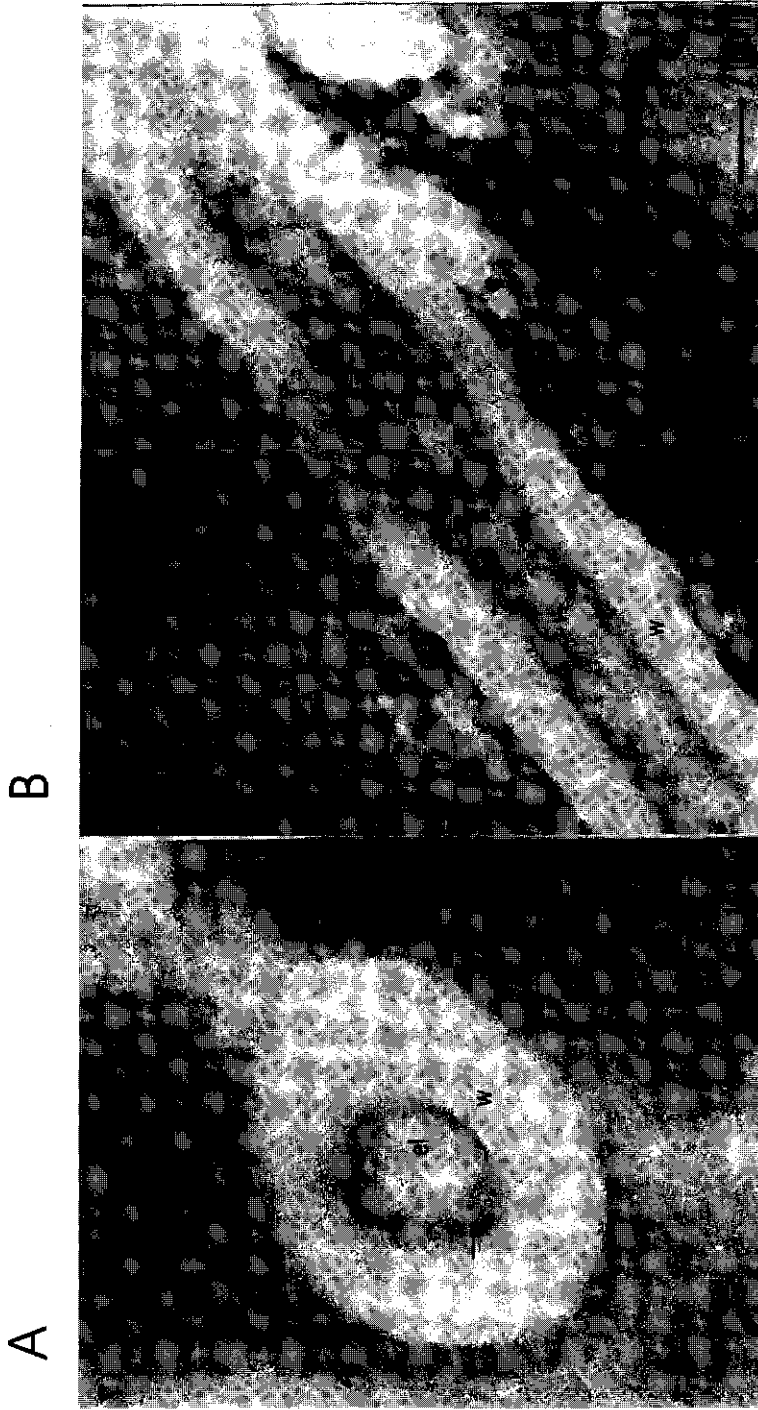
Figure 3.7 Electron micrograph of a cross (A) and longitudinal section (B) of the circular lumen of the oesophagus in parasitic Globodera rostochiensis J₂ in planta. Immunogold labelling (arrow) with MGR48 shows specific localisation in the circular lumen (el) of the oesophagus (procorpus; w = cuticularised oesophageal wall).

subventral oesophageal 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 oesophagus (procorpus) in parasitic J_2 *in planta* with MGR48 indicated also that *svps* of *G. rostochiensis* are transported anterior to the metacorpal pumpchamber. These data from preparasitic and parasitic J_2 under relatively natural conditions suggest that *svps* are secreted through the stylet into plant tissue.

Under natural conditions a J_2 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 preparasitic J_2 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 preparasitic J_2 do not secrete *svps* under natural conditions until the moment that a root of a host plant is reached.

Until now, exposure to DMT was the most obvious means to induce the secretion of oesophageal gland proteins of cyst nematodes ([16], see also chapter 2). In the case of potato cyst nematodes, *svp* containing stylet secretions can be induced in J_2 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* [16] and *M. incognita* [7]. However, the observation that these stylet secretions precipitate at the lip region needs further investigation. For potato cyst nematodes (J_2) 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 preparasitic J_2 for 24 hours gives the lowest immunodetection in homogenates of parasitic J_2. Most *svps* were detectable in preparasitic and in parasitic J_2, and not in J_3, J_4 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 [25], or in feeding site induction [26]. The *svps* are apparently not involved in feeding-site maintenance. Remarkable is the increased expression of the *svps* in adult males. Contrary to J_3, J_4 and females, adult males are mobile and can leave the root. Expression of *svps* in adult males would favour the hypothesis that *svps* are involved in the migratory process. Our findings are in accordance with the accumulation of secretory granules in the subventral oesophageal glands of males in *H. schachtii* [32] and *H. glycines* [1].

The isolation and functional analysis of genes encoding secretory proteins from the oesophageal 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 oesophageal glands was used to isolate a cDNA clone from a *M. incognita* expression library [21]. The cDNA sequence showed similarities with the rod portion of myosin heavy chain of various origins. It was hypothesised 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 [30,32], secretions from the subventral oesophageal glands of cyst nematodes probably play a direct role in the host-parasite interaction as concluded earlier for root-knot nematodes [7]. Analysing various developmental stages strongly suggests that the *svps* are involved in the early events of the interaction. Further purification and characterisation of these *svps* will without doubt be a major step forward toward the understanding of the interaction between plants and sedentary plant-parasitic nematodes.

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Chapter 4

Endogenous cellulases in animals: isolation of β-1,4-endoglucanase genes from two species of plant-parasitic cyst nematodes[1]

Abstract

β-1,4-endoglucanases (EGases, EC 3.2.1.4) degrade polysaccharides possessing β-1,4-glucan backbones such as cellulose and xyloglucan and have been found among extremely variegated taxonomic groups. Although many animal species depend on cellulose as their main energy source, most omni- and herbivores are unable to produce EGases endogenously. So far, all previously identified EGase genes involved in the digestive system of animals originate from symbiotic microorganisms. Here we report on the synthesis of EGases in the oesophageal glands of the cyst nematodes *Globodera rostochiensis* and *Heterodera glycines*. From each of the nematode species, two cDNAs were characterised and hydrophobic cluster analysis revealed that the four catalytic domains belong to family 5 of the glycosyl hydrolases (EC 3.2.1-3.2.3). These domains show 32 to 44% overall amino acid identity with EGases from the bacteria *Erwinia chrysanthemi*, *Clostridium acetobutylicum*, *Pseudomonas fluorescens* and *Bacillus subtilis*. One EGase with a bacterial type of cellulose-binding domain was identified for each nematode species. The leucine-phenylalanine rich hydrophobic core of the signal peptide and the presence of a polyadenylated 3'-end precluded the EGases to be of bacterial origin. Cyst nematodes are obligatory plant parasites and the identified EGases presumably facilitate the intracellular migration through plant roots by partial cell-wall degradation.

INTRODUCTION

The most abundant carbohydrate polymer on earth, cellulose, is mainly produced by terrestrial plants and marine algae. This recalcitrant polymer is used as a food source by many organisms. In plants, cellulose is a main component of the cell walls, in which it predominantly occurs in long rigid microfibrils. These microfibrils each consist of several dozen parallelly oriented linear chains of (1→4) β-linked D-glucose showing a varying and plant species-dependent degree of crystallinity. The cellulose microfibrils are embedded in a matrix of lignin and hemicellulose. The latter includes either xyloglucans (type I primary cell walls) or glucuronoarabinoxylans (type II primary cell walls) [6]. To exploit the energy and carbon available in cellulose, organisms such as fungi and bacteria produce mixtures of synergistically acting cellulases [2,37]. Many cellulases share a common basic architecture composed of a catalytic domain linked to a cellulose-binding domain by a glycosylated, Pro/Thr/Ser-rich peptide [13]. The non-catalytic cellulose-binding domain (CBD) determines the efficiency of degradation of insoluble cellulose [26].

Although cellulose is a major food source for many animal species, most omni- and herbivores do not produce cellulases themselves and live in symbiosis with cellulolytic microorganisms. For example, ruminants have highly specialised digestive tracts where mixtures of bacteria and protozoa degrade cellulose under anaerobic conditions

[1]. In most non-ruminant herbivorous mammals and birds, microorganisms mediate cellulose digestion, too. The ability of animals to hydrolyse cellulose in the absence of microorganisms has been subject of various studies. However, it is often difficult to establish whether cellulase is synthesised by an animal or by microorganisms without isolating the corresponding genes. To date, no animal cellulase genes have been identified and endogenous cellulase production by animals seems exceptional. Indications for the existence of cellulase genes within the animal kingdom have been obtained by measuring cellulase activity in microbe-free digestive tracts of cockroaches, higher termites, grass carps, eri silkworms, and earthworms [7,21,35,41].

This chapter describes the characterisation of four cellulase genes from obligatory plant-parasitic cyst nematodes of the genera *Globodera* and *Heterodera*. These cellulases are thought to play a role during intracellular migration of the larvae through the root cortex to the vascular cylinder where they modify selected plant cells into a multinucleated feeding cell [25]. Nematodes are equipped with a protrusible hollow stylet through which secretions from the oesophageal glands are released. These secretions are subject of intensive study because of their putative roles in plant-pathogenesis [22]. A monoclonal antibody (MGR 48), recognising antigen(s) within the subventral oesophageal glands from the cyst nematodes *Globodera rostochiensis* and *Heterodera glycines* [8,36] was employed to immunopurify endoglucanases (EGases). Evidence is provided that both in *G. rostochiensis* and *H. glycines* EGases are produced endogenously.

MATERIALS AND METHODS

Materials
Nematodes were cultured on host plant roots and preparasitic J_2 were extracted as described previously [8,14]. All reagents were of analytical grade.

Purification and N-terminal sequencing
In homogenates of *G. rostochiensis*, mAb MGR48 reacted on western blots with four protein bands (31, 32, 39, and 49 kDa), whereas in *H. glycines* only one protein band (49 kDa) was recognised. From each nematode species, one protein band was selected for purification and N-terminal sequencing. Approximately 1.0 ml of preparasitic second-stage juveniles (J_2, ~ 2.10^6 J_2/ml) of each *G. rostochiensis* and *H. glycines* were homogenised in 10 mM Tris-Cl pH 7.4 including 3 mM Pefabloc (Boehringer) and the supernatant was separated by preparative continuous flow SDS-PAGE (BioRad) on a 10% denaturing polyacrylamide gel [8]. Immunoblotting with mAb MGR48 was used to identify successive fractions containing the 39 kDa subventral gland protein of *G. rostochiensis* and the 49 kDa subventral gland protein of *H. glycines*. For each protein, these fractions were pooled and subsequently purified on a MGR48 immunoaffinity chromatography column [16]. The immunopurified proteins (*svp*39 and *svp*49) were dialysed in 0.1 M NH_4HCO_3, and lyophilised. The purified proteins were subjected to SDS-PAGE using a Tris-tricine buffer system and

electroblotted on polyvinylidene fluoride membrane [34]. Blotted protein bands were stained with Coomassie Brilliant Blue R-250 and cut out for protein sequencing (ARIAD Pharmaceuticals, Cambridge, MA). O-phthalaldehyde treatment and CNBr digestion circumvented n-terminal blocking of the proteins.

cDNA isolation and characterisation

A degenerative gene-specific primer (HG1f) designed from the N-terminal amino acid sequence of the 49 kDa subventral gland protein of *H. glycines* (Table 4.1) was used in 3' rapid amplification of cDNA ends (3'RACE, GIBCO/BRL). Template RNA for 3' RACE was extracted from J2 in 4 M guanidinium isothiocyanate buffer [33] and purified on a cesium trifluoroacetate cushion (Pharmacia Biotech). Gene-specific primers to the initial 3' RACE products were designed and used in a 5' RACE protocol (GIBCO/BRL). Full-length cDNA clones were obtained by using the 5' RACE products to probe plaque lifts of a *H. glycines* (J$_2$) oligo-dT primed cDNA library (Uni-Zap, Strategene; [33]). Two full-length cDNAs named HG-*eng1* and HG-*eng2* were isolated from *Escherichia coli* and sequenced on an Automated Laser Fluorescent DNA sequencer (Pharmacia Biotech).

Table 4.1. *Partial amino acid sequences of purified secretory proteins and deduced oligonucleotide primers used to amplify and clone EGase genes from G.* rostochiensis *and* H. glycines.

Nematode/ Primer[*]	Protein/DNA	Sequence[*]
		Amino acid sequence
H. glycines	*svp*49 (N terminal)	ASAVAPPFGQLSVSGSNLVGANKQPVQLISNSLFEH
G. rostochiensis	*svp*39 (N terminal)	LTATPPPYGQLSVSGTKLVGSMGQPVQLIGNSLFWPQFQGQYWNA
G. rostochiensis	*svp*39 (internal)	PTATYNLAVAVIEAAISQGMYVIVDTHSTEAHAD
		DNA sequence of deduced oligonucleotide primer[†]
HG1f	*svp*49	5'-GCIGTIGCICCICCITTYGGICA-3'
GR1f	*svp*39 (N-terminal)	5'-ACIGCIACICCICCICCITAYGGNCA-3'
GR2f		5'-CAGTGAAGGCRCTCAAATGCAATTGG-3'
GR3f		5'-CTTCCGTGTCTTCCTCCTCCATG-3'
GR4f		5'-TGGGATCCACTGCCACGCCTCCCCCA-3'
HG2r		5'-ATAGCCGCCCTCGTCCAC -3'
GR5r	*svp*39 (internal)	5'-TGCCARTCIACDATNACRTACTKCC-3'
GR6r		5'-CCAATTGCATTTGAGYGCCTTCACTG-3'
GR7r		5'-GGAAGCTTGCGCAACCACTTTTTTATCATCATC-3'
GR8r		5'-GGAAGCTTCGCAACAATTTTATCGTCAATAAATT-3'
pCDNA5f	PCDNAII	5'-GGTGACACTATAGAATACTCAAGCTATGCA-3'
pCDNA3r	PcDNAII	5'-GACGGCCAGTGAATTGTAATACGACTCACT-3'

[*] The single character amino acid code is used.
[*] The forward (f) or reverse (r) orientation of the oligonucleotide primers is indicated in their names.
[†] I= Inosine, K= G+T, N= A+C+G+T, R= A+G, Y= C+T, underlining indicates endonuclease restriction site.
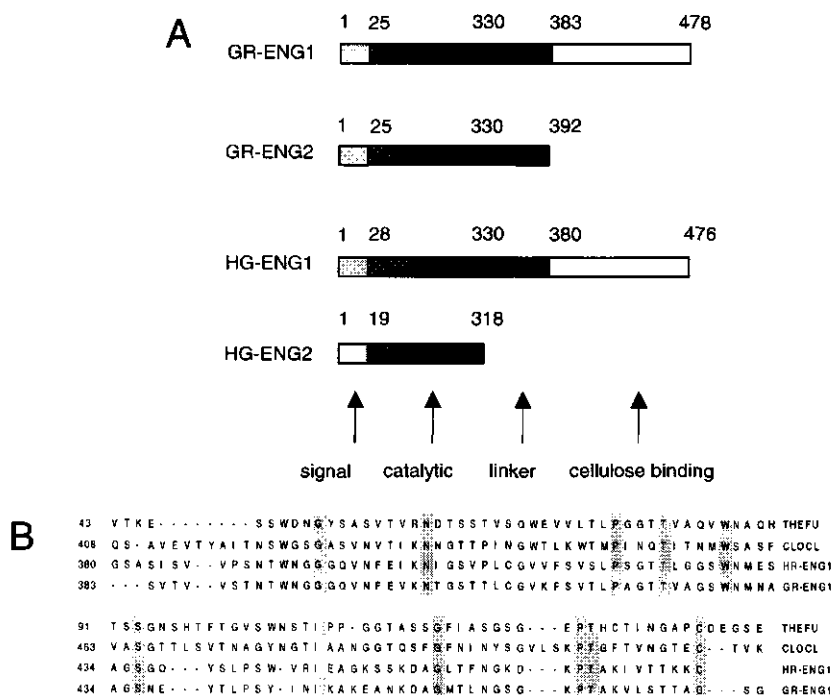
Figure 4.1 A, Global architecture of the four nematode β-1,4-endoglucanase precursor proteins. The amino acid positions of the functional domains are indicated above (see also Fig. 5.3). B, Sequence alignment of the C-terminal cellulose binding domains of G. rostochiensis *ENG1, and* H. glycines *ENG1 with two representatives of the bacterial type of cellulose binding domains (THEFU,* Thermomonospora fusca *endoglucanase E-5, Genbank accession L01577, and CLOCL,* Clostridium cellulovorans, *endoglucanase D, Genbank accession M37434). Residues conserved in all four cellulose-binding domains are shaded in gray.*

Similarly, an oligo-dT primed cDNA library from *G. rostochiensis* was prepared in the vector pcDNAII (Invitrogen). Two gene-specific primers derived from amino acid sequences obtained by protein sequencing were used for initial amplifications (Table 4.1). The 5' and 3' ends of the library inserts were isolated by using gene-specific primers combined with primers derived from flanking sequences in the library vector pCDNAII. Two cDNAs, GR-*eng1* and GR-*eng2*, were isolated from a plasmid prep (Promega) of the cDNA library and cloned into pCR2.1 (Invitrogen). Additional RT-PCR was performed to extent the cDNA at their 5' end, thereby confirming the presence of the ATG startcodon in the largest open reading frames (GIBCO/BRL).

Heterologous expression in *E. coli*

HG-*eng1* and HG-*eng2* were cloned into the expression vector pET-28c and

pET28a, respectively, overexpressed and purified according to the manufacturer (Novagen). GR-*eng1* and GR-*eng2* were cloned into pMAL-c2 expression vector using adapter-primers (GR4f, GR7r, and GR8r; Table 4.1). Overexpression and affinity purification of the fusion proteins was done according to the manufacturer (Novagen and New England Biolabs).

CMC assay

Carboxymethylcellulose (CMC) hydrolase activity in both *G. rostochiensis* and *H. glycines* J2 was assayed with a non-reducing SDS-PAGE gel overlay method [29]. An identical gel was transferred to nitro-cellulose membrane, and probed with MGR48 for comparison of banding pattern [8]. A semi-quantitative CMC cup plate assay was used to determine the CMC hydrolase activity of affinity-purified heterologous fusion proteins [5].

In situ hybridisation

Digoxigenin-11-UTP labelled RNA probes were transcribed from the nucleotide 273 to 447 region of the HG-eng2 cDNA. J_2 of *H. glycines* were fixed overnight in 2% paraformaldehyde, cut into sections and permeabilised as described [8]. The J_2 sections were then hybridised with sense or antisense riboprobe. Following digestion with RNAse A and stringency washings, bound riboprobe was detected by alkaline phosphatase staining (Boehringer Mannheim, Genius kit). The stained J_2 were examined with differential interference contrast microscopy.

Production of antisera

For the subcutaneous immunisation of BALB/c mice, partially purified fusion proteins were mixed 1:1 with Freund's complete adjuvant [16]. All four antisera raised against the fusion proteins were assayed with immunofluorescence microscopy and western blotting for binding to nematode oesophageal gland proteins [8,14].

Hydrophobic cluster analysis (HCA)

HCA plots were made by using the program HCA-PLOT V3.0 (Doriane, Le Chesnay, France). In these plots, the amino acid sequence of the proteins is drawn on a duplicated helical net using the standard one-letter code except for P, G, T and S which are represented by stars, diamonds, squares and pointed stars, respectively. Clusters of hydrophobic residues (V, I, L, F, W, M, and Y) are automatically drawn on the bi-dimensional helical representation. Analysis of the plots was performed as described in the results [28].

RESULTS

Monoclonal antibody MGR48 was used to immunopurify the subventral gland secretory proteins *svp*39 and *svp*49 from *G. rostochiensis* and *H. glycines* respectively. Five to ten micrograms of these proteins were used for amino acid sequencing (Table

4.1). Two sequences (one N-terminal and one internal) were determined for the *G. rostochiensis* protein *svp*39. An N-terminal amino acid sequence was obtained with *svp*49 purified from *H. glycines*. The amino acid sequences presented in Table 4.1 were used to design gene-specific PCR primers.

Cloning of two EGases from *H. glycines*

PCR products from 3' RACE using forward primer HG1f (Table 4.1), and 5' RACE using reverse primer HG2r were used for screening of a cDNA library from preparasitic J$_2$ of *H. glycines*. A large cDNA clone obtained (HG-*eng1*) comprised 1615 bp with an open reading frame of 1428 bp, encoding a putative 49,800 Da protein (HG-ENG1). A smaller cDNA clone comprised 1191 bp (HG-*eng2*) with a 957 bp open reading frame encoding a putative 34,700 Da protein (HG-ENG2). A predicted amino acid segment nearly identical to the N-terminal sequence of the purified *svp*49 protein was identified downstream of the alanines at the positions 108 and 121 in HG-*eng1* and HG-*eng2*, respectively. The regions between the putative ATG start codons (positions 60 and 64, in HG-*eng1* and HG-*eng2* respectively) and the N-termini were identified as signal peptides (at http://www.cbs.dtu.dk/services/SignalP/;[23]). In HG-ENG1, the most likely cleavage site was between amino acids Ser-Leu at the amino acid positions 27-28 and between Asp-Asp at positions 18-19 in HG-ENG2.
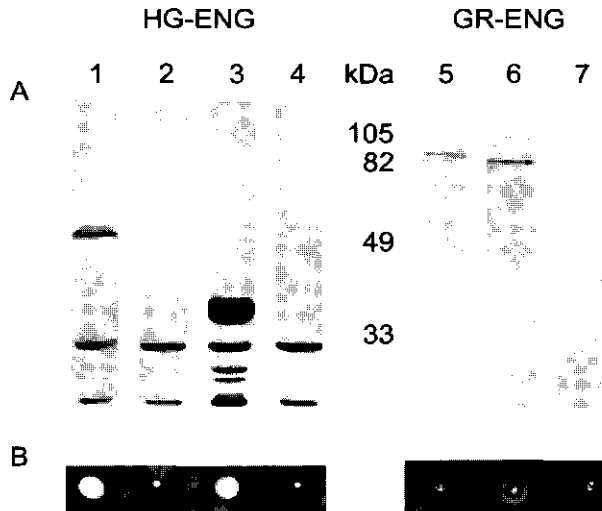
Figure 4.2 Detection of recombinant nematode EGases in E. coli lysates on western blot (A) and carboxymethyl cellulose (CMC) hydrolysis assay (B). A, immunodetection was done with subventral gland specific mAbs (17). Lane 1, HG-ENG-1 produced from pET28c. Lane 2, pET28c control. Lane 3, HG-ENG-2 produced from pET28a. Lane 4, pET28a control. Lane 5, GR-ENG-1 from vector pMAL-c2. Lane 6, GR-ENG-2 from vector pMAL-c2. Lane 7, pMAL-c2 control. B, detection of CMCase activity (halo) in affinity-purified heterologous cyst nematode EGases that corresponds to lanes above.

Cloning of two EGases from *G. rostochiensis*

Two primers (GR1f and GR5r; Table 4.1) were designed from the N-terminal and internal amino acid sequences obtained by protein sequencing of *svp*39. Using these primers, a 300-bp DNA fragment was amplified from a cDNA library. Based on its sequence, two additional gene-specific primers (GR2f and GR6r) were synthesised in opposite orientation. In theory, use of these primers should have resulted in two overlapping DNA fragments when combined with either a reverse (pCDNA3r) or a forward (pCDNA5f) primer specific for the flanking region in the vector pCDNAII. However, two products of 1450 and 1200 bp were amplified using GR2f and pCDNA3r. In the opposite orientation primers GR6r and pCDNA5f only resulted in a single 250 bp PCR fragment. Three additional oligonucleotide primers (Table 4.1) were synthesised with which two cDNAs, 1571 bp (GR-*eng1*) and 1300 bp (GR-*eng2*) were isolated. The largest open reading frames in these two cDNA fragments comprised 1434 bp and 1179 bp respectively, encoding two putative precursor proteins of 50,700 Da (GR-ENG1) and 42,000 Da (GR-ENG2). In both clones, a peptide sequence nearly identical to the N-terminal protein sequence of purified *svp*39 was present. Upstream of this amino acid sequence, a signal peptide was predicted for both proteins [31]. The common most likely cleavage site was between amino acids Ala and Leu at positions 24-25 in both GR-ENG. These predictions correspond with the results of the N-terminal amino acid sequence obtained by protein sequencing of the *svp*39 protein.
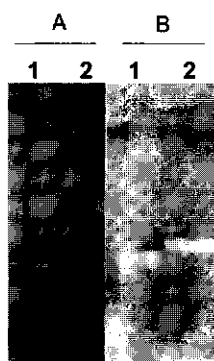
Figure 4.3 *Comparative non-reducing SDS-PAGE of nematode homogenates following either western blotting (A) or CMC overlay (B). A, mAb MGR48 reacting with subventral gland protein of* H. glycines *(lane 1) and* G. rostochiensis *(lane 2). B, Congo red stained agarose bed containing CMC showing hydrolysis activity (halo) by specific proteins of* H. glycines *(lane 1), and* G. rostochiensis *(lane 2).*

Sequence comparison

The two cDNA clones of *G. rostochiensis* shared a high degree of similarity at their 5' ends (95% identity in first 884 nucleotides). The two cDNA clones isolated from *H. glycines* were less similar at their 5' ends (79% identity in first 873

nucleotides). The four encoded proteins were compared to sequences currently available in protein databases using the FASTA search method [32]. The search result showed that the nematode proteins shared an overall identity of 33 to 44% with β-1,4-endoglucanases of various bacterial origins including *Erwinia chrysanthemi* (Swiss-Prot accession P07103), *Clostridium acetobutylicum* (Swiss-Prot accession P15704), *Pseudomonas fluorescens* (PIR accession S56132) and *Bacillus subtilis* (Swiss-Prot accession P07983).

Fig. 4.1A presents the global architecture of the four nematode endoglucanase precursor proteins with the amino acid positions of the functional domains. In HG-ENG1 and GR-ENG1, C-terminal extensions of 96 and 97 amino acid residues, which are missing in HG-ENG2 and GR-ENG2 respectively, show significant similarity to type II cellulose-binding domains (CBD; [38]). The alignment of these C-terminal extensions with two representatives of this bacterial type of cellulose binding domain is illustrated in Fig. 4.1B.

EGase activity assays

Fusion proteins of the predicted sizes, 54 kD for HG-ENG1 and 38 kD for HG-ENG2, expressed in *E. coli* were recognised by mAb MGR48 on western blots. Similarly, fusion proteins of the predicted sizes, 90 kD for GR-ENG1 and 82 kD for GR-ENG2, expressed in *E. coli* were recognised on western blots by a cocktail of subventral gland protein-specific mAbs [8] (Fig. 4.2A).

Applying affinity-purified preparations of the four recombinant ENG proteins in a CMC plate assay showed significant hydrolytic activity (Fig. 4.2B). Similarly, homogenates of both nematode species were analysed in a PAGE gel overlay on an agarose bed containing CMC (Fig. 4.3B). In *G. rostochiensis* homogenate, a distinct band of approximately 50 kDa showed CMC hydrolase activity, whereas in *H. glycines* a slightly smaller protein hydrolysed CMC. Both apparent CMC hydrolases aligned with one of the subventral gland specific antigens (approx. 49 kDa) recognised by MGR48. No CMCase activity was detected for *svp*39 in *G. rostochiensis* homogenates. This is presumably caused by detrimental effects of SDS. When comparing to a standard SDS-PAGE [8], the SDS concentration in the electrophoresis buffer and polyacrylamide gels was significantly reduced. This may account for the minor differences in reaction pattern of MGR48 on western blot (Fig. 4.3A).

Localisation of EGase transcripts and protein

Endoglucanase transcripts were detected by whole mount *in situ* hybridisation in the subventral gland cells of *H. glycines*. Anti-sense RNA probes transcribed from the HG-*eng2* cDNA bound specifically within the subventral glands of preparasitic J_2s of *H. glycines* (Fig. 4.4A and B). The transcripts were abundant within the subventral gland cell lobe, but were not detected in gland extensions and ampullae.

Antisera raised against the four recombinant ENG proteins bound specifically on western blots to the purified recombinant proteins that were used as immunogens and the subventral gland proteins in nematode homogenates (data not shown). Immunofluorescence microscopy revealed a clear binding of these antisera to the

subventral glands of preparasitic J_2 of the two cyst nematode species (shown for *H. glycines* only Fig. 4.4C and D).
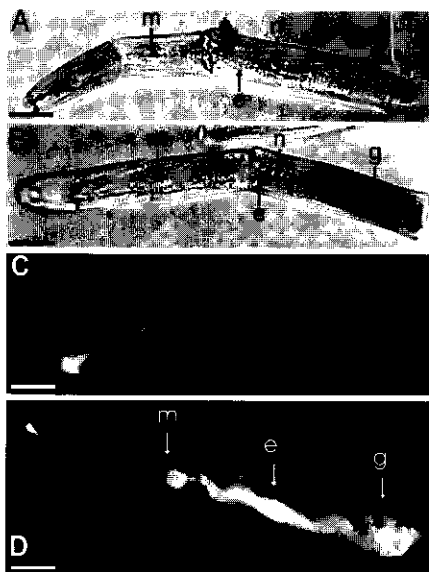
Figure 4.4 *Localisation of EGase transcripts and proteins within cyst nematodes. A and B. Anterior sections of preparasitic second-stage juveniles of* H. glycines *hybridised with digoxigenin-labelled RNA probes transcribed from the HG-eng-2 cDNA. A, no staining is observed with the sense probe. B, specific hybridisation of the antisense probe to the posterior region of the subventral gland cells (g). The gland extensions (e) remain unstained. n, nucleolus of dorsal gland. m, metacorpal pump chamber. C and D. Immunofluorescence of* H. glycines *using anti-serum raised against recombinant nematode HG-ENG2. C, no specific labelling with the pre-immune serum. D, intense labelling of subventral glands with HG-ENG-2 specific immune serum (Bar = 20 μm.).*

Hydrophobic cluster analysis (HCA)

For further characterisation, HCA plots of the catalytic domains of four nematode endoglucanases were compared to various other cellulases. The nematode endoglucanases showed highest similarity to cellulases from the glycosyl hydrolase family 5 [18-20]. Fig. 4.5 shows the HCA plots of HG-ENG1 and GR-ENG1 together with two representatives of glycosyl hydrolase family 5, namely the endoglucanases of *Bacillus subtilis* (Swiss-Prot accession P07983) and endoglucanase A of *Clostridium cellulolyticum* (Swiss-Prot accession P17901). The latter, whose three dimensional structure has been determined [11], was used to identify the catalytic machinery and the secondary structure elements constituting the $(\beta/\alpha)_8$ barrel structure in the corresponding nematode endoglucanases. Thus, the acid/base and the nucleophilic residue can be assigned to Glu_{160} and Glu_{249} in HG-ENG1, Glu_{149} and Glu_{238} in HG-ENG2, and Glu_{159} and Glu_{249} in both GR-ENG1 and GR-ENG2.

DISCUSSION

Cyst nematodes produce β-1,4-endoglucanases endogenously

Several lines of evidence demonstrate the endogenous origin of the four β-1,4-endoglucanase genes isolated from *G. rostochiensis* and *H. glycines*. Firstly, the leucine/phenylalanine rich hydrophobic core (h-region) of the signal peptides [31] and the polyadenylated 3' cDNA ends show that the nematode EGases are of eukaryotic origin, and do not originate from bacterial symbionts. Secondly, contributions from eukaryotic symbionts can be precluded because detailed EM studies have shown that mAb MGR48 antigens, now identified as EGases, are localised within the secretory granules of the nematode's subventral glands and no eukaryotic symbionts were observed in these glands [8,12,36]. Thirdly, the nematode origin of the isolated EGase cDNAs was confirmed by specific binding within the subventral gland of antisera raised to all four active recombinant EGases and of an antisense riboprobe synthesised from the HG-*eng2*.

What kind of β-1,4-endoglucanases are produced by cyst nematodes?

HCA of the catalytic domain allowed the assignment of the acid/base and nucleophilic residues involved in catalysis and revealed that all four EGases belong to the family 5 glycosyl hydrolases, the most numerous family to date. This family includes endoglucanases, mannases and β-1,3-exoglucanases from aerobic and anaerobic bacteria and fungi [18-20]. The substrate specificities of family 5 members can be relatively broad and, apart from β-1,4-endoglucanase activity, GR-ENG1, 2 and HG-ENG1, 2 may have other glycosyl hydrolase activities as well. In two of the four EGases, namely GR- and HG-ENG1, cellulose-binding domains (CBD) were identified. Hence, both cyst nematodes could also have the ability to degrade crystalline cellulose.

The EGases from *G. rostochiensis* and *H. glycines* show an intriguing homology with those from soil bacteria. The catalytic domains of the nematode EGases show 32 to 44 % overall amino acid identity with EGases from *Erwinia chrysanthemi*, *Clostridium acetobutylicum* and *Bacillus subtilis*. In addition, the CBDs show significant sequence similarity to type II CBDs. Type II CBDs have only been encountered in bacteria ([38], Prosite entry PCDOC00485). The close relatedness of the four nematode EGases to those of bacteria raises the question whether nematodes have acquired their cellulases by horizontal gene transfer from a prokaryote. However, too few sequence data are currently available for plant parasitic nematodes to make a firm statement about possible differences in molar G+C content of nematode EGase genes [15] as compared to the hosting nematode chromosome.

Between the catalytic domains and the CBDs, linker sequences of 51 and 50 amino-acid residues are present in HG-ENG1 and GR-ENG1, respectively. In HG-ENG1 the linker almost fully consists of an imperfect $(SGSS)_{12}$-repeat. A similar amino acid repeat of unknown function has been identified from *Dictyostelium discoideum* (Genbank accession U66523). In general, these GlySer-rich regions in proteins may allow flexibility between two functional domains [23]. At the C-terminal
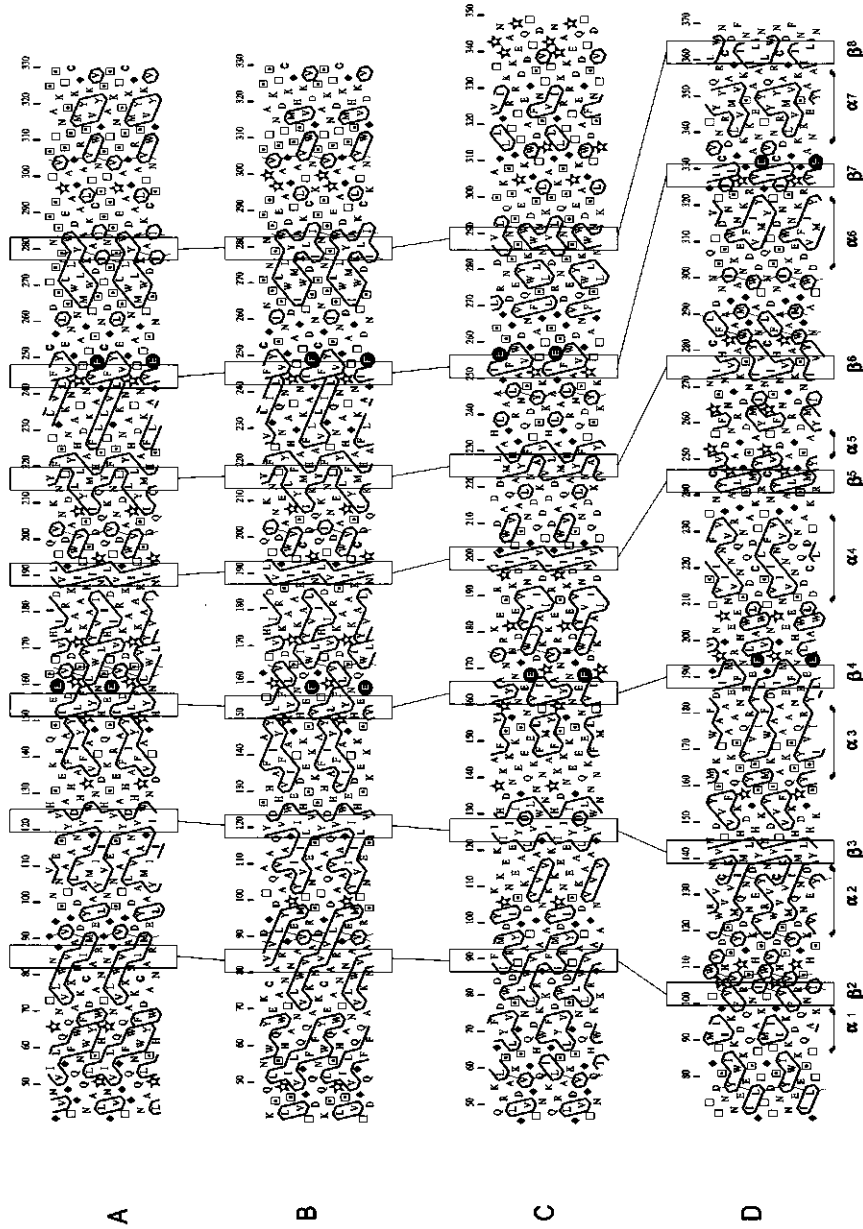
Figure 4.5 Hydrophobic cluster analysis of the N-terminal catalytic domains of HG-ENG1 (A), GR-ENG1 (B), endoglucanase of Bacillus subtilis (Swiss-Prot accession P07983) (C) and endoglucanase A of Clostridium cellulolyticum (Swiss-Prot accession P17901) (D). The assignment of the secondary structure in C. cellulolyticum endoglucanase A is according to Ducros et al. [11]. The correspondence between the b-strands in C. cellulolyticum endoglucanase A and the equivalent strands in the other sequences is shown. The two catalytic residues in family 5 glycosyl hydrolases are printed white in black circles.

extension of GR-ENG2, a sequence (PAAAKK(S)PPAK)$_3$ was found with high similarity to parts of H1 histones of tomato involved in the protein-DNA interaction [24]. Nematode EGases are functional outside the plant cell, and it is hard to give a biological interpretation for this observation.

Putative biological functions of β-1,4-endoglucanases produced by nematodes

The intracellular migration of cyst nematodes within roots was thought to be facilitated by mechanical means only [42]. Our present findings indicate that intracellular migration in host roots by *G. rostochiensis* and *H. glycines* involves a combination of mechanical force and enzymatic softening of plant cell walls. Recently we showed that the secretory proteins of the subventral glands, now identified as EGases, are expressed solely in the mobile stages of *G. rostochiensis, viz.* in preparasitic and parasitic second stage juveniles and in adult males [36]. Both cyst nematode species produce an EGase with a CBD, which could allow for the degradation of crystalline cellulose within the plant cell wall. The two nematode EGases lacking a CBD could enable hydrolysis of soluble polysaccharide fragments that are products of the initial degradation of crystalline cellulose. These latter EGases could also be involved in hydrolysis of xyloglycan, a polysaccharide interconnecting crystalline cellulose microfibrils in type I primary cell walls [17,40]. However, the recombinant nematode EGases did not show activity in a plate assay with tamarind xyloglucan (data not shown). In our view the enzymatic characteristics of the EGases described allow the nematode to weaken the mechanical strength of the cellulose microfibril network. It is likely that cyst nematodes produce other cell wall degrading enzymes to attack the plant cell wall in concert with cellulases, *e.g.* pectinases, and proteases.

Distribution of endogenous EGases among members of the phylum Nematoda

The monoclonal antibody MGR48 employed to immunopurify the EGases from *G. rostochiensis* and *H. glycines*, recognises also proteins from the cyst nematodes *Globodera pallida, Globodera tabacum* [8] and *Heterodera schachtii*. In addition, cellulase activities have been detected in homogenates and secretions of a range of plant-parasitic and fungivorous nematodes [3,4,9,10,27,30,39]. In view of these results, it is assumed that the ability to produce cellulases endogenously is widely distributed within the phylum Nematoda.

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Chapter 5

Genomic organisation of four β-1,4-endoglucanase genes in plant-parasitic cyst nematodes and its evolutionary implications[1]

Abstract

The genomic organisation of genes encoding β-1,4-endoglucanases (cellulases) from the plant-parasitic cyst nematodes *Heterodera glycines* and *Globodera rostochiensis* (HG-*eng1*, Hg-*eng2*, GR-*eng1*, and GR-*eng2*) was investigated. HG-*eng1* and GR-*eng1* both contained eight introns and structural domains of 2151 bp and 2492 bp, respectively. HG-*eng2* and GR-*eng2* both contained seven introns and structural domains of 2324 bp and 2388 bp, respectively. No significant similarity in intron sequence or size was observed between HG-*eng1* and HG-*eng2*, whereas the opposite was true between GR-*eng1* and GR-*eng2*. Intron positions among all four cyst nematode cellulase genes were conserved identically in relation to predicted amino acid sequence. HG-*eng1*, GR-*eng1*, and GR-*eng2* had several introns demarcated by 5'-GC...AG-3' in the splice sites, and all four nematode cellulase genes had the polyadenylation and cleavage signal sequence 5'-GAUAAA-3' -- both rare occurences in eukaryotic genes. The 5'-flanking regions of each nematode cellulase gene, however, had signature sequences typical of eukaryotic promoter regions, including a TATA box, bHLH-type binding sites, and putative silencer, repressor, and enhancer elements. Database searches and subsequent phylogenetic comparison of the catalytic domain of the nematode cellulases placed the nematode genes in one group, with family 5, subfamily 2, glycosyl hydrolases from Scotobacteria and Bacilliaceae as the most homologous groups. The overall amino acid sequence identity among the four nematode cellulases was from 71% - 83%, and the amino acid sequence identity to bacterial family 5 cellulases ranged from 32% - 44%. The eukaryotic organisation of the four cyst nematode cellulases suggests that they share a common ancestor, and their strong homology to prokaryotic glycosyl hydrolases may be indicative of an ancient horizontal gene transfer.

INTRODUCTION

The plant cell wall is a major barrier in the local environment of plant pathogens. Extracellular enzymes that can (partially) degrade this complex of polysaccharides are produced by a number of plant-pathogens. Digestion of plant cell walls by secreted microbial exo- and endoglucanases (cellulases) may enhance direct penetration of plant tissue, facilitate the degradation of associated cell wall components by other enzymes, and enhance the release of plant cell constituents that can be utilised for nutrition of phytopathogens [3,35]. Cellulases and xylanases have been grouped into a number of distinct families based upon amino acid sequence identities and hydrophobic cluster analysis [12,17,18]. Typically these glycanases consist of a catalytic domain and a cellulose-binding domain (CBD) that is connected by a Pro/Thr/Ser-rich peptide linker [12]. The absence of a CBD and associated peptide linker has been observed in some endoglucanases, and this appears to affect substrate specificity [12,36,37].

Cellulose is a major constituent of the plant cell wall and the linear chains of $(1 \rightarrow 4)$ β-linked D-glucose are predominantly present as long rigid microfibrils [5]. β-1,4-

endoglucanases (EGases, EC 3.2.1.4) hydrolyse cellulose, and could be important determinants of pathogenicity [2]. EGases have been cloned from plant-pathogenic bacteria (*e.g. Pseudomonas solanacearum* [21] and *Xanthomonas campestris* [14]), and plant-pathogenic fungi (*e.g. Cochliobolus carbonum* [13], *Macrophomia phaseolina* [36], and *Fusarium oxysporum* [27]).

The first isolation of endogenous cellulase genes from animals has recently been reported for two species of plant-parasitic cyst nematodes, *Heterodera glycines* and *Globodera rostochiensis* [29]. These microscopic worms secrete cellulases that are produced in their oesophageal gland cells. It is hypothesised that these cellulases promote the migration of the nematodes through plant root cells. In both *H. glycines* and *G. rostochiensis* transcripts of two EGases genes[2] were identified, each species contained one larger (approximately 1.6 kb) endoglucanase cDNA (termed HG-*eng1* and GR-*eng1*, respectively), and one smaller (approximately 1.2 kb) endoglucanase cDNA (termed HG-*eng2* and GR-*eng2*, respectively). The endogenous expression of the four endoglucanase genes specifically within the subventral glands of both nematode species was confirmed using polyclonal sera that were raised to heterologous proteins of each of the four isolated genes [29]. Each of the four cyst nematode endoglucanases contained a secretion signal peptide, a cellulase catalytic domain, and a polyadenylated 3' terminus. Both HG-*eng1* and GR-*eng1* also contained a peptide linker and a CBD at the C-terminus. Predicted amino acid sequence homologies and hydrophobic cluster determined that the catalytic domains in the four nematode endoglucanases each contained the glycosyl hydrolases family 5 signature found in some bacterial and fungal endoglucanases [18,29].

The cyst nematode endoglucanases only showed a significant amino acid homology with endoglucanases of (plant-pathogenic) bacteria. In order to determine whether this homology extents beyond the coding sequences we have investigated the exon/intron organisation, and 5'- and 3'-flanking regions of the genes. Although ample coding sequences and regulatory sequences have been identified in the free-living nematode, *Caenorhabditis elegans* [1,23], the genomic organisation and the transcription of coding regions from plant-parasitic nematode genomes have not been studied extensively. This report presents the genomic organisation of HG-*eng1*, HG-*eng2*, GR-*eng1*, and GR-*eng2*, and highlights putative regulatory elements in the flanking sequences of these cyst nematode endoglucanases. In addition, our genomic study strongly suggests that the four nematode cellulases share a common ancestor, and that this putative ancestor may have acquired the cellulases from a prokaryote via horizontal gene transfer.

MATERIALS AND METHODS

Isolation of gDNA from nematodes

Preparasitic second-stage juveniles (J_2) of *Heterodera glycines* and *Globodera rostochiensis* were hatched from eggs extracted from greenhouse cultures as described

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[2] cDNA sequences: HG-*eng1*, Genbank accession no. AF006052; HG-*eng2*, Genbank accession no. AF006053; GR-*eng1*, Genbank accession no. AF 004523; and GR-*eng2*, Genbank accession no. AF004716

previously [8,15]. Total DNA was extracted from J_2 by alkaline/SDS lysis and phenol/chloroform extraction [31].

H. glycines genomic library construction and screening

The *H. glycines* genomic DNA was partially digested with *Sau*3A, *Bgl*II and *Bam*HI and used to produce a genomic library by cloning into the Lambda FIX II/*Xho*I partial fill-in vector (Stratagene, La Jolla, CA). This *H. glycines* genomic library consisted of 76,000 primary recombinants and had an amplified titre of 430,000 pfu/µl. The amplified library was diluted to 10,000 pfu and plated in on 150 mm LB plates at 37°C for 8 hours. Plaque lifts onto nitrocellulose filters and subsequent treatments were conducted as suggested for the Lambda Fix II vector (Stratagene). The DIG-labelled cDNA probes for HG-*eng1* and HG-*eng2* described above were used for hybridisations on plaque lifts at 59°C and subsequent washing temperatures of 68°C. Positive plaques were identified by the color reaction described above and isolated for restriction mapping.
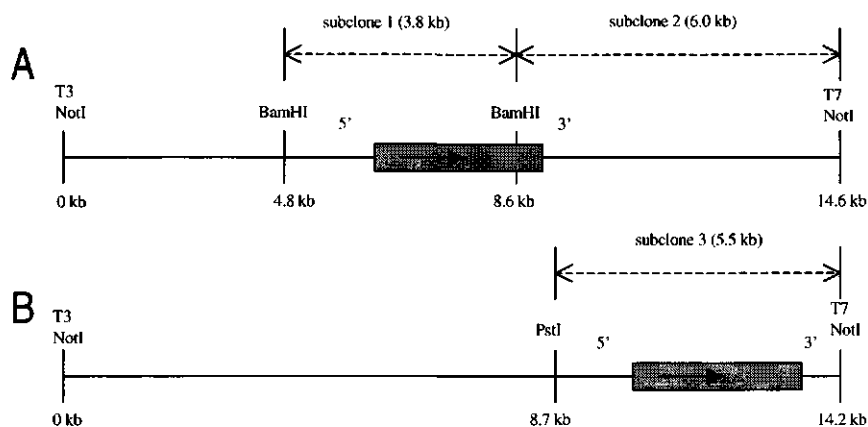
Figure 5.1 Position of H. glycines *endoglucanase genes HG-eng1 (A) and HG-eng2 (B) on* H. glycines *genomic DNA fragments excised from Lambda Fix II clones. Dark boxes with arrows represent coding regions and transcription orientation.*

Lamda DNA purification and HG-*eng1* and HG-*eng2* gene mapping

Lamda Fix II DNA harbouring positive genomic HG-*eng1* and HG-*eng2* clones was amplified in *E.coli* host MRA-P2, lysed, and purified using a Lambda Mini Kit (Qiagen, Valencia, CA). Inserts of *H. glycines* genomic DNA were excised from purified lamda Fix II DNA with *Not* I, and the inserts were digested various restriction endonuclease for agarose gel electrophoresis and Southern blots [26]. The DIG-labeled HG-*eng1* and HG-*eng2* cDNA probes were used in these Southern blots to identify restriction enzymes that yielded *H. glycines* genomic DNA fragments that contained either complete gene for subcloning. Genomic fragments containing entire HG-*eng1* or HG-*eng2* genes were directionally subcloned into pBluescript-KS [+] and the orientation of each clone was determined using T3 and T7 primers that were labelled with the DIG Oligonucleotide 3'-

End Labeling Kit (Boehringer Mannheim Biochemicals, Mannheim). The identified subclones were used as template to amplify full-length genomic clones of HG-*eng1* and HG-*eng2* using inward primers designed from the 5' - and 3'-ends of their corresponding cDNAs. The resulting PCR products were cloned into TA-vectors (Invitrogen, San Diego) and submitted for automated nucleic acid sequencing. The 5'- and 3'-flanking regions of each gene were obtained for sequencing by PCR using one outward primer derived from the sequence of each end of the gene and a second primer derived from the appropriate flanking vector sequence.

Isolation of GR-*eng1* and GR-*eng2* genomic clones and 5'- and 3'- flanking region

Inward primers were designed from the most distal sequences in the GR-*eng1* and GR-*eng2* cDNAs [29] to amplify genomic sequence of each gene using *G. rostochiensis* DNA as template. In order to clone the 5'- and 3'-flanking region of GR-*eng1* and GR-*eng2*, a genomic library of *G. rostochiensis* J$_2$ was constructed in the plasmid vector pZErO-2 (Invitrogen). The *Sau*3A partially digested fragments ranging from 500bp to 4kb were ligated to a *Bam*HI digested pZErO-2 vector. The genomic library contained 10^6 primary recombinants with an average insert of 1kb. The 5'- and 3'-flanking regions of the GR-*eng1* and GR-*eng2* genes were isolated from the library by the same PCR strategy as with Hg-*eng1* and HG-*eng2*. Amplified products were cloned into TA-vectors (Invitrogen) and sequenced from both directions.
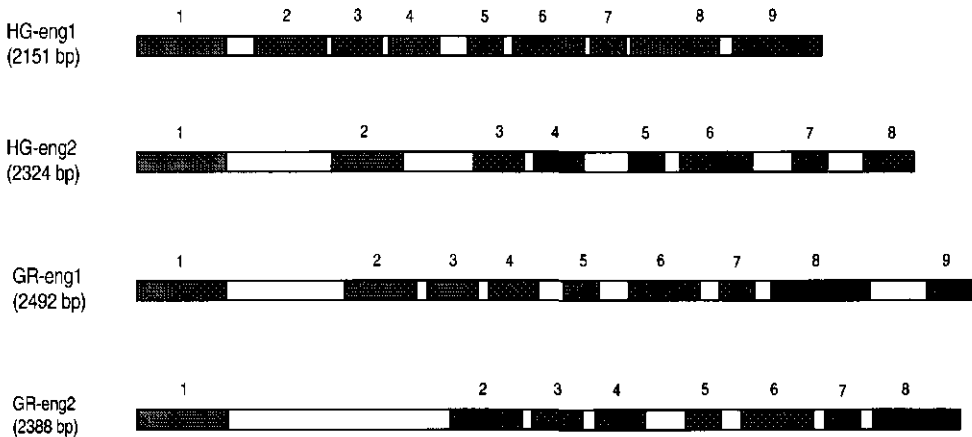

Figure 5.2 *Exon/intron organisation of four cyst nematode endoglucanase genes,* Heterodera glycines *genes HG-eng1 and HG-eng2, and* Globodera rostochiensis *genes GR-eng1 and GR-eng2. Exons (dark boxes) and introns (open boxes) are drawn proportionally to indicate the relative position and size of the introns.*

RESULTS

Genomic clones of cellulase genes from *H. glycines* and *G. rostochiensis*

Five positive genomic plaques were each initially obtained for HG-*eng1* and HG-*eng2*, and after various restriction digestions and hybridisations with either appropriate cDNA on Southern blots, one lambda clone of each gene was selected for further analysis (Fig. 5.1). The location and orientation of the SCN cellulases in the lambda DNA was verified also by PCR using cellulase internal primer and the T3 and T7 primers located in the lambda vector (data not shown). For obtaining the 5'- flanking regions and coding sequence of HG-*eng1*, the 3.8 kb *Bam*HI digested fragment from Lambda DNA was cloned into the pBluescipt KS$^+$ *Bam*HI site. The 6kb fragment containing 3'-flanking regions of HG-*eng1* was cloned into the pBluescript KS$^+$ *Not*I and *Bam*HI site. A 5.5 kb DNA fragment from Lambda DNA excised by *Pst*I and *Not*I, which contained both 5'-and 3'-flanking region of HG-*eng2*, was cloned into the pBluescript-KS$^+$ *Pst*I and *Not*I site. The length of the genomic structural fragments (corresponding to cDNA) of HG-*eng1*[1] and HG-*eng2* are 2151 bp and 2324 bp, respectively. The two genomic structural fragments encoding GR-*eng1* and GR-*eng2* are 2492 bp and 2388 bp, respectively, and they were amplified directly from *G. rostochiensis* J$_2$ genomic DNA.

Cis splicing in cyst nematode endoglucanase genes

The intron/exon boundaries of the genomic sequences of the four cellulase genes were determined by aligning the genomic sequence with the cDNA sequence for each corresponding gene (Fig. 5.2). Both *eng1* genes contained eight introns and both *eng2* genes contained seven introns. Intron size among the four genes ranged from 45 bp to 742 bp, with an average intron size of 128 bp. Exon size among the four genes ranged from 70 bp to 311 bp, with an average exon size of 158 bp. The average G+C contents of the exons in GR-*eng* and HG-*eng* were 53.8% and 50.8% respectively. The average G+C contents of the introns in GR-*eng* and HG-*eng1* were 42.3% and 41.3% respectively. The average G+C content of the intron sequences in HG-*eng2* was 32.4%, which is significantly lower than the other nematode cellulase genes.

The size of the corresponding introns between HG-*eng1* and HG-*eng2* is also quite different, with HG-*eng1* having smaller introns at each position. In contrast, the size of corresponding introns between GR-*eng1* and GR-*eng2* is highly conserved except for intron number 1. All of the intron sequences from *H. glycines* and *G. rostochiensis* have been examined to identify potential homology between introns. No significant sequence homology was detected between the introns from HG-*eng1* and HG-*eng2*, nor was there homology between HG-*eng* introns and GR-eng introns. However, strong sequence homology existed between corresponding introns of GR-*eng1* and GR-*eng2*. Introns number 2-7 of GR-*eng1* and GR-*eng2* share from 87-96% identical internal sequence as well as similarity in size. In contrast, the size of the first GR-*eng1* intron is 308 bp as compared to that of GR-*eng2*, which is 742 bp. The G+C-content of

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[1] Genomic DNA sequences: HG-*eng1*, Genbank accession no. AF052733; HG-*eng2*, Genbank accession no. AF052734; GR-*eng1*, Genbank accession no. AF056110; GR-*eng2*, Genbank accession no. AF056111

```
                   1              15 16          30 31      45 46               60 61              75 76               90
1 hg-eng1  MCRLQATHLLARLFL LLALCTALVSS  LTAV APFFGQLSVSGTNLV GANGQPVQLIGNSLF WHQWYPQFWNADTVK ALKCNWNANVIRGAM   90
2 hg-eng2  ------MFVQLVL    LAIVGITFVDA  ---A APPFGQLSVKGTNLV GSNGKPVQLIGNSLF WHQWYPQFWNAQTVK ALKCNWNSNVVRTAM   79
3 gr-eng1  MSRLQS--LFPQVFL LHGHLHIVFCNA LTAT PPPYGQLSVSGTKLV DSSGQPVQLIGNSLF WHQFQAQYWNAETVK ALKCNWNANVVRAAV   88
4 gr-eng2  MCRLQF--LFRVFL  LHALHIVFCNA  LTAT PPPYGQLSVSGTKLV GSNGQPVQLIGNSLF WHQFQGQYWNNADTVK ALKCNWNANVRAAV   88
          (.......signal-peptide.........)  ↑ (cleavage site)                              ↑ intron 1

           91           105 106          120 121          135 136           150 151           165 166              180
1 hg-eng1  GVD-EGGYLSDANTA YNLMVAVIEAAISNG IYVIVDMHAHNSHPD EA VKFFTRIAQAYGS YPHILYEDFNEPLS- VSWTDVLVPYHKK VI   178
2 hg-eng2  GVE-QGGYLSDANTA YRLTAAVIEAAIAQG IYVIVDMHAHEPNAD KA IEFFTKIAKAYGS NPHLLYETFNEPLD- VSWNDVLVPYHKK VI   167
3 gr-eng1  GVDLERGYMSDPTTA YNQAVAVIEAAISQG LYVIVDMHSHESHYD KA IEFFTKIAKAYGS YPHVLYETFNEPLQG VSWTDILVPYHKK VI   178
4 gr-eng2  GVDLERGYMSDPTSA YNLAVAVIEAAISQG MVVIVDMHSHEAHAD KA VEFFTKIAKAYGS YPHVLYETFNEPLQG VSWTNILVPYHKK VI   178
                         ↑ intron 2    (family 5 signature)                                           ↑ intron 3

           181          195 196          210 211         225 226           240 241           255 256              270
1 hg-eng1  AAIRAIDKKNVIILG TPTWSQDVDVASQNP IKDYQNLM YTLHFYA SSHFTNDLGAKLKTA VNN GLPVFVTBYGTC EASGNGNLNTDSMSS   268
2 hg-eng2  SAIRAIDKKNVIILG TPKWSQDVDVAAQNP IKGFSNLM YTLHFYA SSHFVDGLGNKLKTA VNK GLPVFVTBYGTC EASSNGNLNSGSMSS   257
3 gr-eng1  AAIRALDSKNVIILG TPTWCQDVDIASQNP IKEYKNLM YTFHFYA ATHFVNGLGAKLQTA INN GLPIFVTBYGTC SADGNGNIDTNSISS   268
4 gr-eng2  AAIRALDAKNVIILG TPTWCQDVDLASQNP IKEYKNLM YTFHFYA STHFVNGLGAKLQTA INN GLPIFVTBYGTC AADGNGNIDTNSISS   268
                         ↑ intron 4                                      intron 5 ↑   (E; nucleophilic residue)

           271          285 286          300 301      315 316           330 331           345 346              360
1 hg-eng1  WWTLLDSLKISYANW AISDKSEACSALSPG TTAAN VGVSSRWTSS GNMVASYVKKKST GV SCSGSSSG-SSSGSS SGSGSSSGSSSGGSS   357
2 hg-eng2  WWSLLDQLKISYVNW SITDKSEACAALTGG TSAAN VGTSSRWTQS GNMVASQHKKKST GV KC------------- ---------------   319
3 gr-eng1  WWSLMDNLKISYLNW AISDKSETCSALKPG TPAAN VGVSSSWTTS GNMVADHDKKKST GV SCSGSTSS-GSSSSN SGNSAATTTKKPPS   357
4 gr-eng2  WWTLLDNLKISYLNW AISDKSESCSALKPG TPAAN VGVSAWTTS  GNLVAAHDKKKST GV SCNGATPARGSSSAN SGKPAAA--KK-PPA   355
                                        ↑ intron 6      intron 7 ↑

           361          375 376          390 391          405 406          420 421          435 436              450
1 hg-eng1  SGSSSGSSSGSGSGSS SGSSSGSGSASISVV PSNTWNGGGQVNFEI KNIGSVPLCGVVFSV SLPSG TTLGGSWNME SAGSGQYSLPSWVRI   447
2 hg-eng2  ---------------- --------------- --------------- --------------- ----- ---------- ---------------   319
3 gr-eng1  NSGQTTNQKPSSSAG SSSSSGSASASVTVV STNTWNGGGQVNFEV KNTGSTTLCGVKFSV TLPAG TTVAGSWNMN AAGSNEYTLPSYINI   447
4 gr-eng2  KPAAAKKSPPAKPAA AKKPP-AKSAKAAPA KKPKSKSG------- --------------- ----- CBD.........               392
          (............................ CBD.........                                   ↑ intron 8

           451          465 466          480 481          495 496          510 511          525 526              540
1 hg-eng1  EAGKSSKDAGLTFNG KDKPTAKIVTTKKC- -                                                                   476
2 hg-eng2  --------------- --------------- -                                                                   319
3 gr-eng1  KAKEANKDAGMTLNG SGKPTAKVLSTTACS G                                                                   478
4 gr-eng2  --------------- --------------- -                                                                   392
          (...............CBD...........)
```

Figure 5.3 Intron position with respect to the predicted amino acid sequence of the four cyst nematode endoglucanase genes, Heterodera glycines genes HG-eng1 and HG-eng2, and Globodera rostochiensis genes GR-eng1 and GR-eng2. The location of the secretion signal peptide and cleavage site, glycosyl hydrolase family 5 signature sequence, catalytic domain (nucleophilic residue), peptide linker region (italics), and cellulose-binding domain (CBD) of the endoglucanases are indicated

the first intron in GR-*eng1* is significantly higher (57.8%) as compared to the other introns in GR-*eng*, which is caused by 8 cytosine repeats (6 to 14 bp long). The divergence in the first GR-*eng* introns is not based solely on point mutations or small insertions - as seems the case for intron 2, 3, 4, 5, 6, and 7. Computer analysis revealed that the most conserved region between the first introns is a 41bp stretch demonstrating 80.5% sequence identity. At the other extreme in intron variation, intron number 6 in both GR-*eng1* and GR-*eng2* show hardly any divergence (96% sequence identity over the entire intron sequence). Out of the 51 nucleotides that are contained in intron 6, two point mutations (transitions) have occurred. The point mutations were transitions from A to G at +13 bp downstream of the 5'-splice site, and from C to T at -4 bp upstream of the 3'-splice site. Interestingly no phase shift in the open reading frame occurs from exon 6 to intron 6, and exon 7.

Intron sequences that signal splicing and polyadenylation signal

The *cis*-splicing in the cyst nematode cellulase largely complies with the "GU-AG rule" [4]. However, the 3rd and 5th introns of HG-*eng1*, and the 3rd intron of both GR-*eng1* and GR-*eng2*, use GC as 5'-splicing donor sequence instead of GU. In HG-*eng2* all of the exons and introns are bordered by canonical sequences. Based upon the collective exon/intron border sequences of both cellulase genes in *G. rostochiensis*, the consensus 5'-splice site is M G_{73} ↓ G_{100} U_{87} R A_{60} G_{73} U_{67} U_{47}, and the consensus 3'-splice site is C C_{67} R_{53} U_{73} U_{67} A_{53} A G_{100} G_{100} ↓ G_{87} Y. For both *H. glycines* cellulase genes, the consensus 5'-splice site is M G_{73} ↓ G_{100} U_{87} R A_{60} G_{67} U_{73}, and the consensus 3'-splice site is Y_{73} Y_{73} Y_{73} U_{80} U_{53} A_{53} A G_{100} ↓ G_{87} U_{73} (M=A or C; R=A or G; Y= C or T). The polyadenylation and cleavage signal for the four nematode cellulase genes are all 5'-GAUAAA-3'. The 3'-end formation signals of HG-*eng1* and HG-*eng2* are located at 32 and 17 nucleotides upstream of the cleavage site, respectively. The 3'-end formation signals for GR-*eng1* and GR-*eng2* both occur at 20 nucleotides upstream of cleavage site.

Introns are inserted at the same positions with respect to the encoded cellulases

HG-*eng1* and HG-*eng2* share 78.37% predicted aa identity in the catalytic domain, and the two GR-eng cellulases share 83.03% predicted aa identity in the catalytic domain [29]. The two least similar cellulases of the four, HG-*eng2* and GR-*eng1*, share 71.38% identity in aa sequence over the catalytic domain. When the cellulase protein alignment was compared to the exon/intron data, it was recognized that the intron positions with respect to predicted aa sequence are identical among the four nematode cellulases (Fig. 5.3). Cellulases from *H. glycines* have apparent amino acid deletions in positions 94 and 165, and the deletions occur at the 2nd and 3rd exons, respectively (Fig.5.3).

The location of putative *cis* regulatory elements in nematode cellulase flanking regions

The nucleic acid sequences upstream of the predicted transcription initiator sites (5'-catcaa-3' in both GR-*eng*, 5'-caattc-3' and 5'-caatcc-3' in HG-*eng1* and HG-*eng2*, respectively predicted by NNPP at http:// www-hgc.lbl.gov) were compared among the

four nematode cellulase genes (Fig. 5.4). Comparison of the 5' –flanking regions between HG-*eng1* and HG-*eng2* did not reveal any conspicuous homology. In contrast, the 5'-flanking region of GR-*eng1* and GR-*eng2* demonstrated strong homology, with 67% nucleic acid identity in the 300-bp region preceding the Inr. When the 3'-flanking regions of the four nematode cellulases were compared, no significant homologies were apparent (data not shown).

In the 5'-flanking region of all four nematode cellulase genes, a TATA box is centered at nucleotide positions ranging from –27 to –29 bp upstream of the transcription start sites ([10]; Fig. 5.4). In fact, the 5'-flanking regions of the HG-*eng1*, HG-*eng2* and GR-*eng1* genes all contained two consensus TATA box sites. No putative CAAT box sequences were identified in the 5'-flanking regions.
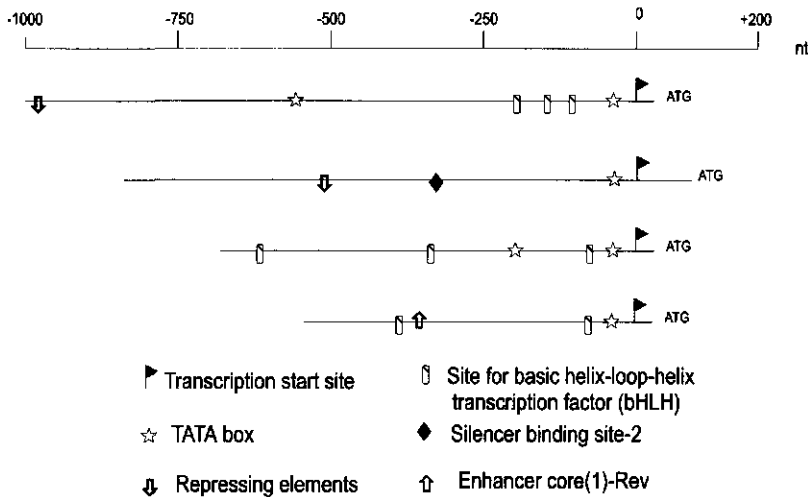
Figure 5.4 *Alignment of the 5'-flanking regions of the four cyst nematode endoglucanase (structural) genes,* Heterodera glycines *genes HG-eng1 and HG-eng2, and* Globodera rostochiensis *genes GR-eng1 and GR-eng2. Consensus of putative transcriptional cis elements and their relative position with respect to the translation start site are depicted by the icons represented above.*

The DNA motifs CAAGTG and CAATTG, which were identical to the consensus core binding site sequence (CANNTG) of the bHLH (basic helix-loop-helix proteins) class of transcription factors, were located at positions -70 and -397 bp relative to the transcription start site of GR-*eng2* ([25]; Fig. 5.4). The bHLH core sequence also occurred at the -72, - 330 and - 688 positions of GR-*eng1*. The bHLH core sequence was located at positions --17, -98, -144, -192, and -975 of HG-*eng1*. The 5'-flanking region of HG-*eng2* did not contain a predicted bHLH core sequence. A putative silencer-binding-site-2 (5'-TATTAAAA-3') was present only in position -414 of HG-*eng2* [39]. Putative repressor elements (consensus sequence 5'-CCWTNTTNNNW-3') were

identified in positions -1001 and -595 of HG-*eng1* and HG-*eng2*, respectively. There were also putative repressor elements in the 3'-flanking sequence of GR-*eng1* at positions +49, +283, +320, and GR-*eng2* at positions +20 and +315 of the cleavage site [28]. In position -382 of GR-*eng2* there is a putative enhancer core-1 element in reverse orientation [38]. In the 3'-flanking region of GR-*eng2* there is a putative enhancer core-2 element (consensus sequence 5'-GGGRHTYYCC-3') located at position +55 of the polyadenylation cleavage site [28].

The G+C contents of the flanking regions in the GR-eng and HG-eng genes are 35% and 34% respectively, which is in close range to the average G+C content of 36% found for *C. elegans* [32] and *G. rostochiensis* (Rouppe van der Voort, pers. comm.).

The relationship between cyst nematode and bacterial cellulases

The first twenty most homologous sequences in TFASTA analyses of the catalytic domain of the four cyst nematode cellulases were those of endoglucanases of bacterial origin. The top ten most homologous cellulases were chosen to construct a phylogenetic tree (Fig. 5.5). The neighbour-joining method was used to establish phylogenetic links among the various cellulases [30]. The fourteen cellulases analysed could be divided into three related groups. The first group is the plant-parasitic cyst nematode cellulases. The second group contained CelE and CelZ from *Pseudomonas* and *Erwinia* respectively, which belong to *Gracilicutes, Scotobacteria*. The third group included cellulases from *Firmicutes, Bacillaceae*. The HG-*eng2* cellulase may be considered to be the most primitive type of cyst nematode cellulase of the four analysed. The HG-*eng2* cellulase has only a catalytic domain consisting of 319 amino acids and no peptide linker or CBD. The HG-*eng2* cellulase shares 71% to 78% identity in predicted amino acid sequence with the other three cyst nematode cellulase (Table 5.1). The HG-*eng2* cellulase shares 33% to 44% identity in predicted amino acid sequence with the ten most homologous bacterial cellulases.

Table 5.1 *Homology of the catalytic domain of the endoglucanase of* Heterodera glycines *(HG-ENG2) to the catalytic domain of other endoglucanases of cyst nematode origin, and the most closely related endoglucanases from bacteria.*

Enzyme	Organism	Accession No.	Homology[•] with HG-eng2	
			Similarity %	Identity %
HG-ENG1	*Heterodera glycines*	AF006052	84.3	78.4
GR-ENG1	*Globodera rostochiensis*	AF004523	82.4	71.4
GR-ENG2	*Globodera rostochiensis*	AF004716	83.1	71.5
CelE	*Pseudomonas fluorescens*	X86798	63.7	44.6
CelZ	*Erwinia chrysanthemi*	Y00540	61.4	41.8
Egl	*Clostridium acetobutylicum*	M31311	62.4	42.8
CelA	*Bacillus spp. (n-4)*	m14729	56.7	39.0
End1	*Bacillus subtilis* (PAP 115)	X04689	60.4	40.5
Cel	*Bacillus subtilis* (CK2)	X67044	60.4	40.5
End2	Bacillus subtilis (N-24)	M28332	60.4	40.5
End2	*Bacillus subtilis* (DLG)	M16185	61.0	41.9
End2	*Bacillus sp.* (KSM-635)	M27420	57.0	32.0
End	*Bacillus sp.* (KSM-64)	M84963	58.0	33.4

• The similarity and identity is calculated by comparing the catalytic domain amino acid sequence using the BESTFIT program in the GCG package.

Scale: ___ is approximately equal to distance of 0.055

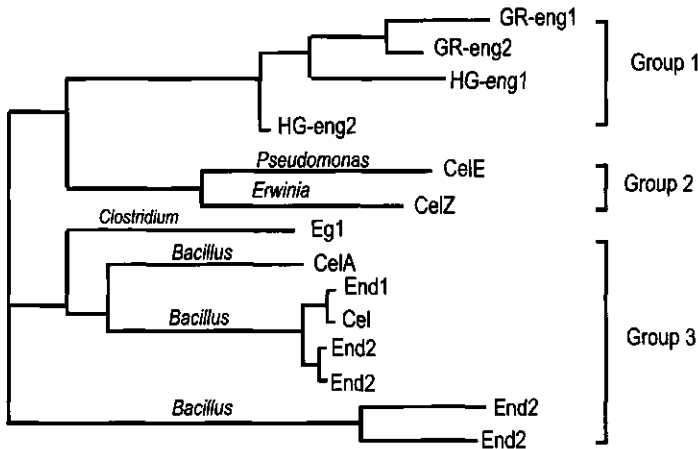***Figure 5.5*** *Phylogenetic tree of the cyst nematode endoglucanase genes,* Heterodera glycines *genes HG-eng1 and HG-eng2, and* Globodera rostochiensis *genes GR-eng1 and GR-eng2, in relation to the most closely-related groups of endoglucanases. The tree was constructed by comparisons of the catalytic domains of each endoglucanase using the neighbor-joining method of Sudhir et al. [30].*

DISCUSSION

Organisation of the cyst nematode β-1,4-endoglucanase genes

We had previously reported several lines of evidence for the endogenous production of β-1,4-endoglucanases from *G. rostochiensis* and *H. glycines* [29]. Herein we showed that these four cyst nematode cellulase genes are interspersed by several introns, thus supporting the eukaryotic organisation of the genes. The relatively small intron size (average intron size 128) of the four cyst nematode cellulase genes suggested that the intron size range is similar to that of *C. elegans* [4]. The average G+C content of coding and non-coding regions further points to a similarity with the genomic composition of *C. elegans*. The 5'–flanking regions of all four cyst nematode cellulase have a TATA box preceding the translation start site in the usual location for eukaryotes [22]. The occasional 5'-GU...AG-3' intron splicing sites found in the cyst cellulase genes, however, are a rare occurrence in *C. elegans* and most vertebrates [4]. In addition, all

79

four of the cyst nematode cellulase genes had the same rarely used polyadenylation and cleavage signal sequence, 5'-GATAAA-3'. By contrast, in the major sperm protein genes of *G. rostochiensis* the polyadenylation signal was 5'-AATAAA-3' [24]. The combined features of the four cyst nematode cellulase genes suggest that while they are indeed similar to other nematode genes, they may represent a unique group of eukaryotic genes.

The *cis* transcriptional elements of the four nematode cellulase genes

Almost no information is available on the transcriptional regulation of gene expression in plant-parasitic nematodes. The HG-*eng1* and HG-*eng2* cellulases were both expressed specifically within the subventral oesophageal glands of infective juveniles of *H. glycines* [29], yet there was little homology between their putative 5' regulatory regions. However, significant homology did exist in the 5'-flanking region of the two *G. rostochiensis* cellulase genes. Computer analyses of the 5'-flanking regions of each cellulase gene revealed consensus elements of eukaryotic promoters including at least one TATA box in each gene, and the presence of bHLH-type binding sites, silencers, repressors, and enhancer elements in several of the cyst nematode cellulase genes. The existence of multiple silencer, repressor and enhancer elements in the flanking regions of the nematode cellulase genes may suggest potential regulatory roles in gland-specific gene expression and plant parasitism. Additional studies are needed to fully characterise these putative promoters from plant-parasitic nematodes and the potential *trans*-acting factors that interact with them.

Introns mark the cyst nematode cellulases at conserved locations

The intron size and sequence in any given position is quite dissimilar overall among the four cyst nematode cellulases. However, the intron marking positions are exactly the same with regard to the encoded cellulase peptide sequence. The striking agreement of all of the intron positions among the nematode cellulases suggests that the intron positions were established before a proposed division of *H. glycines* and *G. rostochiensis* from a common ancestor. The "intron-early" view holds that exons should represent functional or folding elements of proteins, whereas the "intron-late" view suggests that the insertion of introns might occur at specific DNA sequences, but may not be correlated with protein sequence [7].

Intron 6 in de EGases of *G. rostochiensis* shows exceptional little divergence (4%) as compared to the other introns in GR-*eng*, which points to some kind of evolutionary constraint. In addition, intron 6 is symmetrical and could be included in the transcript without a shift in the open reading frame. Alternative splicing of intron 6 would lead to translation of 16 additional aa at C-terminus of the catalytic domain, folding into an additional (β/α) repeat [6]. Apparently this addition is either redundant with respect to the protein function or under strict developmental control, because EGases 2 kDa larger than the ones described by [29] have not been observed in *G. rostochiensis* J_2. Alternative splicing probably may have been maintained longer in *G. rostochiensis* than in *H. glycines,* because the marked intron position for all four nematode EGases is the same, while the divergence of intron 6 in *H. glycines* is larger.

The reaction mechanism of glycosyl hydrolases either leads to an overall retention (*e.g.* family 5) or an inversion of the anomeric configuration at the hydrolysis site. The regions around the acid catalyst and the nucleophile in nematode cellulase as well as in family 5 cellulases in general are particularly conserved. Henrissat et al. [19] have used these regions to define "motifs" in the active site. These motifs can be found consistently in many glycosyl hydolases that show the overall retention mechanism. Like in other cellulases, nematode cellulases have these motifs, that is a motif near the proton-donor (*e.g.* α-helic, loop, and β-strand; Asn_{159}-Glu_{160} in HG-*eng1*), and one near the nucleophilic residue (*e.g.* α-helic, loop, and β-strand; Glu_{249} in HG-*eng1*). Surprisingly both motifs are not contained in a single exon each. Intron 2 is located in the middle of the α-helix of the first motif, whereas intron 5 is between the α-helix, and the β-strand of the second motif. Thus, in nematode cellulase genes the exons are not correlated to distinct motifs in the secondary amino acid structure.

The tertiary structure predicted for the catalytic domain in the nematode cellulase is a $(β/α)_8$-barrel, linked to a CBD by an 50 aa long extended loop [29]. The catalytic domains in the nematode cellulases are separated from the linker and CBD by an intron. The catalytic domain results from joining the exons 1 to 7, whereas the linker region and the CBD are spliced from exons 8 and 9. These findings are in contrast to the cellulase gene *cel3* of the fungus *Agaricus bisporus* in which the exons are not only correlated with functional domains, but also with parts of the secondary structure regions of the encoded protein [40]. The alignment of intron position with protein structure has also been reported for triose-phosphate isomerase genes (encoding $(β/α)_8$ –barrel) between organisms separated by great evolutionary distances such as maize and vertebrates [11]. Moreover, some intron positions were found to be conserved in glycosyl hydrolases cloned from *Trichoderma reesi*, *Phanerochate chrysosporium*, and *A. bisporus* [40]. If conservation of intron position is considered as an evolutionary event, this may provide strong evidence that the cellulase genes from *H. glycines* and *G. rostochiensis* share a common ancestral gene.

The relationship among the four cyst nematode cellulases

Evidence that HG-*eng2* is more distantly related to the other three cyst cellulases is not only apparent in the phylogenetic tree based upon the catalytic domain sequence, but also in the absence of a rare donor splice site (GC instead of GU), and bHLH binding sequence that were present in HG-*eng1*, GR-*eng1*, and GR-*eng2*. In addition, both the size distribution and G+C content of the introns further illustrate the exceptional position of HG-*eng2* among the nematode cellulases. The two *G. rostochiensis* cellulases were the most closely related, and several lines of evidence support the hypothesis that the two GR cellulases might have been derived from recent gene duplication and exon shuffling; a) the overall amino acid sequence identity of the catalytic domain of the GR-*eng1* and GR-*eng2* is 83%, b) their 5'-flanking region share 88% sequence identity in the 322 bp region preceding the translation start site and, c) the intron sequences between GR-*eng1* and GR-*eng2* tend to be very conserved except for the first intron.

The cyst nematode cellulases and family 5 glycosyl hydrolases

Interestingly, all of the bacterial cellulases with the highest homology to cyst nematode cellulases belong to glycosyl hydrolase family 5, subfamily 2 [20]. In the phylogenetic tree (Fig.5.5), the nematode cellulases and family 5 (sub 2) cellulases were classified into three closely-related groups. It has been suggested that cellulases within the same families are not only structurally related, but they have probably evolved from a common ancestral gene [19,34]. Evidence for the interspecific transfer of cellulase genes between bacteria has been reported [16].

The most rigorous criteria for establishing a case for ancient horizontal transfer is to compare a phylogenetic tree based upon a specific protein or DNA from distantly related organisms with that of the known phylogeny for the species [33]. If a significant incongruency is observed between the " protein tree" and the "species tree", then it is possible that horizontal gene transfer has occurred. In no other nematodes, including *C. elegans*, and indeed in no other eukaryotes, was significant homology found at the nucleic acid or aa level with the cyst nematode cellulases. Although functional convergence is common, and enzyme structural convergence may have occurred in nematodes, there is no convincing data for genuine sequence convergence from eukaryotic genes at either the protein or DNA level [9]. A strong case can be made here that a common ancestor of the cyst nematode may have acquired the cellulases via horizontal transfer from a prokaryote. However, this proposed event must taken place at least tens of millions of years ago thereby allowing the foreign genes to adapt to their new genomic environment.

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Chapter 6

Summary and concluding remarks

Stylet secretions are considered to be important pathogenicity factors in the plant-parasitism of cyst nematodes. Despite the many attempts that have been undertaken in the past to characterise stylet secretions, the identity of pathogenicity factors in these secretions has remained obscure. In this thesis we present for the first time evidence for basic-compatibility factors in the secretions of plant-parasitic nematodes. We describe the identification and characterisation of cellulases in the stylet secretions of two cyst nematode species. This conclusive chapter discusses the structure and function of the nematode cellulases in relation to their importance for pathogenicity, and their evolutionary origin.

From *svps* to endoglucanases

Purified proteins from some nematode species may act as an immunodominant antigen when used for immunisation of mice. The immunodominancy is caused by a rare terminal cap on the N-glycosylations of secreted proteins [11]. For example, several proteins in excretions/secretions from *Trichinella spiralis* share such an immunodominant carbohydrate epitope, and elicited a set of cross-reactive antibodies [38]. An immunodominant 38.0 to 40.5 kDa-protein fraction from the potato cyst nematode *G. rostochiensis* was used for the immunisation of mice. The immunisation resulted in a set of 11 cross-reactive mAbs (MGRs) that recognised four proteins (*svp*49, *svp*39, *svp*32, and *svp*31; chapter 2). The cross-reactivity of antibodies on the *svps* is not based on a similar pattern of N-linked glycosylation attached to all four proteins, because the epitopes recognised by the mAbs are not carbohydrates (chapter 3). Therefore, the *svps* may arise from a single gene-product differentiated in to several proteins by proteolytic post-translational modifications. Alternatively, the shared epitopes could be parts of highly conserved stretches of amino acids in the primary sequences transcribed from more than one gene.

mAb MGR48 was used to immunopurify *svp*39 from homogenates of *G. rostochiensis*. The partial amino acid sequences obtained from N-terminal sequencing resulted in the cloning of two β-1,4-endoglucanase genes. The N-terminal catalytic domains encoded by the EGases genes were highly conserved (95% identity in 883 bp). The predicted molecular masses of the encoded mature proteins are 47.9 kDa and 39.1 kDa. Polyclonal sera raised against both EGase genes recombinantly expressed in *E. coli* recognised both *svp*49 and *svp*39 in homogenates of *G. rostochiensis*. In conclusion, at least two genes contribute to the EGase activity found in *G. rostochiensis* (chapter 4).

The expression of the *svps* is confined to subventral oesophageal gland cells in *G. rostochiensis*. Three *svps* (*svp*49, *svp*39, and *svp*31) could be identified in collected stylet secretions from *G. rostochiensis* (chapter 3). Among these *svps* the smallest form *svp*31 predominated. Although the smaller *svps* show hardly any detectable CMCase activity in homogenates, they represent the majority of the CMCase activity found in stylet secretions (data not shown).

On two-dimensional western blot prepared from protein extracts of *G. rostochiensis* *svp*31 and *svp*32 appeared as a cluster of four protein spots (*svp*30, *svp*31a, *svp*31b, and *svp*32). These proteins were also subjected to immunopurification and N-terminal sequencing, however, the mixture of proteins was too complex to result in a reliable amino acid sequence of the N-termini (data not shown). Therefore, it remains unsolved whether the immunogenic and enzymatic similarity between *svp*30, *svp*31a and b, and *svp*32, and the two cloned EGases is the consequence of yet another homologous EGase gene.

Proteolytic processing of glycosyl hydrolases into smaller molecular mass forms has been shown for fungal endoglucanases [13,30] and cellobiohydrolase [23], and bacterial endoglucanases [19]. In several cases the proteolytic processing of bacterial and fungal cellulases occured in the exposed peptide linker between two functional domains in the protein structure. Instead of encoded by a third EGase gene the smaller

svps could arise form post-translational modified *svp*39 and *svp*49. This kind of protein processing of *svp*39 and *svp*49 is then most likely to happen in the secretory granules of the regulated secretory pathway of nematode EGases [4]. In theory, the smallest catalytic protein fragment that could be processed from GR-*eng1* and GR-*eng2* is 31,9 kDa and 31,6 kDa respectively. This is well within the range of the molecular masses of the smaller *svps*, but could only explain two protein spots. Moreover, the expression of a single gene encoding a pectin-degrading endopolygalacturonase from *Fusarium moniliforme* resulted in the secretion of four glycoforms (38 to 48.5 kDa) of peptide backbone [3], which is yet another biochemical pathway to get multiple forms of a single protein precursor. Thus a combination of proteolytic processing and protein glycosylation could be a valid explanation for the predominance of the smaller svps in stylet secretions, but additional effort is required to finally determine how *svp*30, *svp*31a and b, and *svp*32 relate to the two cloned EGase genes.

The situation in *H. glycines* seems less complicated as mAb MGR48 only reacted on western blot with a single protein of approximately 49 kDa, which is detected in homogenates (chapter 4) and in collected stylet secretions (data not shown). This protein is solely expressed in the subventral oesophageal gland cells of the nematode. *Svp*49 from *H. glycines* was immunopurified to homogenity, and the partial N-terminal sequence enabled the PCR-based cloning of two homologous EGase genes. Polyclonal sera raised against EGases expressed in *E. coli* reacted with two bands of approximately 39 kDa and 49 kDa. In addition, an anti-sense probe transcribed from one of the clones hybridised with RNA in the subventral oesophageal gland cells of *H. glycines*, thereby providing conclusive evidence for the endogenous transcription and translation of the genes. Similar to *G. rostochiensis* only *svp*49 showed EGase activity in *H. glycines* homogenates, whereas enzyme activity was shown for both *H. glycines* genes expressed in *E. coli*.

Structure and function of cyst nematode β-1,4-endoglucanases

The predicted tertiary structure of the nematode endoglucanases contains a catalytic domain shaped as a classical $(\beta/\alpha)_8$-barrel (HG-*eng2*), an approx. 50 amino acid long linker peptide (GR-*eng2*), and a bacterial type of cellulose-binding domain (HG-*eng1* and GR-*eng1*). The $(\beta/\alpha)_8$-barrel was first found in triose–phosphate isomerase (TIM), and since then identified in many families of glycosyl hydrolases (PDB entry 1EDG;[7]). The nematode endoglucanases are categorised into the family 5 glycosyl hydrolases which includes 40% of all known cellulase sequences. The homology within the glycosyl hydrolase families is based on primary amino acid sequences and secondary structure prediction [14,16,17,33].

The prediction of the tertiary structure of the nematode endoglucanase is deduced from a representative of the family 5 glycosyl hydrolases, endoglucanase A from *Clostridium cellulolyticum* (CelCCA), whose crystal structure has been determined (chapter 4; [10]). The structural analogy with CelCCA enabled the localisation of the two catalytic residues (Glu_{159} and Glu_{249} in GR-*eng1*) at C-terminal ends of the β-strands 4 and 7, at approximately 90 amino acid distance from each other. Seven

amino acid residues are strictly conserved within the family 5 – Arg_{85}, His_{126}, Asn_{158}, Glu_{159}, His_{220}, Tyr_{222}, and Glu_{249} (in GR-*eng1*). These conserved residues are all located on the same side of the β-barrel, which is situated at the bottom of the large cleft on the protein surface. In addition, some of the conserved aromatic residues that are also highly conserved, such as Phe_{58}, Trp_{59}, Trp_{79}, Trp_{166}, Tyr_{173}, Phe_{221}, and Trp_{283} (in GR-*eng1*), line up to form a hydrophobic surface in the active site of CelCCA [10]. Particularly the positions of the side-chains of tryptophanes in the cleft may allow for the hydrophobic interaction with the hydrophobic faces of the pyranose ring in the substrate, *e.g.* cellulose [10].
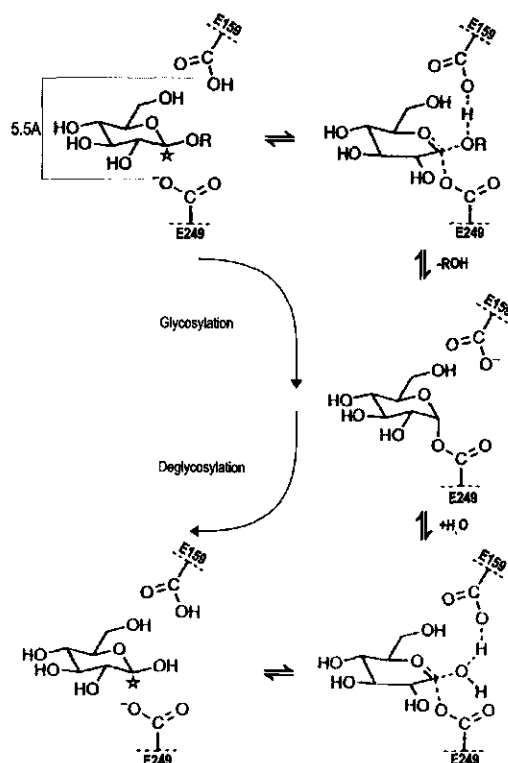
Figure 6.1 *General acid hydrolysis of the β-1,4-glycosidic bond in cellulose leading to a rentention of β-configuration of the anomeric carbon (★). The reaction mechanism involves an initial attack of the bond by a proton donating glutamic acid (E159), which leads to an intermediate glycosylation of the endoglucanase at the nucleophilic glutamic acid at position 249 (E249). The cellulose fragment carrying the non-reducing end is then replaced by a water molecule. The water molecule provides the OH that subsequently releases the covalent bond between remaining cellulose fragment and E249 (deglycosylation). The H$^+$ from the water molecule regenerates the proton lost by E249 ([26, 37]; indicated amino acid positions are of GR-ENG).*

The hydrolysis by glycosyl hydrolases is expected to take place via general acid catalysis [27]. Depending on the distance between two catalytic residues in the enzyme the outcome of the acid hydrolysis can either result in an overall retention or a conversion of the anomeric carbon [7,18,27,37]. The distance of the catalytic residues in enzymes that show a retaining mechanism of glycan hydrolysis is on average 5.5Å, whereas those showing an inverting mechanism have a distance of 10Å between the catalytic residues. Because the protein structure of the family-5 glycosyl hydrolase is so conserved, and the catalytic residues can be localised within this conserved structure, the catalytic mechanism is expected to be the same for all members of this family. For CelCCA it has been shown that the outcome of the acid hydrolysis is a retention of the anomeric carbon, therefore it is concluded that hydrolysis of the β-1,4-glycan linkages by nematode endoglucanases will also result in the same retained stereochemical outcome (Fig. 6.1;[10]).

At the C-terminus of the catalytic domain in three nematode endoglucanases there is a predicted extended loop maintained by a stretch of approximately 50 amino acids (chapter 4). In both nematode endoglucanases carrying a cellulose-binding domain (*engl*) this linker peptide is rich in hydroxyamino acids that have a high O-glycosylation potential (data not shown). The linkers in nematode endoglucanase are reminiscent of the hydroxyamino acid, proline, alanine, and glycine rich linkers that have been found in many multi-domain proteins [14]. The extensive hydrogen bonds that are enabled by the arrangement of the prolines and hydroxyamino acids in the linker regions result in structural stability while still allowing for sufficient flexibility between the two functional domains [33]. O-linked glycosylation will further contribute to the stability of the linker region and increase the resistance to proteolysis [21].

Cellulose-binding domains (CBDs) are classified into structurally related families based on similarities in the primary amino acid sequence [2,14,33]. So far, the majority of the CBDs are designated into two main types. The first type that is known as the fungal type of CBD is characterised by its relative small size of approximately 35 amino acids containing a highly conserved pattern of at least four conserved aromatic residues, and four cysteines that form two disulfide bridges [33] Prosite Prodoc entry 00486]. A fungal type of CBD found in the *cbhl* gene of *Trichoderma reesi* has a wedge shape composed of three anti-parallel β-strands with a flat hydrophilic and hydrophobic side [20]. The aromatic residues are exposed to the solvent of the CBD and are presumably involved in protein-carbohydrate interactions [33].

Although the nematode EGases do not fully comply to the pattern derived from bacterial type of CBDs they clearly have the signature of this type of CBD (Prosite Database:Prodoc 00485). Typically these CBDs consist of approximately 100 amino acids with two conserved cysteines residues at either extremity of the domain, and several regularly spaced aromatic amino acid residues. The cysteines are involved in a disulfide bridge spanning the entire domain. CBDs in nematode endoglucanases also have two conserved cysteines, one at the C-terminus and a second cysteine at 33

amino acids from the N-terminus (Cys_{411} in GR-ENG1 and HG-ENG1 respectively). The recently resolved tertiary structure of a bacterial CBD in *Thermonospora fusca* endoglucanase E-4 shows two β-sheets, of four and five anti-parallel β-strands respectively, projected on a large helical structure. Because of the larger variation in primary amino acid sequence among the bacterial CBDs as compared to the fungal type CBDs, and because of the significant difference in the position of the two cysteines, a tertiary structure prediction of the nematode CBDs is not opportune.

There is no evidence pointing to a definite role of the CBD in glycosyl hydrolases other than that it binds cellulose, and thereby increases the affinity of an enzyme for its substrate [33]. The individual domains in cellulases can function independently, but the activity can be greatly enhanced by the presence of a CBD. Some cellulases show catalytic specificity for the physical structure of cellulose, and the CBD may be involved in directing the specificity. At present there is no conclusive evidence that CBDs disrupt the crystalline structure of cellulose, and that CBDs are essential for hydrolysis of crystalline cellulose [29].

Both nematode species studied in this thesis have a cellulase with a CBD, and second one without a CBD. The occurrence of several cellulases in a single organism is common to many plant pathogens [1]. What evolutionary drive would favour the existence of more than one gene whose product is involved in the same chemical reaction? Henrissat [16] explained the differentiation in specificity of cellulases as a consequence of the emergence of new carbohydrate metabolites either by evolution, or by change of habitat. Gene duplication could provide enzymes an escape from two counteracting selective forces in these situations. Enzymes that are more efficient and specific in their catalytic activity to the original substrate may result in a higher fitness for the hosting organism. By contrast, enzymes that are less specific and can hydrolyse new substrates too, may also result in fitness advantage. Gene duplication would allow for divergence of one copy of the gene, while the duplicated gene still maintains its activity towards the original substrate. A good example of a putative gene duplication followed by enzyme divergence is given by GR-*eng1* and GR-*eng2* which are highly homologous in their catalytic domain (93% identical amino acids), but only one enzyme carries a CBD. A similar situation seems to exist in *H. glycines*, where the homology between the catalytic domains in the cellulases is significantly lower (80% identical amino acids), possibly indicating an earlier duplication.

It is not clear what the degree of variation in substrate specificity is among the cyst nematode EGases, or to what extent the nematode EGases comply to the specificity map of family 5 endoglucanases [6]. The catalytic activity of the enzymes in homogenates and stylet secretions is only tested on CMC (chapter 4). Substantial quantities of soluble purified enzyme are required to perform additional substrate mapping with chromogenic and fluorogenic substrates. It is, however, not feasible to collect such quantities from nematodes, hence specificity mapping of nematode cellulases depends on heterologously expressed protein. Nematode cellulases that are expressed in *E. coli* are consistently sequestered to inclusion bodies, and therefore necessitate aggressive denaturation and renaturation steps. Such a protocol is used in chapter 4, and only resulted in minimal amounts of active protein. Thus, alternative

eukaryotic expression systems, *e.g.* yeast (*Pichia pastoris*) or baculovirus, have to be explored for their potential to produce substantial amounts of active soluble protein that will allow for the determination of the catalytic properties and specificity of the enzymes. Once the specificity of the four enzymes is clear it will be possible to determine what the primary target substrate of a particular enzyme species is.

The role of β-1,4-endoglucanase in pathogenicity

The β-1,4-endoglucanases from the two nematode species described herein are the first cellulase genes identified in animals. Based on experiments done in the 60ies and 70ies it is likely that many other nematode species express cellulases genes. It is expected that endogenous cellulases occur in other animal species, too [36]. In general, the fitness cost involved in the expression of structural genes makes it likely that cellulase genes are a benefit for the hosting plant-parasitic nematode. This means that cellulases will somehow contribute to the success of the parasitic strategy adopted by cyst nematodes.

A direct approach to gain insight in relative importance of genes in the parasitic strategy of an animal is to eliminate the effect of the gene during parasitism. In theory two methods should be applicable to obtain elimination of the gene. First, virulent plant-nematodes may be transformed in such a way that a putative pathogenicity gene is inactivated, *e.g.* by anti-sense inactivation, co-suppression, or dsRNA interference [12]. A major drawback in this approach is that transformation of cyst nematodes is seriously complicated by the fact that they are obligatory plant feeders with an amphimictic mode of reproduction. At present the only nematode species transformed so far are the hermaphroditic *Caenorhabditis elegans* and *C. briggsae*. Reports on the stable transformation of plant-parasitic nematode species have not been published yet.

A second approach to eliminate the function of a gene product in plant-parasitic nematodes uses inhibitory antibodies [26]. Monoclonal antibodies or derivatives thereof can be used for heterologous expression in the host-plants of plant-parasitic nematodes. By selecting antibodies that have shown inhibition of the activity of the gene product *in vitro* heterologous expression may allow for the assessment of the relative importance of the gene product in parasitism.

Several endoglucanase and cellobiohydrolase genes have been cloned from plant-pathogenic bacteria and fungi [1,35]. In many of these pathogens glycosyl hydrolases are secreted as mixtures of several types of enzymes with overlapping substrate specificity. For instance, in *Fusarium oxysporum* five cellulase genes were identified belonging to four separate families, including β-1,4-endoglucanases and cellobiohydrolases. In spite of the attempts to determine the role of bacterial and fungal cell wall-degrading enzymes in pathogenicity there is no equivocal picture arising from these experiments. Single disruptions of individual genes in many cases failed to show that they are essential for pathogenicity. *Pseudomonas solanacearum* mutants deficient in β-1,4-endoglucanase production only showed a quantitative effect on pathogenicity [25]. Moreover, CelV, a β-1,4-endoglucanase of *Erwinia carotovora* subsp. *carotovora* was demonstrated to be contributive but not essential for the ability to cause soft rot in potato tuber tissue [34]. Endoglucanases, cellobiohydrolases, and

β-glucosidases have overlapping substrate ranges with a major preferred substrate. Disruption of a single class of cellulase genes could easily be compensated by the overlapping activity of another class of enzymes, therefor complicating the assessment of the importance of such a class of genes in pathogenicity.

Immunolocalisation studies using the polyclonal sera against the heterologous nematode cellulases will clarify where the target of nematode cellulases is localised in the plant cells. In chapter 3, a similar attempt using the mAb MGR48 was hampered by poor fixation of the epitope at strongly diluted concentration in plant tissue. From these experiments it became clear that the nematodes secrete endoglucanases during migration through the roots, but the exact location of the substrate could not be identified due to these experimental limitations. Still, several additional lines of evidence in this thesis indicate that the cellulases are involved in the root penetration and subsequent migration through cortical tissue (chapter 1). First, extensive stylet thrusting and cellulase secretion by pre-parasitic J_2 is observed when the nematodes have been exposed to root exudates presumably at concentration similar to that in the rhizosphere (chapter 3). A significantly increased stylet activity was previously observed in juveniles in the rhizosphere of tomato, just prior to invasion of the root [31]. Second, the expression of the cellulase genes in *G. rostochiensis* is confined to the migratory developmental stages (chapter 3). Third, in general cellulases in plant-pathogens are primarily associated with cell wall degradation, because the carbohydrates that are potential substrates are major constituents of cell walls.

It seems unlikely that the nematode endoglucanases described in this thesis are involved in the degradation of cell walls in syncytial cells, because the temporal expression pattern of the cellulase genes does not correlate with the degradation of syncytial cell walls. In addition, the polar shape of syncytia and specific localisation of cell wall degradation in syncytial cells would then implicate a selective transport of nematode cellulases through syncytial cells to those specific sites. Such a mechanism mediating the transport of nematode cellulases over long distances in plant cells is hard to imagine.

Horizontal gene transfer

Catalytic domains in glycosyl hydrolases that are categorised into the same family are thought to have evolved from a common ancestor. Members of the same family share their secondary and tertiary protein structure, the stereochemical outcome of the hydrolysis reaction, and biochemical specificity. In April 1998 the total number of protein database entries of cellulases (EC 3.2.1.4) was 275.

These 275 enzymes have been examined for any homology with the nematode cellulases. In Figure 6.2, a comprehensive phylogenetic tree illustrates how the nematode cellulases relate to those cellulases found in bacteria, fungi, and plants. The nematode cellulases are clustered significantly closer to bacterial cellulases than to cellulases from any other eukaryotic origin. A similar line of reasoning holds true for the CBDs identified in nematode cellulases.

Table 6.1 Summary of the organisms from which endoglucanases have been identified (Genbank release of June 1998).

Organism			# cellulases genes cloned
Prokaryotes:	Eubacteria		188
Eukaryotes:	Mitochondrial eukaryotes;		
		Fungi	44
		Viridiplantae	38
		Dictyostelia	1
		Metazoa; Nematoda	4

In theory two evolutionary pathways may have led to the current phylogenetic position of the nematode cellulases. First, the genes encoding the nematode cellulases and bacterial cellulases may have evolved from an ancient cellulase ur-gene in a common ancestor of bacteria and nematodes. Cellulase genes in both nematodes and bacteria are then extremely conserved. This implicates the existence of a strong evolutionary constrain on these enzymes. An unprecedented sequence convergence to the extent as is found in the cellulases described in this thesis is a very unlikely explanation [8]. These observations lead us to speculate on a horizontal gene transfer of the nematode cellulase from a bacterial source.

It is theoretically impossible to provide conclusive evidence for a horizontal gene transfer from one organism to the germ line of another organism. Therefore various lines of evidence are usually necessary the build a convincing case. Several examples of putative cases of horizontal gene transfer were described from prokaryote to prokaryote, from eukaryote to prokaryote, and from prokaryote to eukaryote [9,22,28,32]. However, there is no example of acquisition of a gene by horizontal gene transfer from bacteria or fungi to an animal.

Interestingly, some of the tentative cases of horizontal gene transfer between bacteria, fungi, and plants included glycosyl hydrolases. Based on the phylogenetic analysis the β-1,4-endoglucanase CelA in *Myxococcus xanthus* may have been evolved through shuffling of domains independently acquired by lateral transfer from Gram⁻ and Gram⁺ bacteria [24]. CelY in the Gram⁻ bacterium *Erwinia chrysanthemi* shows a high level of identity with an endoglucanase from the Gram⁺ bacterium *Cellulomonas uda* [15]. By comparing molar G+C content of both coding regions and hosting chromosomes the authors speculated that a horizontal gene transfer may have occurred from *E. chrysanthemi* to *C. uda*. In the anaerobic fungus *Neocallimastrix pastriciarum* an intronless *celB* endoglucanase gene has been identified with significant homology with anaerobic rumen bacteria [39]. The fact that other fungal cellulase genes are interspersed by introns, and that the bacteria and the fungus coexist in the rumen could be an indication for horizontal gene transfer [39]. Similarly, an intronless 1,3-1,4-β-D-glucanase gene from the *Orpinomyces* sp. is homologous with bacterial glucanases, though the flanking regions are of fungal origin [5].
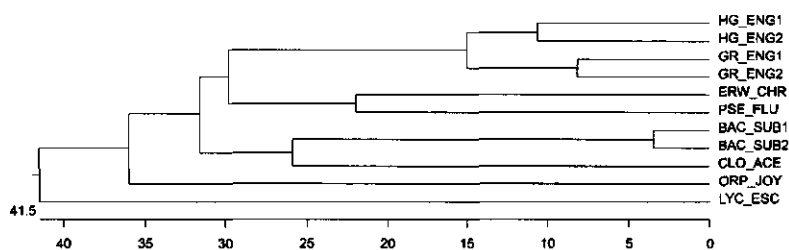
Figure 6.2 *Phylogenetic tree of endoglucanase amino acid sequences as calculated by Clustal-method in the software package DNASTAR (ERW-CH is* Erwinia chrysanthemi *endoglucanase Z [accession no. P07103], PSE_FLU is* Pseudomonas fluorescens *endoglucanase A [accession no. S56132], BAC_SUB1 and 2 are* Bacillus subtilis *endoglucanase 1 and 2 [accession nos. X04689 and M16185], CLO_ACE is a* Clostridium acetobutylicum *endoglucanase [accession no. P15704], ORP_JOY is an endoglucanase cloned from the fungus* Orpinomyces joyonii *[accession no. U59432], and LYC_ESC refers to a tomato (*Lycopersicon esculentum*) endoglucanase [accession no U12055]).*

The nematode EGases clearly show the highest similarity with bacterial EGases, which could also point to a horizontal gene transfer from bacteria to a common ancestor of the cyst nematodes species. It is not likely that several independent transfers have taken place after the divergence of the two species *G. rostochiensis* and *H. glycines*, because of the conserved intron positions in the genes. No additional clues for a horizontal gene transfer are present in the current data. The molar G+C content of the coding regions of the nematode EGases are within the range of that in bacterial EGases (*Erwinia* sp. and *Pseudomonas* sp.), but is not deviant from molar G+C content normally found in nematodes (chapter 5). In addition, the nematode EGase genes have introns, splicing features, and flanking regions that show an eukaryotic signature (chapter 5). This means that any speculation on a lateral transfer of the cellulase into the nematode genomes is based on primary amino acid sequence only. In conclusion, a lateral transfer of the cellulases from a bacterial origin can not be excluded, but if such is the case the cellulase genes have adapted extensively to their new genomic environment. Sequence data from other endogenous cellulase in unrelated animal and nematode species is necessary to determine if the phylogenetic position of the cyst nematode cellulase genes is exceptional, or if it just discloses a distinct evolutionary pathway of highly conserved cellulase genes in the old phylum Nematoda.

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Samenvatting

Deze Nederlandstalige samenvatting is bedoeld voor hen die niet ingewijd zijn in het taalgebruik en gedachtegoed van biologen en plantenziektekundigen. Vakgenoten worden verwezen naar de 'Abstracts' van de afzonderlijke hoofdstukken en de 'Summary and concluding remarks'.

Doel van dit proefschrift

Cystenaaltjes zijn kleine draadwormen (nematoden) die zich uitsluitend kunnen vermeerderen door planten te parasiteren. Dit doen ze door op vernuftige wijze de ontwikkeling van cellen in de wortels te manipuleren zodanig dat daaruit voedingscellen ontstaan. Aan deze voedingscellen onttrekken de nematoden het voedsel dat zij nodig hebben voor hun groei en ontwikkeling. Een dergelijk voedingsrelatie tussen een waardplant en een cystenaaltje duurt enkele weken. De gevolgen van een infectie met cystenaaltjes uiten zich in de bovengrondse delen van de plant als ontwikkelingsachterstand en verwelking.

Een van de kernvragen in de Nematologie heeft betrekking op de moleculaire instrumenten die door cystenaaltjes worden gebruikt om voedingscellen te induceren in planten. Centraal hierbij staat wat voor stoffen worden door cystenaaltjes uitgescheiden tijdens het parasitisme van planten? En wat het effect van deze stoffen is op de ontwikkeling van cellen in de wortels van waardplanten? In het lichaam van de cystenaaltjes zijn diverse organen aanwezig die stoffen (secreties) uitscheiden. Het onderzoek dat in dit proefschrift beschreven is, was gericht op de identificatie van de secreties uit de slokdarmklieren. Deze slokdarmklieren zijn eencellige organen die via de slokdarm verbonden zijn met een holle stylet. Via de stylet komen de secreties van de slokdarmklieren terecht in de nabije omgeving van de kop van de aaltjes.

Cystenaaltjes hebben twee soorten slokdarmklieren. Afhankelijk van de plek in de lichaamsholte van het cystenaaltje worden de klieren dorsale dan wel subventrale slokdarmklieren genoemd. Analoog aan zoogdieren worden de slokdarmklieren in cystenaaltjes ook wel speekselklieren genoemd. In het vervolg van deze samenvatting zal de triviale term speekselklier als synoniem gebruikt worden voor slokdarmklier. De specifieke doelstelling van het onderzoek in dit proefschrift was vervolgens de identificatie van eiwitten in de secreties uit de subventrale speekselklieren van cystenaaltjes. Hiervoor zijn twee soorten cystenaaltjes gebruikt, namelijk het aardappelcystenaaltje (*Globodera rostochiensis*) en het sojabooncystenaaltje (*Heterodera glycines*). Het aardappelcystenaaltje is een belangrijke plaag in de aardappelteelt in Nederland. Het sojabooncystenaaltje komt in Nederland niet voor, maar is een groot probleem in de teelt van sojabonen in de Verenigde Staten en Brazilië.

Het gereedschap

Jonge cystenaaltjes komen uit het ei nadat ze zijn geactiveerd door stoffen die uit de wortels van een waardplant lekken. De geactiveerde aaltjes maken een opening in de buitenste celwand van een wortel, en kruipen vervolgens door de wortel naar een geschikte plek om een voedingscel te induceren. In deze fase zijn cystenaaltjes nauwelijks te zien met het blote oog (lengte x breedte is ca. 0,5x0,02 mm). Het analyseren van secreties uit deze minuscule nematoden vereist een krachtig gereedschap, dat specifiek is voor eiwitten uit de secreties en met grote kracht bindt aan deze eiwitten.

Het gereedschap dat bij uitstek geschikt is voor dit werk zijn monoklonale antilichamen. Antilichamen zijn een onderdeel van het immuunsysteem van gewervelde dieren. Ze worden geproduceerd door een bepaald type witte bloedcellen. Deze cellen produceren antilichamen als reactie op de aanwezigheid van een lichaamsvreemde stof (het antigeen) die bijvoorbeeld in het dier is geïnjecteerd. Wanneer deze antilichaam producerende cellen uit het lichaam worden gehaald gaan ze snel dood. Er is echter een techniek die ons instaat stelt dit te voorkomen. Daarin worden antilichaam producerende cellen gefuseerd met tumorcellen die wel in een reageerbuis willen groeien. De gefuseerde cellen delen zich snel en vormen dan een kloon van identieke cellen die allemaal hetzelfde antilichaam produceren: monoklonale antilichamen.

In hoofdstuk 2 wordt beschreven op welke wijze preparaten van cystenaaltjes zijn gemaakt voor de immunisatie van muizen. Er werden hoofdzakelijk antilichamen opgewekt die bonden aan eiwitten uit de subventrale speekselklieren van het aardappelcystenaaltje. De truc die wij gebruikt hebben, bestond uit het scheiden van een compleet eiwit extract van miljoenen vermalen cystenaaltjes in fracties met een toenemend molecuulgewicht. Een van deze fracties heeft na injectie in muizen 11 monoklonale antilichamen opgeleverd die specifiek bonden met 4 eiwitten uit de subventrale speekselklieren. Uit nader onderzoek bleek dat deze antilichamen specifiek reageerden met de secreties die geproduceerd worden in deze speekselklieren. Dit is vastgesteld door de weg die deze eiwitten afleggen naar de stylet te onderzoeken op aanwezigheid van de eiwitten. Daarnaast bleek dat deze secretie-eiwitten terug te vinden zijn in chemisch geïnduceerde precipitaten van speeksel bij de mondopening van cystenaaltjes. Deze antilichamen waren niet alleen specifiek voor speekseleiwitten van het gele aardappelcystenaaltje (*Globodera rostochiensis*), maar reageerden ook met de subventrale speekselklieren van het soyabooncystenaaltje, het tabakcystenaaltje (*Globodera tabacum*), en het witte aardappelcystenaaltje (*Globodera pallida*).

Zijn speekseleiwitten uit de subventrale speekselklieren van belang voor het parasitisme?

Monoklonale antilichamen zijn vanwege de combinatie van specificiteit en hoge bindingskracht geschikt om relatief kleine hoeveelheden van een bepaald eiwit te zuiveren uit een complex mengsel van eiwitten. Het is niettemin erg bewerkelijk om

een dergelijk procedure uit voeren. Vandaar dat gezocht is naar additionele informatie over de speekseleiwitten die een dergelijke inspanning zou rechtvaardigen. In hoofdstuk 3 zijn de experimenten beschreven, die inzicht hebben gegeven in het belang van de subventrale speekseleiwitten voor de het succes van het parasitisme van cystenaaltjes.

Uit de experimenten bleek dat de secretie van de speekseleiwitten beïnvloed wordt door wortelexudaten van aardappelplanten. Tevens werd duidelijk dat de secretie van deze eiwitten weliswaar kan worden gestimuleerd door toevoeging van specifieke chemicaliën, maar dat het niet noodzakelijk is voor het aantonen van de eiwitten in speeksel. Kortom de waarnemingen waren niet het gevolg van de stress die de nematoden ondervonden bij de experimenten. De expressie van de speekseleiwitten was sterk gecorreleerd met de levensfasen waarin het aaltje door de wortel van een waardplant migreert. Met name de expressie in de vroege fasen van de infectie illustreerde het belang dat deze speekseleiwitten zouden kunnen hebben voor de aaltjes. Vandaar dat op grond van deze resultaten is vervolgd met de identificatie van de speekseleiwitten.

Speekseleiwitten ontmaskerd

Het vierde hoofdstuk beschrijft hoe duidelijk werd dat de speekseleiwitten uit de subventrale speekselklieren cellulases zijn. Uit het panel van 11, is een monoklonaal antilichaam (MGR48) geselecteerd om twee speekseleiwitten te zuiveren, namelijk één uit het aardappelcystenaaltje en één uit het sojabooncystenaaltje. Door nogmaals miljoenen aaltjes te vermalen en het totale eiwit extract uit deze homogenaten opnieuw te scheiden in molecuulgewicht-fracties konden uit enkele fracties met behulp van MGR48 twee subventrale speekseleiwitten gezuiverd worden.

Eiwitten zijn opgebouwd uit aminozuren. De volgorde van de aminozuren bepaald de eigenschappen van een eiwit. De aminozuurvolgorde wordt gestuurd door de genetisch informatie die ligt besloten in het DNA van een organisme. De aminozuursamenstelling van een deel van de gezuiverde speekseleiwitten leverde de gegevens op die nodig waren om de onderliggende genetisch informatie (cDNA) van de cystenaaltjes te kunnen analyseren. Uit de initiële data bleek al een overeenkomst tussen de speekseleiwitten en endoglucanases van bacteriële herkomst, die later bevestigd werd door het kloneren van vier unieke cDNAs – twee uit het aardappelcystenaaltje (GR-*eng1* en GR-*eng2*) en twee uit het sojabooncystenaaltje (HG-*eng1* en HG-*eng2*). Deze cDNAs zijn kopieën van de informatie die is vastgelegd in de genen in ieder van de twee onderzochte soorten cystenaaltjes.

De enige significante overeenkomst van de cDNAs bleef beperkt tot bacteriële β-1-4-endoglucanases (cellulases), en niet met cellulases van schimmels of planten. Cellulases breken enzymatisch celwanden in planten af door twee hoofdcomponenten, cellulose en hemicellulose, uit de celwanden te knippen. In andere diersoorten is ook cellulase activiteit aangetoond, zoals bijvoorbeeld in runderen die gras verteren, maar deze activiteit is afhankelijk van endosymbiontisch bacteriën of schimmels. Vanwege de overeenkomst met bacteriële cellulases bestaat hoofdstuk 4 voor een aanzienlijk deel uit het bewijs voor de endogene oorsprong van de cellulases in cystenaaltjes. Het

bewijs bestaat uit een vergelijking van de enzymatische activiteit van de gekloneerde cDNAs met de enzymatische activiteit in vermalen aaltjes. Daarnaast is vastgesteld of de cDNAs corresponderen met specifieke transcripten van de subventrale speekselklieren. Tot slot is gekeken naar immunologische overeenkomsten tussen de eiwitprodukten van de cDNAs met de cellulases in aaltjes.

De conclusie van hoofdstuk 4 is, dat cellulases van cystenaaltjes de eerste zijn waarvoor is vastgesteld dat het dierlijke cellulases zijn. Ondanks dat ze erg op bacteriële cellulases lijken worden de nematode cellulases autonoom geproduceerd, zonder hulp van bacteriën of schimmels, in de speekselklieren van cystenaaltjes. De productie van de cellulases is gecorreleerd met die levensfasen waarin de cystenaaltjes door de wortel kruipen. Vermoedelijk verweken cystenaaltjes met behulp van de cellulases de celwanden die ze bij hun migratie door de wortel tegenkomen. Dit betekent dat, naast brute kracht, subtiele enzymatisch activiteit van belang is bij de migratie in wortelweefsel.

Over de grens

De uitzonderlijke overeenkomst van de cellulases van cystenaaltjes met bacteriële cellulases roept vragen op ten aanzien van de evolutionaire achtergrond van deze enzymen. Een verklaring voor deze overeenkomst zou kunnen zijn dat de nematoden op een of andere wijze cellulase-genen van bacteriën hebben overgenomen. Dit zou via een horizontale gen overdracht hebben moeten plaatsvinden waarbij de grenzen tussen prokaryoten en eukaryoten zijn overschreden. Een dergelijk vorm van acquisitie van genen door dieren is totnogtoe niet eerder waargenomen en vereiste nader onderzoek.

In hoofdstuk 5 is de DNA-structuur van de cellulase genen inclusief de flankerende sequenties in cystenaaltjes onderzocht. Met dit onderzoek is getracht te bepalen in hoeverre de overeenkomsten met bacteriële cellulases ook aanwezig waren buiten de coderende stukken van de genen. In eukaryoten (bijv. nematoden) wordt de informatie die is weergegeven in cDNAs samengesteld uit de coderende stukken van een gen. De coderende stukken (exons) van een gen worden van elkaar gescheiden door niet-coderende stukken DNA (introns). In prokaryoten (bijv. bacteriën) is er slechts een enkel exon dat codeert voor het complete enzym zonder dat het gesplist wordt door introns. De cellulase genen in cystenaaltjes bevatten wel introns. De grootteverdeling van deze introns evenals de exons correspondeerde met die van de veel onderzochte nematodensoort *Caenorhabditis elegans*. Daarnaast bleken andere moleculair biologische parameters die betrekking hebben op de transcriptie en translatie van de genen ook overeen te komen met die van *C. elegans*.

De cellulase genen uit de nematoden zijn voor 70 tot 80 % identiek. Daarnaast zijn de posities van de introns in relatie tot aminozuurvolgorde van de enzymen sterk geconserveerd in de vier gekloneerde cellulase genen. Dit duidt erop dat de cellulases uit beide soorten van cystenaaltjes een gemeenschappelijk vooroudergen hebben. Een horizontale gen overdracht kan hebben plaatsgevonden, echter wel in een gemeenschappelijke voorouder van de beide soorten cystenaaltjes. Dit is dan wel zo

lang geleden, tenminste enkele tientallen miljoenen jaren, dat de genen zich volledig hebben kunnen aanpassen aan hun nieuwe genomische omgeving.

List of abbreviations

aa	amino acid
kb	kilo bases
bp	base pair
CBD	cellulose binding domain
cDNA	DNA complementary to RNA
CMC	carboxymethylcellulose
Da	Dalton
2-DGE	two-dimensional gel electrophoresis
DMT	5-methoxy-N,N-dimethyltryptamine-hydrogen-oxalate
EGase	endoglucanase
FITC	fluorescein isothiocyanate
HCA	hydrophobic cluster analysis
HEPES	N-[2-hydroxyethyl]piperazine-N'-[4-butanesulfonic acid]
IEF	isoelectric focussing
IEM	immunoelectronmicroscopy
IF	immunofluorescence
J_n	Juvenile stage n
mAb	monoclonal antibody
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PRD	potato root diffusate
PVDF	polyvinylidene fluoride
RACE	rapid amplification of cDNA ends
SDS-PAGE	sodiumdodecylsulfate-polyacrylamide gel electrophoresis
Tris	tris[hydroxymethyl]aminomethane
UTR	untranslated region

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Nawoord

Na vier jaar lang de blik strak vooruit, is nu het moment van bezinning gekomen. Een van de gevoelens die dan boven komen drijven heeft betrekking op een tegenstelling. Namelijk, dit proefschrift draagt alleen mijn naam, echter voor de totstandkoming van dit werk, was de samenwerking van diverse personen nodig. Vandaar dat ik op deze plaats een aantal mensen met naam wil bedanken voor hun waardevolle bijdrage aan dit proefschrift.

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Het was reeds in 1991 dat ik inging op het verzoek van Jaap Bakker om een promotie-onderzoek te doen bij de vakgroep Nematologie. Ik was toen ongeveer halverwege mijn studie, en dus nog groener dan gras. Sinds de start van het onderzoek in 1994 is hij een belangrijke initiator en inspirator gebleken. Ik ben verheugd over het feit dat dit een van de eerste proefschriften is die door hem als nieuwe hoogleraar in de nematologie worden gepromoot.

Mijn kompaan uit de eerste jaren was Aska Goverse. Samen zijn we met het secretiewerk begonnen, en hebben we bijvoorbeeld eindeloos veel versies van het DMT-verhaal gemaakt totdat we begrepen hoe het niet moest. Ik wil haar bedanken voor de vele discussies, de reflectie, de relativering en haar hulp tijdens de afgelopen jaren.

Hans Helder's analytisch vermogen heeft met name tijdens Jaap's afwezigheid een belangrijke rol gespeeld. Zijn kritische bijdrage is vooral van invloed geweest bij het schrijven van de publicaties en geven van richting aan het onderzoek.

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De beslissing van Arjen maakte mij kamergenoot van Jack Stokkermans alias 'De Stok', 'Stokje', en 'MacinStock'. Hij was degene die mij de fijne en de minder fijne kneepjes van de moleculaire biologie heeft bijgebracht. Gedurende twee jaar heb ik heel veel plezier beleefd aan zijn enthousiasme en wijze van werken. Ik ben er van overtuigd dat onze samenwerking een cruciale factor is geweest bij de totstandkoming van diverse publicaties, en natuurlijk dit proefschrift. Vanzelfsprekend waren er minder leuke momenten, zoals zijn nog steeds onbegrepen wens alle praktische problemen op het lab te reduceren tot het vermeende superioriteitsverschil tussen Groningers en Brabanders. Of bijvoorbeeld de onbeheersbare drang om eigenhandig

de slecht-verende tegeltjes uit onze 'hall of table tennis' te verwijderen. Kortom, 'de Stok' wordt nog altijd gemist.

Rikus Pomp heeft samen met mij de opmars van de Brabanders bedwongen. Daarnaast heeft hij getracht me in te wijden in de programmatuur van de 'Biopilot' hetgeen nodig was voor alle eiwitzuiveringen die we hebben gedaan. Voorts heeft zijn wijze van organiseren van het lab er voor gezorgd, dat lastige experimenten op een efficiënte wijze konden worden uitgevoerd. En natuurlijk niet te vergeten de strijd die we hebben gevoerd op de 'ping-pong'-tafel.

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Curriculum vitae

Geert Smant, geboren op 5 maart 1968 te Winschoten, behaalde in 1986 het HAVO diploma aan de Winschoter Scholengemeenschap. Na de middelbare school studeerde hij Nederlandse Akkerbouw aan het Van Hall-instituut (AHS) te Groningen. Hij behaalde hiervoor in 1990 het diploma, en kreeg bovendien *de Sloetprijs* uitgereikt. Tijdens deze studie heeft hij onderzoek verricht naar adaptatie van planten en bodemorganismen aan langdurige blootstelling van herbiciden. In hetzelfde jaar startte hij met de studie Plantenziektekunde aan de Landbouwuniversiteit in Wageningen en volgde daarin de Ecologische en Epidemiologische oriëntatie. Tijdens zijn doctoraalstudie deed hij onder meer onderzoek naar de isolatie van secretiegranules uit de speekselklieren van cystenaaltjes. In 1994 rondde hij *cum laude* de studie af. Aansluitend werd hij aangesteld als Assistent in Opleiding bij het Laboratorium voor Nematologie, van de Landbouwuniversiteit Wageningen. Dit proefschrift is het resultaat van vier jaar onderzoek uitgevoerd tijdens deze AIO-aanstelling. Sinds juni 1998 is hij aangesteld als post-doc medewerker bij hetzelfde laboratorium.