The identification of cell wall degrading enzymes in
Globodera rostochiensis

Herman Egbertus Popeijus

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
prof. dr. ir. L. Speelman,
in het openbaar te verdedigen
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Stellingen

1. Degradation of plant cell walls by endo-parasitic nematodes is symbiont-independent and requires the disruption of both covalent and non-covalent bonds.

   Dit proefschrift; L. Qin, 2001. Molecular genetic analysis of the pathogenicity of the potato cyst nematode Globodera rostochiensis. Thesis Wageningen University. Pp. 96

2. The discovery of various distinct classes of beta-1,4-endoglucanases within the order Tylenchida is not in concordance with the intriguing hypothesis of horizontal gene transfer of pathogenicity factors from bacteria to nematodes.

   Dit proefschrift, Davis et al. 2000. Nematode parasitism genes. Ann. Rev. Phytopathol. 38:365-396

3. The dual specificity of the resistance gene *Mi-1* indicates that aphids and root knot nematodes have developed highly similar mechanisms to parasitise plants.

   Rossi et al. (1998). The nematode resistance gene Mi of tomato confers resistance against the potato aphid. *Proc. Natl. Acad. Sci.* USA, 95, 9750-9754. Dangle and Jones, 2001. Plant pathogens and intergrated defence responses to infection. Nature 411:826-

4. The division of phenotypic responses to changing environmental conditions into passive and active plasticity is artificial and results in dogmatic views on the evolution of reaction norms.

   Pigliucci (1996). How organisms respond to environmental changes: from phentype to molecules (and vice versa). Tree 11: 168-173.

5. The inability to produce active recombinant expansin in bacterial and fungal heterologous expression systems is due to a total lack of knowledge on the biochemical mode of action of this class of proteins.

   Rochange and McQueen-Mason, 2000. Expression of a heterologous expansin in transgenic tomato plants. Planta 211:583-586.

6. Promoveren is als dansen: leiden en laten leiden.

7. Economische drijfveren en het op een menswaardige manier omgaan met onze medeschepselen op aarde, vormen antipolen van elkaar. Het (voortdurend) voorrang geven aan de economische drijfveren knaagt aan ons geweten. Een bewijs voor deze stelling is te vinden in het verbloemende taalgebruik tijdens de afgelopen landbouwcrisis: "op een verantwoorde manier ruimen van....".

Stellingen behorende bij het proefschrift getiteld: "The identification of cell wall degrading enzymes in *Globodera rostochiensis*", door Herman Popeijus

Wageningen 23 september 2002

Bibliographic Abstract:

This thesis describes the identification of cell wall degrading enzymes of the potato cyst nematode *Globodera rostochiensis*. A robust method using expressed sequence tags (ESTs) was applied to identify novel parasitism related enzymes. One of the ESTs revealed the first pectate lyase from a metazoan origin. Another tag shared a strong identity towards a previously determined N-terminal amino acid sequence. Further analysis of corresponding cDNA sequence and the gene revealed two closely related beta-1,4-endoglucanases. Heterologous expression of the pectate lyase and both beta-1,4-endoglucanases showed that they are active enzymes towards their appropriate substrates (*e.g.* polygalacturonic acid for the pectate lyase and carboxy methyl cellulose for both beta-1,4-endoglucanases respectively). The application of *in situ* hybridisation predict that these cell wall degrading enzymes are produced in the subventral oesophageal gland cells. Evidence is provided that nematodes use mixtures of cell wall degrading enzymes in order to penetrate and migrate in the plant root.

Voor mijn grootouders

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The identification of cell wall degrading enzymes in
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Chapter 1:

INTRODUCTION

NEMATODES

Nematodes are the most abundant multicellular animals on earth. They are found in practically all environments that sustain life. The name Nemata refers to their thread-like body, which is made of approximately 1,000 somatic cells in the adult life stage. The more than 20,000 nematode species that have been described so far show strikingly little variation in morphology. The alimentary tract that extends from the head to tail and the reproductive organs are the two most prominent structures in the anatomy of nematodes.

Nematodes are frequently grouped according to the way they acquire their food i.e. microbial feeders, predacious species, animal parasites and plant parasites. The world's most famous nematode, *Caenorhabditis elegans*, is a microbial feeder. *C. elegans* has proven to be an excellent model for studies on animal genetics and development. It was the first animal whose genome was completely sequenced (Consortium, 1998). *Enoploides longispiculosus* is an example of a predacious nematode species that lives in marine sediments, whereas *Onchocerca volvulus* (mammalian parasite), *Oncorhynchus nerka* (fish parasite) and *Deladenus siridicola* (insect parasite) are examples of species that infect other animals.

The plant parasitic nematodes obtain their nourishment from the cytoplasm of plant cells and as such cause substantial losses in arable crops. Measures to control nematode populations in the field and in greenhouses include crop rotation, cultivation of resistant varieties, and the application of chemical pesticides. Due to their undesirable side effects pesticides have recently become more controversial and therefore the focus in nematode control currently shifts to nematode resistant host-plants. Insight in the nematode genes that are essential in the plant-nematode interaction is a prerequisite to make productive use of both natural and bio-engineered resistance in host plants. The objective of this thesis is to identify genes in nematodes that are pivotal in the interaction between parasite and host-plant at the onset of parasitism.

PLANT PARASITISM

The plant parasitic nematodes have specialised to distinct feeding strategies. First, the ectoparasites (*e.g. Trichodorus* and *Xiphinema*) that mainly feed on epidermal cells and root hairs or on the outer cortex cells underneath the epidermal cell layer using their stylet. The migratory endoparasites form the second group of nematodes that have either specialised to herbaceous (*e.g. Aphelenchoides*) or woody plants (*e.g. Bursaphelenchus*). They are able to penetrate plant tissue, migrate intracellularly through several cell layers and use the cytoplasm of cells they come across as food source. Finally, the sedentary endoparasites (*e.g. Meloidogyne* and *Nacobbus*) establish

a so-called feeding site in the plant from which they feed for weeks to follow. The potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*), subjects of study in this thesis, also belong to the sedentary endoparasites.

THE POTATO CYST NEMATODE – PLANT INTERACTION

The potato cyst nematodes have a relatively narrow host range including *Solanceae* only. Presumably, they originate from the Southern-American Andes were they live on wild relatives of crop plants such as potato (Evans & Stone, 1977). Fully developed second stage juveniles (J2s) go into diapause to synchronise their life cycle with that of their host plants. The potato cyst nematodes can survive in the soil for many years as dormant J2 inside an eggshell contained within the protective cover of the cyst. The cyst is the remnant of the adult female body that has died following fertilisation. To this purpose, the cuticle of the female hardens to protect her offspring against all kinds of harsh conditions like cold, dehydration, and infections of bacteria or fungi.

The J2 only hatch from the egg following exposure to root diffusates from host plants (Perry, Veech, & Dickson, 1987; Perry, Zunke, & Wyss, 1989). Components in root diffusates are known to initiate transcription and translation as well as various other physiologically changes in the dorsal and subventral oesophageal gland cells (Fig. 1) (Atkinson, Taylor, & Fowler, 1987; Blair, Perry, Oparka, & Jones, 1999; Perry et al., 1989). The hatched juveniles migrate to the root system and penetrate the root preferentially near the growing tip. To penetrate the epidermal cell layer of the root the juveniles use their hollow retractable stylet (Fig. 1) to puncture the cell walls (Steinbach, 1972). Enzymes that weaken the structural components of the cell walls probably enhance the effect of the vigorous stylet thrusts (De Boer et al., 1999; Smant et al., 1998; Wang et al., 1999). While leaving behind a track of disrupted cells (Rice, Leadbeater, & Stone, 1985) the J2 migrate intracellularly in the root in search for a proper cell to transform into a feeding site. Anti-oxidation enzymes like peroxiredoxins are found at the nematode surface and may protect the nematode against reactive oxygen released by necrotic cells during migration (Robertson et al., 2000).

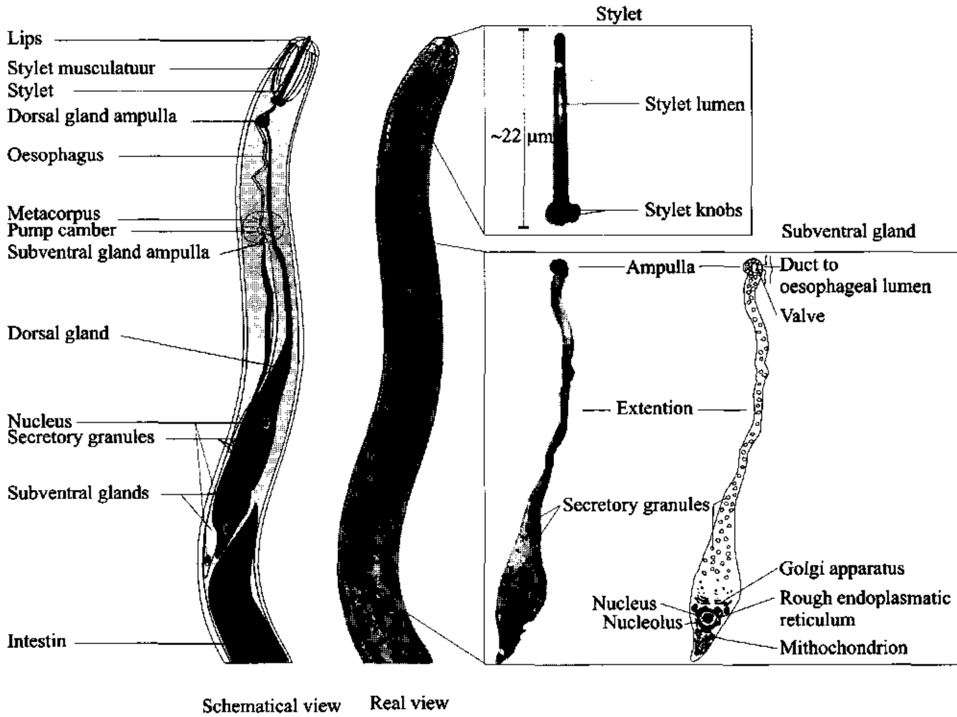

Figure 1. *A view of the anterior part of G. rostochiensis. The real view picture is made using Leica optics in combination with a CCD camera*

The nematode settles in the root when a plant cell is found that is eligible to be transformed into an initial feeding cell. The exact mechanism by which nematodes redirect the developmental program of root cells in order to change them into transfer cells-like structures is not known. Circumstantial evidence points to a role for the proteins secreted from oesophageal glands (Goverse et al., 2000; Williamson & Hussey, 1996). Nevertheless, the effects of the putative pathogenicity factors in nematode secretions are profound. The recipient plant cell responds with a renewed cell cycle activation heading for mitosis, however, a short-circuit that is presumed to act just prior nuclear division redirects it into repeated cycles of endoreduplication. The endoreduplication of the genome in the initial feeding cell causes the plant cell to adopt its typical morphology i.e. increase in cell size, highly dense cytoplasm filled with subcellular organelles, small vacuoles, large nucleus (Cole & Howard, 1958; Gheysen, Almeida-Engler, & Montagu, 1997; Jones & Northcote, 1972; Rice et al., 1985). In the

case of cyst nematodes feeding cell growth is accompanied by local cell wall degradation at the most distal position from the nematode's head. The local cell wall degradation gives rise to fusion of the cytoplasm ultimately leading to a large multicellular feeding cell complex, the so-called syncytium. The syncytium continuously expands as long as the nematode feeds from its cytoplasm.

When the parasitic J2 starts feeding the longitudinal muscles that are required for intracellular migration in the plant gradually degrade. For its development the immobile juvenile thus becomes totally dependent on the food supply available in the syncytium. The food uptake proceeds through a repeating pattern with three distinct phases *i.e.* nutrient ingestion, retraction and reinsertion of the stylet, and salivation (Steinbach, 1973; Wyss, 1992; Wyss & Zunke, 1986). The third phase presumably involves the release of secretory fluids from the ampulla of the dorsal oesophageal gland. Each salivation phase is accompanied by the formation of a feeding tube, which has been shown to act as molecular sieve during nutrient ingestion (Bockenhoff & Grundler, 1994).

During the course of weeks the juveniles develop via three moults into adult males and females. The males have a lesser nutritional demand and stop feeding earlier in their development than female nematodes. Adult males regain their mobility and migrate out of the root in order to fertilise the females. The females continue to feed from the syncytium until fertilisation is completed. Then the female dies leaving behind 100 to 800 juveniles well protected within her hardened cuticle (Arntzen, Muller, & Visser, 1994).

PLANT CELL WALLS

The plant cell wall is a dynamic compartment that largely dictates the shape of a plant. It determines growth of plant cells and thereby influences the differentiation of tissues into specialised structures and organs. The rigidity and strength of cell walls also provide the plant cell with strong armour against pathogens and parasites (*e.g.* migratory nematodes). Furthermore, cell walls are involved in positional signalling, cell-to-cell communication, and cell wall fragments may elicit the release of defence molecules (Cote & Hahn, 1994; Ridley, O' Neill, & Mohnen, 2001).

Three layers – the middle lamella, the primary cell wall and the secondary cell wall – determine the cell wall's make-up in differentiated cells. The highly organised composite nature of the plant wall layers is of many different polysaccharides, proteins and aromatic polymers. The core structure that provides most of the strength is the cellulose-hemicellulose network in the primary cell wall, which accounts for about 50% of the total cell wall mass. This cellulose-hemicellulose network lies embedded in a

second independent but interacting network of the pectic matrix (Carpita, McCann, & Griffing, 1996; Carpita & Gibeaut, 1993).

The primary cell walls of neighbouring cells are separated by the middle lamella, which is mainly a continuation of the pectic matrix. Once growth of a cell ceases the primary cell wall is locked into its final shape presumably by structural proteins such as hydroxy-proline-rich-proteins (*e.g.* extensins) and proline-rich-proteins. The cross-linking by proteins coincides with the elaboration of the secondary cell wall within the primary cell wall. A distinguishing feature of the secondary cell wall is the incorporation of aromatic *p*-phenylpropanoids such as lignins. These lignins are bonded to cellulose and hemicellulose in the secondary cell wall thereby rendering it more refractory to chemical and enzymatic degradation. The structural proteins and the lignins are often regarded as independent structural networks.

Cellulose, the principal scaffolding polymer in plant cell walls, is a homopolymer of D-glucose linked by β-1-4-glycosidic bonds. These linear glucan polymers may incorporate up to 10,000 glucosyl residues. The individual β-1-4 glucan chains adhere to each other via numerous hydrogen bounds and Van der Waals-forces and assemble into strong insoluble microfibrils. Depending on the pattern of hydrogen bounds two types of cellulose can be distinguished. Type I is the highly crystalline form whereas the second type comprises a more amorphous structure.

The cellulose microfibril scaffold is interlocked by hemicelluloses via hydrogen bonds. Hemicellulose is the non-cellulolytic polysaccharide fraction extracted from plant cell walls by alkali and after removal of pectic polysaccharides. Hemicelluloses comprise of cross-linking polysaccharides with a (1→4)-β linked backbone (*e.g.* D-glucan, D-xylan or D-mannan), which are decorated with complex carbohydrates. The two major cross-linking components of hemicellulose in flowering plants are xyloglucans and glucuronoarabinoxylans.

The two principal constituents of the pectin matrix are homogalacturonan and rhamnogalacturonan I (RG I) (reviewed by (Ridley et al., 2001)). Smooth regions in pectins consist of backbone of homogalacturonan, which is a polymer of α-1-4 linked D-galacturonic-acid residues. The smooth regions are interspersed by ramified RG I regions composed of alternating rhamnose and galacturonic acid residues with several neutral polymers arabinans, arabinogalactans, and unbranched galacatans attached to it (Catoire, Goldberg, Pierron, Morvan, & duPenhoat, 1998). There are two types of structurally modified homogalacturonans, xylogalacturonan and rhamnogalacturonan II. In xylogalacturonan the galacturonic acid residues are substituted with xylose. The RG II is a complex homogalacturonan characterised by unusual kinds of sugar linkages and the presence of rare sugars such as fucosyl, apioseaceric acid (Vidal et al., 2000).

In addition to structural diversity created by side chains the galacturonic acid residues can be esterified by methanol or, albeit at a lesser frequency, by acetic acid. The degree of esterification is defined as the number of moles of methanol and acetic

acid per 100 moles of galacturonic acid. Pectins are regarded to be highly methylated or high methoxyl pectins when the value for the degree of methylation is 50 or higher. In all other cases, the pectin is called low methoxyl pectin or pectate. The degree of esterification of pectin varies with the plant species from which it is isolated. It determines to a considerable extent the degradability of the pectins by enzymes.

CELL WALL DEGRADATION

The complex chemical composition and physical organisation of the cell wall protects the plant protoplasm against many invasive pathogens and parasites. The tensile strength of the cell wall as well as its resistance against chemical and enzymatic degradation have directed the evolution of complex mixtures of cell wall degrading enzymes in invaders. These mixtures frequently comprise synergistically acting enzymes active against the cellulolytic and pectic networks in the plant cell walls (Annis & Goodwin, 1997).

In spite of its simple chemical composition the elaborate secondary and tertiary structure of native cellulose make it a complex and insoluble heterogeneous substrate. The efficient solubilisation and hydrolysis requires the presence of many different enzymes that all share the chemical specificity for the β-1-4-glycosidic bonds. The different modes of action of the cellulolytic enzymes on their polymeric substrate are commonly described as an endo- and exo acting type of attack. Typically, the endo-acting cellulases (also called endoglucanases, endo-1,4-β-D-glucanases or carboxymethylcellulases; EC 3.2.1.4) efficiently decrease the degree of polymerisation by cleaving the bonds along the length of the cellulose chains. The exoglucanases (also called cellobiohydrolases; EC 3.2.1.91) are thought to be the processive enzymes that exhibit their actions at the non-reducing ends of the cellulose chains produced by the cellulases. The cellobiohydrolases primarily produce cellobiose, a dimer of β-1-4-linked D-glucose residues. These cellobiose units are the subsequent substrates for the β-1-4-glucosidase (also called gentobiase, cellobiase, amygdalase; EC 3.2.1.21) that yields D-glucose as its final product (Beguin, 1990; Beguin & Aubert, 1994).

The classification of cellulolytic enzymes into three groups is probably a simplification (Beguin, 1990). Ample evidence points to more diversity in the catalytic action of cellulose degrading enzymes, which is not only related to endo- or exo activity. For instance, the substrate specificity may also reflect the degree of polymerisation and crystallinity of the cellulose.

Pectic enzymes (pectinases) are classified according to the substrate specificity and the mode of action of their enzymatic activity, too. The first broad division is made between those that de-esterify pectins (pectic esterases) and those that degrade the galacturonan backbone (depolymerases). The pectin esterases de-esterify methylated

(EC 3.1.1.11) and acetylated pectins (EC 3.1.1.-) producing either methanol or acetic acid and pectate. Clearly, the pectin esterases change the degree and the pattern of acetylation and methylation of pectins thereby influencing its accessibility for degradation by depolymerases. The pectic depolymerases are grouped according to their specificity to the degree of methyl and acetyl esterification of the galacturonan backbone and their mode of action (endo- or exo). Endo-depolymeryzing enzymes that act on the non-esterified galacturonan backbone, pectate, and pectin respectively are named polygalacturonase (endoPG; EC 3.2.1.15), pectate lyase (PeL; EC 4.2.2.2) and pectin lyase (PnL; EC 4.2.2.10). Similarly, exo-enzymes that act on the backbone at the non-reducing ends of the polygalacturonic acid and pectate polymers are named exopolygalacturonase (exoPG; EC 3.2.1.67) and exopolygalacturonate lyase (exoPeL; EC 4.2.2.9) respectively.

CELL WALL DEGRADING ENZYMES IN PLANT-PARASITIC NEMATODES

The infective potato cyst nematodes have three large single-celled oesophageal glands - two subventral glands and one dorsal gland (Fig. 1) (Endo, 1984). In active gland cells the large nucleus resides within an elaborate rough endoplasmic reticulum that has at its perimeter numerous Golgi-bodies. Large numbers of secretory granules constantly bud from the trans-Golgi networks in Golgi-bodies at the perinuclear region in the cell. Microscopic observations have shown that these granules exhibit extensive movement predominantly to a so-called ampulla at the apical region of the gland (Wyss & Zunke, 1986). Proteins destined to be secreted from the oesophageal glands are presumed to be released from the secretory granules into the end-sac in the ampulla by exocytosis.

A distinguishing feature for the two types of oesophageal glands is the site in the nematode where they connect to the oesophagus. The two subventral gland cells release their secretions into the oesophagus just posterior to the pump chamber in the metacorpus. The dorsal gland cell is connected to the oesophagus close to the stylet base (Hussey, 1989b). It has been shown that secretions from both gland types are emanating from the stylet orifice following transport through the oesophagus (Goverse, Davis, & Hussey, 1994; Hussey, 1987, 1989a; Smant et al., 1997).

The temporal activity pattern of the two types of glands also show striking differences in potato cyst nematodes (Wyss, 1992). The subventral gland cells are only active in preparasitic and parasitic J2s. The dorsal gland cell is active in these stages too, however, its activity continues into more advanced developmental stages. As opposed to the subventral glands cells, the active release of secretions of the dorsal gland is indirectly observed during feeding from the syncytium (Williamson, Ho, & Ma, 1992; Wyss & Zunke, 1986). The temporal activity pattern of the subventral glands

points to a role in plant invasion and intracellular migration at the initial stages of parasitism. The observation that these subventral glands regain their activity in migratory male nematodes supported this hypothesis (De Boer et al., 1999).

In the last five years ample evidence has been found for the endogenous production of cellulases in the subventral oesophageal gland cells of cyst nematodes and root-knot nematodes (Goellner, Smant, De Boer, Baum, & Davis, 2000; Rosso et al., 1999; Smant et al., 1998; chapter V of this thesis). Furthermore, Wang and co-workers have shown that cellulases produced in the subventral glands are released by the nematode while migrating through the host plant root (Wang et al., 1999). These leads support the hypothesis that one of the primary roles of the subventral oesophageal glands is related to cell wall degradation at the onset of parasitism.

Pectinase activities have been detected in homogenates of several plant-parasitic nematodes too, *e.g. Meloidogyne sp., Pratylenchus sp., Rhadopholus sp., Aphelenchoides sp., Globodera sp., Heterodera sp.,* and *Ditylenchus sp.* (Chitwood & Krusberg, 1977; Giebel, 1974; Krusberg, 1967; Myers, 1965). In plant-pathogenic bacteria and fungi, cell degrading enzymes occur in mixtures with partially overlapping specificities towards the cellulose/hemicellulose and the pectic networks. Taking the complexity of structural components of the plant cell wall into account it was reasonable to expect that plant-parasitic nematodes produce mixtures of cell wall degrading enzymes, too.

OUTLINE OF THE THESIS

The cloning of the first cellulase genes from the plant-parasitic nematodes was ensued following an extensive and slow experimental procedure including production of secretions specific monoclonal antibodies, protein purification, protein sequencing, and PCR-amplifications using degenerate oligonucleotide primers (De Boer et al., 1996; Smant et al., 1997; Smant et al., 1998). The recent fall of prices for DNA sequencing issued an alternative approach using expressed sequence tags, which is in the case of nematodes in principle far more efficient than proteomics.

The second chapter of this thesis describes a small-scale cDNA-sequencing project with the potato cyst nematodes *G. pallida* and *G. rostochiensis*. Among the 1,000 ESTs produced in this project several candidate parasitism genes were tagged *e.g.* putative cellulase, cellulose binding protein and pectinase (Popeijus, Blok et al., 2000). Chapter 3 includes the report of the first metazoan pectate lyase gene (Popeijus, Overmars et al., 2000). This finding provides the evidence that nematodes produce cell wall degrading in mixtures in order to facilitate their migration through plant tissues. The structural, functional and phylogenetic characterisation of this gene is described in chapter 4. The cloning and characterisation of a novel and most abundant cellulase in

preparasitic juveniles is presented in chapter 5. Chapter 5 illustrates that secretory proteins refractory to amino acid sequencing may be efficiently identified using the EST approach. In chapter 6 the application of EST analysis in relation to the identification of novel parasitism related genes is discussed.

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ABSTRACT

Expressed sequence tag (EST) projects offer a rapid route to the discovery of novel genes. Genes expressed in a wide range of parasitic nematodes of medical or veterinary importance have been investigated using EST analysis but these techniques have not yet been applied to plant parasitic nematodes. We describe a small scale EST project using cDNA libraries made from the two species of potato cyst nematode, *Globodera rostochiensis* and *G. pallida,* and assess the utility of this approach to identify mRNAs encoding abundantly expressed secreted proteins and other proteins present in the nematode at the onset of parasitism. Approximately 1000 sequences were obtained from *G. rostochiensis* and 100 from *G. pallida.* A variety of genes was characterised and approximately 11% of the cDNAs sequenced were apparently PCN specific. Secreted proteins identified included a novel PCN homologue of chorismate mutase, a cDNA recently cloned from the gland cells of *Meloidogyne javanica.* The results obtained justify a much larger scale application of this technology to these parasites.

INTRODUCTION

Practical problems in obtaining large quantities of nematode material have meant that molecular biological studies on plant parasitic nematodes have progressed relatively slowly compared to similar work on animal parasitic nematodes. Whereas relatively few protein-encoding genes have been isolated or characterised from plant parasitic nematodes, more than 25,000 cDNA sequences from nematode parasites of animals are present in the databases (dbEST). These sequences have been generated largely as expressed sequence tags (ESTs), single pass sequences of cDNA clones selected randomly from libraries made from life cycle stages of interest (*e.g.* (Blaxter et al., 1996); see the Blaxter Lab EST pages - http://www.ed.ac.uk /~mbx/small_genomes.html for details of current parasite EST projects). This EST approach offers a rapid and cost-effective route to the discovery of novel genes (Blaxter, 1997) and provides a background of information about the sequences of genes expressed in an organism. A similar database of expressed genes from plant parasitic nematodes may provide a variety of new genes for investigation as potential targets for novel control methods.

Although EST projects are apparently random in nature, it is possible to target this type of project at genes involved in specific processes. For example, genes expressed at the onset of parasitism and which may be important in the host parasite interaction can be targeted with an EST project by using a cDNA library constructed

from the hatched infectious second stage juvenile (J2). Changes occur in J2s during hatching indicative of the transition to a parasitic mode of existence. These changes include activation of transcription in the gland cells (Atkinson et al., 1987; Perry et al., 1989; Blair et al., 1999) as well as behavioural changes (Perry, 1989) and changes in gene expression (Jones, Robertson, Perry, & Robertson, 1997; Qin et al., 2000). It is also feasible that an insight into the molecules secreted by potato cyst nematodes (PCN) may also be gained using ESTs. The gland cells of PCN are large and packed full of secretory granules and rough endoplasmic reticulum (*e.g.*, (Hussey & Mims, 1990)). Furthermore, proteinaceous secretions are produced in great quantities from the parasite surface (Robertson et al., 2000). mRNAs encoding secreted molecules are therefore likely to be present in abundance in a representative cDNA library. When this approach was used with a library made from the invasive stage of the animal parasite *Toxocara canis,* over 15% of the sequences obtained encoded homologues of known secreted proteins (Tetteh, 1997). Secreted molecules are likely to have important roles in several aspects of the host-parasite interaction (reviewed by (Jones & Robertson, 1997)). They may be responsible for the changes induced by these parasites in the roots of their hosts (Hussey, 1989a) and they assist in invasion and migration through the root (Smant et al., 1998). Secretions may also mask the parasite from the host (Forrest, Robertson, & Milne, 1989) or down-regulate host defence responses (Molinari & Miacola, 1997) and, as the parasite molecules in most intimate contact with the host, are likely to be the 'avirulence factors' detected when a resistant response is initiated.

In order to determine the utility of the approach and in an attempt to identify abundantly expressed mRNAs encoding secreted molecules we have undertaken a small-scale EST project from both species of PCN, *Globodera rostochiensis* and *G. pallida.* The results obtained are sufficiently encouraging to justify a much larger scale application of this technology to these and other plant parasites.

MATERIALS AND METHODS

cDNA libraries

cDNA libraries from *G. rostochiensis* (Smant et al., 1998) and *G. pallida* (Jones, Curtis, Wightman, & Burrows, 1996) were used in this project. Both libraries included J2 as their primary source of biological material and contained more than 10^6 primary recombinants with an average insert size of more than 1kb. These libraries were therefore considered representative of the genes expressed in the J2 of the parasites.

Template preparation

The *G. rostochiensis* library was prepared in a plasmid vector (pcDNA 2.1 – Invitrogen, Groningen, NL). Preliminary experiments showed that over 95% of the

plasmids contained inserts, eliminating the need for pre-screening of colonies. To prepare template DNA for sequencing, approximately 100 colony forming units were spread on 15 cm LB-Ampicillin (LB-Amp) plates and incubated overnight at 37°C to allow growth of the bacteria. Individual, well-separated colonies were transferred to 3 ml of LB-Amp broth, which was then incubated at 37°C overnight in a shaker incubator. Plasmid DNA was prepared from these cultures using a Wizard plasmid preparation kit (Promega, Wisconsin, USA). Approximately 250 ng of this DNA was used as template in sequencing reactions. For long-term storage, aliquots of purified plasmid DNA were stored at -20°C.

The *G. pallida* library was made in a phage vector (lambda gt11). This made it necessary to use a different method for preparing template DNA for sequencing. Approximately 100 plaque-forming units were grown on 15 cm LB-Amp plates overlaid with top agarose using standard protocols (Sambrook, 1989). Individual, well-separated plaques were removed using a sterile Pasteur pipette and left in 100 µl of SM (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin) overnight at 4°C. Ten µl of the phage plug eluate was then used as template in a PCR reaction with the lambda gt11 forward and reverse primers which bind to sites immediately flanking the cloning site in this vector. Fifty µl reactions were carried out in 1X reaction buffer (5 mM KCl, 10 mM Tris-HCL pH 9.0, 1% Triton-X 100) containing 1.5 mM $MgCl_2$, 200 µM each of dATP, dGTP, dCTP and dTTP, 1 µM each primer and 1 unit of *Taq* DNA polymerase (Promega, Wisconsin, USA). An aliquot of the reaction products was run on a 1% agarose gel (Sambrook, 1989) and reactions yielding a single band of greater then 500 bp (by comparison with DNA standards) were chosen for further analysis. The products of these reactions were cleaned using a Wizard PCR preps kit (Promega, Wisconsin, USA). DNA concentration was estimated by comparison with DNA standards on ethidium bromide stained agarose gels. Approximately 100 ng of purified DNA was used as template in DNA sequencing reactions. For long-term storage, aliquots of the amplified DNA and of the phage plug eluate were kept at -80°C as previously described (Sambrook, 1989).

DNA sequencing

Sequences were obtained from the *G. rostochiensis* library using the SP6 primer, while the lambda gt11 forward primer was used when sequencing products from the *G. pallida* library. A Big Dye Terminator cycle sequencing kit (Perkin Elmer Applied Biosystems, Warrington, UK) was used, following the manufacturers instructions, to generate sequences. Sequencing reactions (1/4 size) were carried out on a Gene Amp System 2400 PCR machine and reaction products were purified by ethanol precipitation before being run on an ABI 373 Stretch DNA sequencing system.

Sequence analysis

DNA sequences were edited to remove vector sequences using Sequence Navigator software (Applied Biosystems, Warrington, UK). Sequence comparisons were carried out using the BLASTX program at The National Centre for Biotechnology Information (NCBI), with sequences submitted in bulk using the Baylor College of Medicine (BCM) Search-Launcher perl script (http://www.hgsc.bcm.tcm.edu/SearchLauncher/), a program written to allow bulk submission of search requests. Some sequences were analysed in more detail using programs available through the ExPASy web pages (http://expasy.hcuge.ch/tools/). Signal peptide predictions were made using the SignalP server (http://www.cbs.dtu.dk/services/SignalP - (Nielsen, Engelbrecht, Brunak, & von, 1997)). Sequence alignments were generated using MultAlin software (http://www.toulouse.inra.fr/multalin.html - (Corpet, 1988)).

Primers

The primers used in this study were:

Lambda gt11 forward	GTGGCGACGACTCCTGGA
Lambda gt11 reverse	TTGACACCAGACCAACTG
SP6	ATTTAGGTGACACTATAG

Results

In initial experiments, 100 sequences were obtained from each cDNA library. Work then focused on the *G. rostochiensis* library and approximately 1000 sequences have been obtained from this library to date. A catalogue of the sequences generated during this project can be viewed at (http://www.scri.sari.ac.uk/nematode/). This page provides a list of keywords for each sequence as well as links to the sequences themselves. Links to the search results generated by BLASTX searches against non-redundant protein databases for each sequence are also provided. The ESTs have also been deposited in dbEST.

Table 1. *Classification of ESTs sequenced.*

Category	Definition	%
Housekeeping genes	ESTs with matches from nematodes and other organisms	59
Nematode specific genes	ESTs only matching sequences from other nematodes	22
PCN specific genes	ESTs with no matches or only matching sequences from non-nematode organisms	11
Other	Vector, poor quality sequence, bacterial genomic DNA etc	8

The sequences obtained were first placed into one of four categories. The distribution of the genes amongst the various categories is summarised in Table 1. 'Housekeeping genes' were defined as genes that had significant matches to genes in the

database from nematodes and other organisms. Although this was not a precise definition, proteins encoded by such genes were likely to be involved in general cell or whole organism metabolism and were not usually likely to have a role in specific nematode physiology or in parasitic processes. These genes were further subdivided as shown in Table 2. Although any classification of this type is to some extent arbitrary, the analysis demonstrates the relative proportions of the various protein types present in freshly hatched *G. rostochiensis* J2. 'Nematode specific genes' only produced matches to genes in the databases from other nematodes (most often predicted proteins from the *C. elegans* sequencing project or ESTs from filarial nematode parasites). These genes were considered to produce proteins likely to be important in nematode physiology and which may be adapted for a role in parasitic processes. 'PCN specific genes' had no significant matches in any of the databases or only produced matches against non-nematode sequences (see below) and were considered of particular interest as having a potential role in PCN physiology or parasitism. 'Other genes' were those, which did not fall into any of the above categories. These genes included sequences resembling parts of transposable elements, fragments of cloning vector and very short sequence reads with which it was not possible to perform meaningful database searches.

The proportion of sequences falling into each of the above categories was similar from both libraries. Since the dataset for *G. rostochiensis* is currently so much larger than that for *G pallida* only these sequences are considered in detail below.

Table 2. *Subdivision of ESTs classified as encoding housekeeping proteins. Categories were chosen initially following those of (Blaxter et al., 1996), with additional categories added where appropriate.*

Category	Definition or examples	Number
Ribosomal proteins	Self explanatory	81
Structural and muscle	Muscle proteins, cytoskeletal components, cell wall components	104
Enzymes and metabolic	Proteins involved in standard cell or body metabolic processes	170
Transport	Lipid transport proteins, membrane transporters	33
Gene expression	Proteins involved in gene expression or RNA processing transcription factors, splicing proteins, tRNA synthesis proteins	80
Neuron function	Proteins involved Specifically in neuronal functions, neuropeptiáe precursors, ion channels	27
Unknown	Proteins from a wide range of organisms for which no function has been described	39
Others	Proteins not fitting into any of the above categories, *e.g.*, cell surface receptors, galectins, heat shock proteins	42

Discussion

59% of the *G. rostochiensis* sequences were classified as housekeeping genes and since 8% sequences fell into the 'other' category the remaining 33% of the sequences can be classified as being specific to nematodes. Analysis of the entire

complement of genes encoded in the *C. elegans* genome suggests that 58% of these genes are specific to nematodes (Blaxter, 1998). The difference between this figure and that reported here is almost certainly due to the fact that the *C. elegans* analysis utilises the genomic sequence and therefore reports matches for each gene in the genome no matter how restricted its expression pattern. Genes expressed at extremely low levels are also included in this analysis. By contrast, analysis of the *G. rostochiensis* genes is currently restricted to those genes present in the J2 and, given the relatively small numbers of cDNAs sampled, to those genes expressed abundantly enough to be detected amongst the first 1000 genes sampled.

Housekeeping genes sequenced covered many of the proteins expected in a functional eukaryotic organism. Although the vast majority of genes in this group are present to allow normal cell or body functions to be performed, genes of potentially greater interest were also placed in this category. Several different proteases which are considered important targets for control of plant parasitic nematodes (e.g. (Atkinson, Urwin, Hansen, & McPherson, 1995; Urwin, Lilley, McPherson, & Atkinson, 1997)) were detected including serine protease (ge1391), cysteine protease (ge1346), leucine aminopeptidase (ge1232) and a matrix metalloprotease (collagenase - ge1498). Similarly, a variety of antioxidant proteins including peroxiredoxin (ge1468) and internal and secreted forms of glutathione peroxidase (ge1229 and 1134) were detected. Proteins encoded by these genes are important for parasitism of animals by nematodes (e.g. (Clark, Hunt, & Cowden, 1986)) and may also provide protection for plant parasitic nematodes against active oxygen species generated as part of the host defence response (e.g. (Molinari & Miacola, 1997; Waetzig, Sobczak, & Grundler, 1999)).

The majority of genes placed into the 'nematode specific' category, like the vast majority of the predicted genes from the *C. elegans* genome, have no known function. Exceptions to this are the lipid binding proteins, which have been extremely well characterised from nematodes. Representatives from several different classes of lipid binding protein were sequenced as ESTs from *G. rostochiensis*, including PCN homologs of Ov20 (Tree et al., 1995; Kennedy et al., 1997; Prior, 1998 - ge1466), the nematode polyprotein allergen ((Kennedy, Britton, Price, Kelly, & Cooper, 1995) - ge1408) and *Ascaris suum* FABP-1 ((Mei, Kennedy, Beauchamp, Komuniecki, & Komuniecki, 1997) - ge1258). It has been suggested that some of the lipid binding proteins of animal parasitic nematodes are adapted for a role in the parasitic process (e.g. (Kennedy et al., 1997)). The cDNA encoding GPSEC-2, the PCN homolog of Ov20, is particularly abundant in both the *G. rostochiensis* and *G. pallida* datasets suggesting an important role for this protein in PCN biology; this protein is currently under investigation in one of our laboratories (Prior et al., 1998).

Perhaps the most interesting of the genes were those classified as PCN specific genes. Genes which produced no matches in any databases, including the *C. elegans* genome sequence and the extensive animal parasite EST databases, were included in

this category as well as other genes which gave matches only against non-nematode sequences. Some of the genes in this category, such those encoding cell wall degrading enzymes (Smant et al., 1998), had an obvious role in the host-parasite interaction. Others did not. For example, one sequence (ge236) gave a good match against an EST from tomato (*Lycopersicon esculentum*) ovary but, despite extensive searching of many databases using a variety of search types with both the nematode and tomato sequences, no clue as to the function of either of these genes could be found. Other ESTs gave no matches in any databases. Since these ESTs often had sequence characteristics which suggested they represented real genes (*e.g.*, a poly-A tail) and since they never produced matches against bacterial DNA fragments, it is likely that these fragments represent genuine *G. rostochiensis* genes and that they may have important functions in parasitism of plants by these nematodes. As with all such 'pioneer' genes, investigating the function of the proteins encoded by these genes is extremely difficult. A good starting point may be to develop systems for analysing spatial expression patterns of large numbers of novel genes using techniques such as *in situ* hybridisation. Those genes expressed in tissues likely to be responsible for generating secretions important in the host-parasite interaction can then be selected for further analysis. A system for large-scale *in situ* hybridisation which is amenable to some degree of automation has been developed for *C. elegans* ESTs (Tabara, Motohashi, & Kohara, 1996); similar procedures are currently being developed for PCN (G. Smant, *pers. comm.*).

One of the aims of this work was to isolate genes encoding secreted proteins of PCN, based on the hypothesis that secreted proteins were likely to be abundantly expressed. Proteins known to be secreted by plant parasitic nematodes such as GPSEC-2, cell wall degrading enzymes, proteases and antioxidant proteins, were relatively abundant in the dataset suggesting that the hypothesis was correct. One of the ESTs (pal458) from *G. pallida* matched the chorismate mutase cDNA, recently cloned from *M. javanica* (Fig. 1) (Lambert, Allen, & Sussex, 1999). The protein encoded by this gene is expressed in the gland cells of *M. javanica* and is thought to be secreted into host tissues. Although the function of this protein in the host-parasite interaction is unclear, the fact that both cyst- and root-knot nematodes contain this gene, which has not been described from any other animals, suggests it plays an important role in the parasitic process.

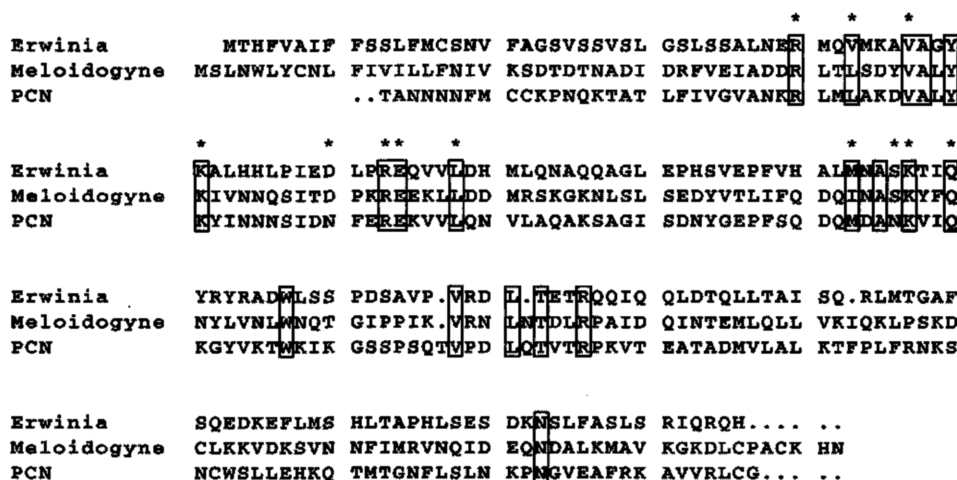

Figure 1. Alignment of predicted amino acid sequences of G. pallida pal458 EST (frame +1 translation) with monofunctional chorismate mutase AroQ from Erwinia herbicola ((Xia, Song, Zhao, Aldrich, & Jensen, 1993) - top non-nematode match with pal458 in database) and NC30 chorismate mutase from Meloidogyne javanica. Absolutely conserved residues are boxed; these are frequently active site residues (as identified from E. coli CM mutase domain – (Lee, 1995)) which are marked with asterisks.

Identifying entirely novel secreted proteins with an important role in the host-parasite interaction proved more difficult. Signal sequence prediction programs (Nielsen et al., 1997) can be used to identify potentially secreted proteins but relying solely on signal peptide predictions can lead to several problems. First, even when sequencing directional libraries, not all EST sequences will be complete at the 5' end, making it impossible to test all ESTs for the presence of a predicted signal sequence. Second, many proteins with a predicted signal sequence will not have a function outside the nematode but will be extracellular proteins which are kept within the nematode body. In the absence of any functional data, screening out these cDNAs is an extremely difficult task. Finally, evidence is emerging that some proteins without an apparently cleavable signal sequence can find their way to the parasite surface (Lu, Egerton, Bianco, & Williams, 1998; Robertson et al., 2000). Clearly, such proteins will not be detected when screening sequences using a signal peptide predictor. Despite these problems, there will be cases where interesting cDNAs can be identified on the basis of their

sequence alone with cell wall degrading enzymes, antioxidant proteins and the chorismate mutase providing examples of such cDNAs identified in the present study. Perhaps the greatest utility of the EST dataset will be its use in combination with other approaches. The collection of PCN sequences and corresponding plasmid clones will be a resource for projects generating fragments of genes. This offers the powerful possibility of going from a short DNA or amino acid sequence directly to a full-length clone without the need for further cloning experiments. The EST dataset also allows functional characterisation of potential target genes to begin immediately.

Projects aimed at isolating genes of plant parasitic nematodes involved in pathogenic processes have previously relied on methods which take many years to complete. One approach favoured by many laboratories world-wide has been to generate panels of monoclonal antibodies and to use these for cDNA library screening or protein purification (*e.g.* (Atkinson et al., 1988)). Despite many years endeavour by many laboratories very few genes involved in pathogenesis have been isolated using this approach. EST projects offer an alternative, complementary approach, which can be used alongside more targeted experiments. This small-scale project is convincing evidence that a much larger scale application of this technology to PCN and to other plant parasitic nematodes, particularly *Meloidogyne* species, is a worthwhile investment of time and resources.

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                                                          I
U13050   68   SKDAVFILEEGATLRNVIIGANQR-EGIHCK-GSCNIEFAWFEDVCEDAISIL
U13051   65   EKDASFILEDGATLSNVIIDRSSG-EGVHCK-GTCTLNNVWWADVCEDAATFK
Y13340   148  DKQKPLIILKNATVKNLRISASGRADGIHCDSGNCTIENVIWEDICEDAATNN
132172   151  DKQPPVITLENATIKNLRISEKGGSDGIHCKSGNCRIENVIWEDICEDAATNL
Pel-1    64   KMKYLMVLKHGVTIKNAIINTPGL--GIYCE-GSCVLENIYYKKLCYHATGFG
              . .  .:  ...*: *  *.      *::*. *.* ::   : .:* .*

                         II                        III
G------SGTANIIGGGAYHASDKVIQHNGCGHVNIVN---FYANDYGKVYRSCGNCKGNTNCKR
QK-----SGTSTINGGGAFSAQDKVLQFNGRGTLNVND---FYVQDYGKLVRNCGNCEGNG-GPR
GKTMTIVGGIAHNAKDGYGGKPDKVLQHNSKNSTTVVKGNFTLTGEHGKLWRSCGDCSNNGGPRF
GKTMTIVGGVAHNTTNGPGGKPDKVLQQNAKNSHTIVQGKFTLTGQHGKLWRSCGDCTNNGGPRN
YKSTG-TSYTYQVIGGAGQGSPDKYFTQSGRGTTIIKN--FCAEGKYGKVWCSCGNCIDQM-PRS
       .       ..     ** :  .. .   : .       .:**:  .**:* .:

                    IV
SVHMEGTTAVKGGELIGINTNYGDKATYSNNCYPKT---------QCQGYKGCD 220 U13050
NINIKGVVAKNGGELCGVNHNYGDVCTITDSCQNKGK--------SCQAYTGND 218 U13051
LTVTSATVNGTIDSIAGVNRNYGDVATISGLKIKNYKEG---KPPVCEEFKGVV 318 Y13340
LTIISATVNGTIDSIAGVNRNFGDVAEIRDLRIKGYKEG---KPPVCEEFNGVE 320 132172
VQISNTKIQGPGLAIISANSNYGDKISISGLTLYGQGSPNTLTKYICQSYNGLT 229 Pel-1
    .         : . * *:**     .            .   *: :.*
```

Figure 1. *Predicted amino-acid sequence of Globodera rostochiensis PEL-1 (Gen bank accession no. AF127915) aligned with bacterial (accession no. Y13340, Erwinia chrysanthemi Pel I, accession no. L32172, P. carotovorum Pel A) and fungal (accession no. U13050, Nectria haematococca Pel D, and accession no. U13051, N. haematococca Pel B) class III pectate lyases. Boxed shaded residues are conserved regions I to IV, as described for class III pectate lyase (Shevchik et al., 1997). The alignment was made by using the program ClustalW and the BLOSUM62 substitution matrix. Asterisks, identical or conserved residues in all sequences in the alignment; colons, conserved substitutions; single dots, semiconserved substitutions (Thompson, Higgins, & Gibson, 1994).*

Classification of PEL-1 as a class III pectate lyase markedly reduces the number of invariant amino-acid residues in this group (Fig. 1). Class III pectate lyases have four highly conserved regions and are characterised by the presence of several cysteine residues (eleven in PEL-1). On the basis of comparison of the PEL-1 sequence with other class III pectate lyases, only four conserved amino-acid residues with charged side chains (two aspartate residues at positions 130 and 199, and two lysine residues at positions 137 and 160) are present in the conserved regions. It is therefore likely that

one or more of these invariant residues are involved in the catalytic machinery of PEL1 and other class III members.

Symbiont-independent degradation of plant-cell walls by animals is now recognised as being possible. An endogenous cellulase gene was first isolated from cyst nematodes (Smant et al., 1998); cellulases are also produced by termites (Watanabe, Noda, Tokuda, & Lo, 1998) and the redclaw crayfish *(Cherax quadricarinatus)* (Byrne, Lehnert, Johnson, & Moore, 1999). Our current finding demonstrates that, like bacteria and fungi, cyst nematodes are genetically equipped to secrete a mixture of depolymerising cellulase and pectinase enzymes that allow the basic framework of plant cell walls to be dismantled.

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Chapter 4:

CHARACTERISATION OF A NOVEL PECTATE LYASE FROM *GLOBODERA ROSTOCHIENSIS*

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Manuscript in preparation

ABSTRACT

The first animal pectate lyase (Gr-pel-1) was discovered previously as the result of expressed sequence tag (EST) analyses from the nematode *Globodera rostochiensis.* Pectate lyases belong to the pectinolytic enzymes that degrade pectate which is one of the basic components of plant cell walls. Gr-pel-1 was actively transcribed in both subventral glands as shown by *in situ* mRNA detection. We found at least two homologous copies of Gr-pel-1 in the genome of *G. rostochiensis.* Differential expression analysis showed elevated mRNA levels in the water-soaked and potato root diffusate-hatched preparasitic juveniles. Sequence comparison and hydrophobic cluster analysis assigned Gr-pel-1 to the polysaccharide lyases family 3. Pectinolytic activity towards polygalacturonic acid was observed in cell lysates of *P. pastoris* cells which heterologous expressed the Gr-pel-1 open reading frame. In the recent years several endoglucanases have been identified in cyst and root knot nematodes. The combined effect of the pectate lyase described herein and these endoglucanases facilitate migration of the nematode through plant tissues.

INTRODUCTION

Cell walls of plants comprise a dynamic compartment of several independent but interacting structural networks (Carpita & Gibeaut, 1993). In this composite the cellulose/hemicellulose scaffold and pectic matrix are the two fundamental components. Their physical organisation in plant cell walls is often depicted as if the cellulose microfibrils coated with hemicelluloses lay embedded in a gel of pectic polysaccharides. Evidence for this model comes from microscopic observations (Morris et al., 1997; McCann et al., 2001).

The pectin matrix in the cell wall co-determines the mechanical and rheological properties of cells. As a pre-existing barrier it contributes to the protoplast's strong armor against invaders. In addition to these structural functions, pectins determine cell wall porosity, modulate cell wall pH and ion balances, regulate cell-cell adhesion at the middle lamella and serve as recognition molecules that signal the plant cells to the presence of symbiontic organisms, pathogens and parasites (Cote & Hahn, 1994; Ridley et al., 2001).

Pectins are probably the most complex class of cell wall polysaccharides (reviewed by (Ridley et al., 2001)). In the pectic matrix, homogalacturonan and rhamnogalacturonan I form the main polymeric chains. The α-D-(1$\rightarrow$4)-galacturonan in homogalacturonan may be substituted with xylose to form the xylogalacturonan. The richest structural diversity, however, is generated in rhamnogalacturonan II. This latter

pectic polymer is also a modified homogalacturonan polymer linked via unusual linkages to rare sugars, including apiose, aceric acid, methyl fucose and methyl xylose (Vidal et al., 2000). As opposed to the aforementioned pectic polysaccharides, rhamnogalacturonan I is heteropolymer made of α-L-(1→2) rhamnose and galacturonic acid disaccharide units with distinct polysaccharide side chains containing mostly neutral sugars such as arabinans, galactans and arabinogalactans. Furthermore, the main chains of pectic polysaccharides are methylated or acetylated with varying degree thereby further altering the degradability by pectinolytic enzymes. The proportion of each of the constituents in the pectin matrix differs among subcellular locations, among cell types and among plant species.

Plant pathogens produce complex mixtures of cell wall degrading enzymes in order to invade plants or to use the degraded components as energy and carbon sources (Annis & Goodwin, 1997). Complete breakdown of pectic polysaccharides requires a suite of pectinolytic enzymes. First, the elaborate side chains are cleaved from the main chain by enzymes such as galactanases, arabinofuranosidases, methyl and acetyl esterases. The main chains that are made accessible are substrate for the depolymerising enzymes, which are grouped according to their mode of action viz. hydrolases and lyases. Smooth regions in pectins are attacked by polygalacturonases, pectate lyases and pectin lyases, whereas the so-called hairy regions are degraded by rhamnogalacturonases and rhamnogalacturon lyases.

By far the most studied pectinolytic system involves plant pathogenic bacteria. Deletion studies in these pathogens show that in spite of the capacity of individual enzymes to macerate plant tissue their separate activities are not crucial for pathogenicity. The presence of multiple pectinolytic enzymes in plant pathogens with partially overlapping activities suggests that rather their combined effects determine virulence and pathogenicity.

At the onset of parasitism potato cyst nematodes (*Globodera rostochiensis*) invade host plants from the soil. Inside a host plant they migrate intracellularly through tissues leaving behind a trail of dead cells (Rice et al., 1985). Each cell is taken following fierce thrusts of their stylet – a hollow buccal spear – on the protective cell walls (Steinbach, 1972). For cellulases, produced in the salivary glands in these animals, it has been shown that they facilitate cell wall degradation during this migration phase (Wang et al., 1999). Recently, a preliminary report was made of the finding of a pectate lyase in this plant-parasite (Popeijus, et al., 2000). It was the first documented case of a metazoan gene encoding a pectin-degrading enzyme. In this study we present the genomic, functional and phylogenetic analysis of this novel protein.

EXPERIMENTAL PROCEDURES

Nematodes

Globodera rostochiensis, pathotype Ro1-Mierenbos, was propagated on green house cultures of *Solanum tuberosum* cv. Bintje. Cysts were harvested from roots approximately 3 months post infection and stored at 4°C until further use. Second stage juveniles (J2s) of *G. rostochiensis* were hatched from eggs by soaking cysts on a 100 µm sieve in potato root diffusate (Clarke & Perry, 1977; Goverse et al., 1994). Suspensions of freshly hatched juveniles were separated from root debris in 5 minutes at 1,000 g on a 35% sucrose layer. The juveniles were washed three times in sterile water and used for further experiments.

Cloning of full-length cDNA and corresponding genomic region

Sequences of the 5'-end cDNA were generated of approximately one thousand clones from a library of *G. rostochiensis* J2s (Popeijus, Blok et al., 2000). One of the expressed sequence tags (ge98) showed homology with pectate lyases in sequence databases (Popeijus, Blok et al., 2000). The 3'-end of the insert of clone ge98 was sequenced using the M13 universal primer (Invitrogen). Preliminary analysis of the encoded protein revealed that the insert of clone ge98 did not represent a full-length transcript. Therefore, an oligonucleotide primer PelcDNA-r (Table 1) was designed to amplify the 5'-region of the transcript using RACE technology (GIBCO/BRL).

In order to amplify the corresponding genomic region, total genomic DNA was extracted from hatched J2s by alkaline/SDS lysis and phenol/chloroform extraction (Sulston & Hodgkin, 1988). Inward primers (PelGen-f and PelGen-r) were designed from the most distal sequences in the transcript to amplify the coding sequences from genomic DNA extracts. Both the 5'- and 3'-end flanking regions were isolated from a genomic DNA library (Qin et al., 1998; Yan et al., 1998) by using the oligonucleotides PelProm-r and Pel3end-f (Table 1) as gene specific primers in the amplification reactions.

DNA sequencing was done using the dye terminator technology for cycle sequencing on an Automated Laser Fluorescent DNA sequencer (Amersham-Pharmacia, Upsala). The trace files were analysed in the ALFwin SA2.01 software package (Amersham-Pharmacia) and sequence contigs were assembled in DNAstar (Lasergene).

***Table 1.** Primers*

Primer	Primer sequence
PelcDNA-r	5'-GCCACGGCCAGATTCG-3'
PelGen-f	5'-ATTAGTAAACGACCAACACCAACAG-3'
PelGen-r	5'-GTTTAACTTTCATCAGAAAATGC -3'
Pel-EcoRI-f	5'-AGTGAATTCCTGTGCACCTTTCCTTCATCG-3'
Pel-NotI-r	5'-TAGCGGCCGCTTAGTTGACAATTTTAATAGC-3'
PelInsitu-f	5'-TGCACCTTTCCTTCATCG -3'
PelInsitu-r	5'-GCCACGGCCAGATTCG-3'
PelSou-f	5'-ACGGCGCCTGTGATGTGAAGAAC-3'
PelSou-r	5'-CCCGGCCCCGTGCTACC-3'
Pel3end-f	5'-CCATTGAAGCATTCGTTTATGG-3'
PelProm-r	5'-GCACTGTTGGTGTTGGTCG-3'
PCDNA5-f	5'-GGTGACACTATAGAATACTCAAGCTATGCA-3'
PCDNA3-r	5'-GACGGCCAGTGAATTGTAATACGACTCACT-3'

Sequence analysis

The sequences were compared with protein sequence databases using the Basic Local Alignment Search Tool algorithm (BLAST-2.0 with default settings) at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov /BLAST/). The probability for the presence of a N-terminal signal peptide for secretion at the start of the open reading frame was calculated with the program Signal-P using a WWW-interface (http://www.cbs.dtu.dk/services/SignalP-2.0/). The complete genomic DNA fragment of 2582 bp was analysed for transcription start sites (TSSW and TSSG) and polyadenylation cleavage sites (POLYAH) at the Sanger Institute (http://genomic. sanger.ac.uk/gf/gf.html).

Hydrophobic cluster analysis 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 (Lemesle Varloot et al., 1990).

cDNA-AFLP

cDNA-AFLP analysis was performed essentially as described (Qin et al., 2000). RNA was extracted from five developmental stages of *G. rostochiensis*: (D-dormant) dehydrated unhatched J2s in cysts (in diapause); (S-soaked) rehydrated unhatched J2s in one-year-old cysts after exposure to sterile tap water for two days; (H-hatched) pre-parasitic J2s (dry cysts were incubated in sterile tap water for one week, tap water is replaced by potato root diffusate (PRD) in the second week); (U- undifferentiated into

J2s) developing nematodes in gravid females two months post-inoculation; (P-pre-diapause) developing nematodes in gravid females three months post-inoculation. In the combination of *Eco*RI – *Taq*I restriction enzyme originally used by (Qin et al., 2000), the rare cutter *Eco*RI was replaced by *Ase*I. The adapters and the primers were replaced accordingly. The primers corresponding to the *Ase*I and *Taq*I adapters (without extension) are indicated as 'A' and 'T', respectively. The *Ase*I / *Taq*I derived fragments were amplified with the following primer combinations: A+G/T+AC.

Phylogenetic analysis

Phylogenetic trees, based on protein sequences from all database accessions that showed a significant match (E-value < 0.01) with the Gr-pel-1 sequence, were constructed using maximum parsimony (ProtPars in Phylip at http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-uk.html) and maximum likelihood (Puzzle). Except for Genbank accessions gi2133293 and gi595570 the putative signal peptide, as predicted by the SignalP-2.0, were deleted from all sequences. An initial alignment of the amino acid data was made in Clustal-W version 1.8 (http://www2.ebi.ac.uk/clustalw/). Removing unambiguously aligned positions and gaps further optimised the alignment. A character set including a total of 240 amino acid residues was used to infer the unrooted trees. For maximum parsimony analysis the character set was jumbled 7 times and relative support for the nodes was assessed using 1000 bootstrap replicates with 7 random number seeds. For the maximum likelihood analysis, trees were inferred using the JJT model of substitution with 1000 quartet puzzling steps. Three models of rate heterogeneity were used: uniform, two rate (1 variable and 1 invariable) and gamma distributed.

DNA blot analysis

The 327 to 964 bp region of Gr-pel-1 cDNA was amplified using oligonucleotide primers PelSou-f and PelSou-r (Table 1) and digoxigenin-11-dUTP in a PCR (Roche Molecular Diagnostics). The probes were checked on 1.5% agarose gel and purified using the Rapid Gel Extraction System (Life Technologies, Grand Island). In each hybridisation reaction 10 ng DIG-labelled DNA probe was used per millilitre DIG Easy hybridisation solution (Roche Molecular Diagnostics). Genomic DNA was isolated from J2s with alkaline/SDS lysis and phenol/chloroform extraction (Sambrook, Fritsch, & Maniatis, 1989). Prior separation on a 0.6% agarose gel was the genomic DNA digested with *Eco*RI, *Bam*HI, *Kpn*I, and *Bgl*II restriction endonucleases. The separated DNA was transferred onto a positively charged Nylon Membrane (Roche Molecular Diagnostics). Pre-hybridisation (in DIG Easy Hyb at 38°C for 30 min.), hybridisation (in DIG Easy Hyb at 38°C overnight) and stringency washes (in 0.1% SSC and 0.1% SDS at 68°C) were performed as recommended by the manufacturer (Roche Molecular Diagnostics). The immunological detection of the digoxigenin-11-

dUTP -labelled DNA probe with CSPD (Roche Molecular Diagnostics) solution preceded exposure of the membrane to X-Ray films (Konica, Tokyo, Japan) for 6, 12, and 20 minutes at room temperature.

In situ hybridisation

Single strand cDNA synthesised with linear amplification (Tabara et al., 1996) was used to obtain digoxigenin-11-dUTP (Roche Molecular Diagnostics) labelled sense and anti-sense probes. The probes were amplified from the 106 to 483 nucleotide region of the Gr-pel-1 cDNA using the PelInsitu-f and PelInsitu-r primers (Table 1) in two separate reactions. The probes were checked on a 1% agarose gel and purified through Mini Quick Spin DNA Columns (Roche Molecular Diagnostics). Freshly hatched nematodes were fixed overnight in 2% paraformaldehyde (in M9 buffer; (De Boer et al., 1999)) and cut into 3 to 4 pieces. The permeabilisation and hybridisation (at 50°C) were performed as described previously (De Boer et al., 1999). Alkaline phosphatase activity was detected with X-phosphate and 4-nitrobluetetrazolium chloride (Roche Molecular Diagnostics). The juveniles were examined using differential interference contrast microscopy (Leica, Dfeerfield).

Heterologous expression

Using PCR an *Eco*RI restriction sequence was introduced at the predicted cleavage site of the signal peptide in the open reading frame of Gr-pel-1. Similarly, a *Not*I site was introduced directly following the stop codon in the cDNA sequence in order to allow directional cloning into the pPICZalpha plasmid (Invitrogen, San Diego). For heterologous expression the plasmid was introduced into *Pichia pastoris* GS115 cells using electroporation. Ten transformants with the Mut^{++} phenotype were taken from plates containing methanol and were analysed for enzyme activity.

Enzyme assays

Cup-plates were prepared by mixing 0.25% polygalacturonic acid from citrus fruit (P-9135, Sigma Aldrich Chemie Gmbh, Steinheim, Germany) in 0.05 M Tris-HCl/1 mM Ca^{2+} (pH 8.5) with 0.5% agarose. Pectinolytic activity in the plates was assessed following incubation for 1 day at room temperature by staining with Ruthenium Red (0.02% (w/v) Sigma R-275D) for 20 minutes according to (Hagerman, Blau, & McClure, 1985).

RESULTS

Cloning of pectate lyase

PCR based cloning and DNA sequencing resulted in a full-length cDNA sequence of 980 bp, which contains an open reading frame of 783 bp (molecular mass of 27.9 kDa). The first 18 amino acid residues of the open reading frame are predicted to encode a signal peptide for secretion with a most likely cleavage between Ala^{18} and Leu^{19}. The predicted isoelectric point of the mature protein of 25.9 kDa is 9.08 including eleven cysteine residues. PCR with two primers designed from the most distal sequences of the transcript was used to amplify the gene from genomic DNA. The genomic fragment from the transcription start site to terminator site included 1794 bp. The coding sequence is interspersed with 6 introns; 5 relatively small introns (150-350 bp) and one larger intron (500 bp). All 5'- and 3'- donor and acceptor splice sites of the introns comply with the "GU-AG"-rule. Computer analysis of the 5'-flanking region using algorithms that recognises transcription start sites predicted a putative TATA-box consensus at –30 bp and a predicted start site at –7 bp relative to the 5'-end of the cDNA. In addition, good support was found for a 3'-end cleavage and polyadenylation region at the position 944 bp (AATAAA).

Sequence comparison

The Gr-pel-1 open reading frame was compared with amino acid sequences of corresponding proteins in the public database using the BLASTP program at NCBI. The open reading frame revealed significant (E-value between e^{-17} and e^{-8}) similarities with various members of the polysaccharide lyase family 3 (PL 3).

The amino acid sequences of all PL 3 family members that have shown pectate lyase activity were aligned using the computer algorithm ClustalW-1.8 (Fig. 1). Five regions (I, II, III, IV and V) in the alignment appear to be conserved among the majority of the sequences; however, none of the consensus regions is conserved in all sequences. Region I is lacking in two pectate lyase accessions from *Pectobacterium carotovorum* (GenBank accession numbers AAA57140 and S44995) and one from *P. chrysanthemi* (GenBank accession number CAA73784). These latter three accessions are nearly identical to each other at this position. Similarly, region V is present in all sequences except for two accessions from *Bacillus* sp. (GenBank accession numbers BAA8792 and CAB40884). Gr-pel-1 is the only exception on the core of sequence (WEDVCED) of conserved region II, which is a recognised signature for pectate lyases beyond PL 3 family. Interestingly, the conserved amino acid residues in the PL 3 family flanking this core sequence are indeed present in Gr-pel-1.

Two out of seven amino acids that proved to be crucial for the activity of the pectate lyase in *Bacillus* sp. (see asterisks in Fig. 1) are conserved in all PL 3 members (Hakamada et al., 2000). At two other apparent crucial sites Gr-pel-1 is the only

exception to the conservation viz. at position 108 the conserved glutamic acid is replaced by a tyrosine and at position 163 the arginine is replaced by a cysteine.

Figure 1. *Alignment of pectate lyases from PL family 3 using the computer algorithm ClustalW-1.8. Blocks of conserved residues. Asterisks indicate one of the seven conserved amino acids that are shown to be important for activity in Bacillus sp. pectate lyase. The question mark points to a contradiction in the alignment. Large shaded rectangles cover the five domains indicated with the Roman numbers I, II, III, IV, and V. The black bars indicate the β-strands and the grey bar indicate an α-helix as derived from the structure analysis of the Bacillus pectate lyase KSM P-15. The sequences shown are: G. rostochiensis (GenBank accession AF127915), Streptomyces coelicolor (GenBank accession CAC1306), P. chrysanthemi (GenBank accession CAA73784), Fusarium solani (GenBank accession S68364 and A45724), F. solani f. sp. pisi (GenBank accession AAA87382, AAC49420, and AAA87383), F. oxysporum f. sp. lycopersici (GenBank accession AAC64368), P. carotovorum (GenBank accession AAA57140 and S44995), Bacillus sp. (GenBank accession CAB40884 and BAA8792).*

```
                                                                          Consensus
         . . . . . . - - - - - - - - - - - - - G . . . . .                
46   -----------------------------TTFVG-----GSGILNGACDV                   AF127915
42   TPNGSEGVSSTLSVSG------------TKDYGMKRL-YGTGDLGSGGQD                   CAC13062
99   VSNAVTTAPNAGPLRAAK---ASSECKPGATFENRTVDCGGVTIGTSCPN                   CAA73784
12   KPTDHISNSKVIEVKA------------GQVYDGKWAKYDRGSGACKGQN                   S68364
12   KPTDHISNSKVIEVKA------------GQVYDGKWAKYDRGSGACKGQN                   AAA87382
23   KSAGATSFPTAVPVK--------------GSYDGGMKRFERSPSVCQGQS                   AAC64368
25   KSAGATSFPTAVPVK--------------GSYDGGMKRFEREPKVCKGQD                   A45724
101  KSNAASTAP--APLRAAPLKAASPECKAGAVIKDKTVDCGGITLGLSCSG                   AAA37140
101  KSNAAQTAP--APLRAAPLKAASSECKAGAVIKDKTVDCGGITLGLSCTG                   S44995
28   KATGSKSLSAPKTLKK------------GEVFDAGWVRYDRGV-KCSGQA                   AAC49420
24   ASAGVQSEPTAIPVRK------------GDKYNGGMKRFVRNPTTCKDQY                   AAA87383
23   AAAAPTVVNSTIVVPK------------GTTYDGQGKTFVANPSTLGDGS                   CAB40884
25   AEAAPTVVHETIRVPA------------GQTFDGKGQTYVANPNTLGDGS                   BAA87892

                      I                                 II

     . . . . . . . . . L . . G A . L . N V I I . . . . . . G . H C K - G . C . . . N V . W E D V C E D   Consensus
62   KNGKMKYLMVLKHGVTIKNAIINTPGL-GIYCE-GSCVLENIYYKKLCYH                   AF127915
79   E--DQGPILELAPGAVLKNVIIGAPAADGVHCK-GSCTLQNVWWEDVGED                   CAC13062
146  DSDKQKPLIILKNATVKNLRISASGRADGIHCDSGNCTIENVIWEDICED                   CAA73784
50   EGGDKDAVFLLHEGATLKNVIIGKDQSEGVHCK-GHCTLEFVWFEDVCED                   S68364
50   EGGDKDAVFLLHEGATLKNVIIGKDQSEGVHCK-GHCTLEFVWFEDVCED                   AAA87382
59   ETGEKDAMFILENGATLSNVIIGASQAEGVHCK-GTCTLNNVWWADVCED                   AAC64368
61   ETGEKDAMFILENGATLSNVIIGASQAEGVHCK-GTCTLNNVWWADVCED                   A45724
149  DSDKQPPVITLENATIKNLRISEKGGSDGIHCKSGNCRIENVIWEDICED                   AAA57140
149  DSDKQPPVITLENATIKNLRISEKGGSDGIHCKSGNCRIENVIWEDVCED                   S44995
65   EGGSKDAVFILEEGATLRNVIIGANQREGIHCK-GSCNIEFAWFEDVCED                   AAC49420
62   ETGEKDASFILEDGATLSNVIIDRSSGEGVHCK-GTCTLNNVWWADVCED                   AAA87383
61   QAENQKPVFRLEAGATLKNVIIGAPAADGVHCY-GSCNISNVVWEDVGED                   CAB40884
63   QAENQKPIFRLEAGASLKNVVIGAPAADGVHCY-GDCTITNVIWEDVGED                   BAA87892
        *              ?             *                  *

                                         III

     A . T . . . . . . . I - - - . G G G A . . A . D K V . Q . N . . G T . . I . . - - F - .   Consensus
110  ATGF-GYKSTGTSYTYQVIGGAGQGSPDKYFTQSGRGTTIIKN--FCAEG                   AF127915
126  AATFRGSSSSNVYTV---SGGGAKEADDKVFQFNGAGTLNISG--F-AVK                   CAC13062
196  AATNNGKTMTIVGGIAHNAKDGYGGKPDKVLQHNSKNSTTVVKGNFTLTG                   CAA73784
99   AISIKEDKAGKESWI---IGGGAYHASDKVVQHNGCGTVNIIN--F-YVE                   S68364
99   AISI----AGKESWI---IGGGAYHASDKVVQHNGCGTVNIIN--F-YVE                   AAA87382
108  AVTLKQTSGT--SYI---NGGGAFHASDKIVQFNGRGTVQIKD--F-YAE                   AAC64368
110  AVTLKQTSGT--SYI---NGGGAFHASDKIIQFNGRGTVHVKD--F-YAE                   A45724
199  AATNLGKTMTIVGGVAHNTTNGPGGKPDKVLQQNAKNSHTIVQGKFTLTG                   AAA57140
199  AATNLGKTMTIVGGVAHNTTNGPGGKPDKVLQQNAKNSHTIVQGNFTLTG                   S44995
114  AISILGSGTAN---I---IGGGAYHASDKVIQHNGCGHVNIVN--F-YAN                   AAC49420
111  AATFKQKSGT--STI---NGGGAFSAQDKVLQFNGRGTLNVND--F-YVQ                   AAA87383
110  ALTLKSSGTVN---I---TGGAAYKAYDKVFQMNASGTINIKN--F-RAD                   CAB40884
112  ALTLKSSGTVN---I---SGGAAYKAYDKVFQINAAGTINIRN--F-RAD                   BAA87892
                                 * *

          IV                                            V

     . . G K L . R S C G . C . . . . . R . . . . . . . . . . . . . . . . G . N . N Y G D . . . .   Consensus
157  KYGKVWCSCGNCIDQM--PRSVQISNTKIQGPGLAIISANSNYGDKISIS                   AF127915
170  NFGTFVRSCGNC--STQYRRTINLNGIEVNWKGGRIAGINTNYGDSATLR                   CAC13062
246  EHGKLWRSCGDCSNNGG-PRFLTVTSATVNGTIDSIAGVNRNYGDVATIS                   CAA73784
143  DYGKLYRSCGNC--SKQCKRNVYIEGVTAK-NGGELAGINANYGDTATLK                   S68364
139  DYGKLYRSCGNC--SKQCKRNVYIEGVTAK-NGGELAGINANYGDTATLK                   AAA87382
150  DYGKLVRSCGNCKDNGG-PRNVVISGSVAV-DGGVLCGINTNYGDTCKIT                   AAC64368
152  DYGKLSRSCGNCKDNGG-PRNVIVENSVAV-DGGVLCGINTNYGDTCKVI                   A45724
249  QHGKLWRSCGDCTNNGG-PRNLTIISATVNGTIDSIAGVNRNFGDVAEIR                   AAA57140
249  QHGKLWRSCGDCTNNGG-PRNLTIISATVNGTIDSIAGVNRNFGDVAEIR                   S44995
155  DYGKVYRSCGNCKGNTNCKRSVHMEGTTAV-KGGELIGINTNYGDKATYS                   AAC49420
153  DYGKLVRNCGNCEGNGG-PRNINIKGVVAK-NGGELCGVNHNYGDVCTIT                   AAA87383
151  DIGKLVRQ----NGGTSYAVNMTLDNSN-------ISNVKDSIMRTDSSV                   CAB40884
153  DIGKLVRQ----NGGTTYKVVMNVENCN-------ISRVKDAILRTDSST                   BAA87892
          *  *

     . . . . . . . . - - - C . . G . . . . . . . . . . . . . C .   Consensus
205  GLTLYGQGSPNTLTKYICQSYNGLTTMATMQPNAKFRPTQSGTGTCSYST                   AF127915
218  NITIVGDSSKKIVP---CQKYIGND---DGDEPDSNGS-GADGTYCKYSS                   CAC13062
295  GLKIKNYKEGKPPV---CEEFKGVV---KGQQGSTEKYGEKWDTTNCKVSR                  CAA73784
190  NV------CADAKQK---CTMYNGCA-------G----GCEPKKIGACPA                   S68364
186  NV------CADAKQK---CTMYNGCA-------G----GCEPKKIGACPA                   AAA87382
198  SS------CQNKGKY---CDRYEGNS---SGAEPSKIGS-GPDGKYCTTSG                  AAC64368
200  NS------CQDKGKY---CDRYEGNS---SGKEPTKIGS-GPDGKYCTVTG                  A45724
298  DLRIKGYKEGKPPV---CEEFNGVE---KGKGKSDKYGEFWDTKNCKVSR                   AAA57140
298  DLRIKNYKAGNPKI---CEEFKGIE---KGKGKTEKYGEFWDSKNCKVSR                   S44995
204  NN------CY-PKTQ---CQGYKGCD---KSKG-----ECEPSKAAKC                     AAC49420
201  DS------CQNKGKS---CQAYTGND---QKKEPPKFGPAGDNGKSCLVKS                  AAA87383
190  SQGKITNTRYSKVP-----TLFKGFA---SGK-----------------TSQS                CAB40884
192  STGRIVNTRYSNVP-----TLFKGFK---SGN-----------------TTAS                BAA87892
```

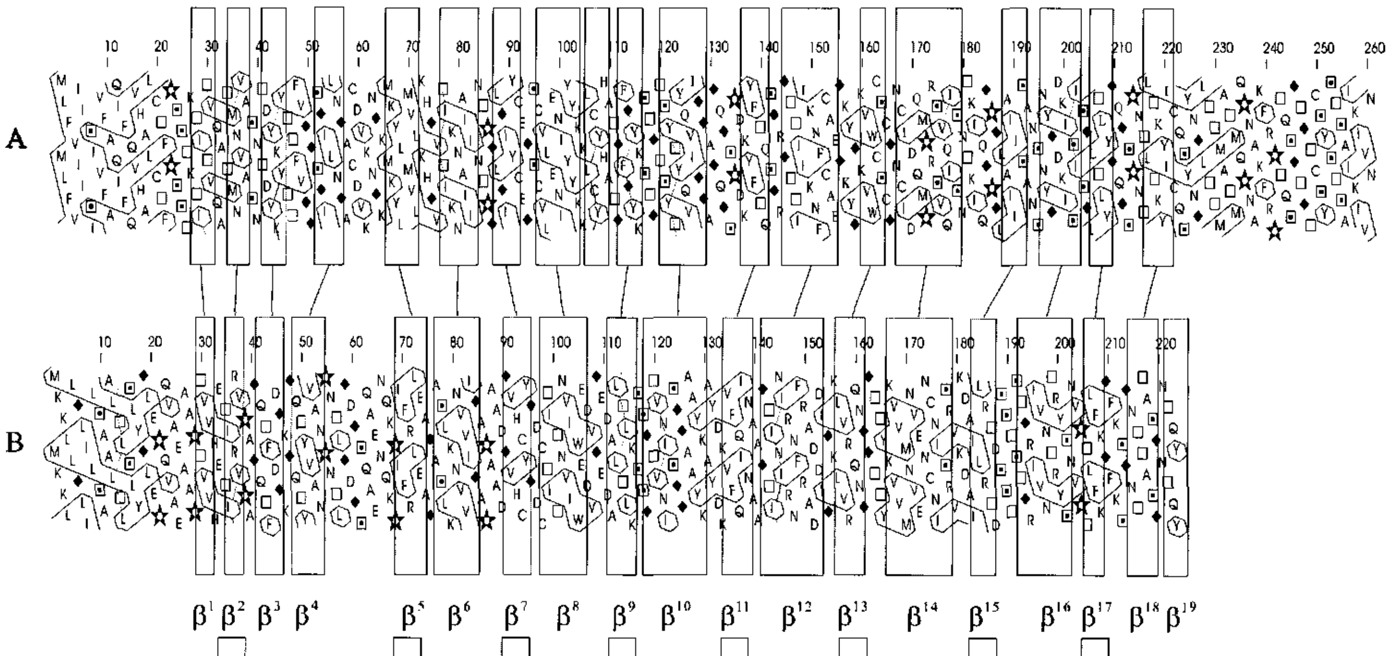

***Figure 2.** Hydrophobic cluster analysis of Gr-pel-1 (A) (GenBank accession AF127915) and pectate lyase of Bacillus sp. KSM P-15 (B) (GenBank accession AF127915). The open boxes indicate the beta-sheets that support the asparagine ladder (Yoder, Lietzke et al. 1993). The alignment of the beta-sheets resolved in Bacillus sp. KSM P-15 with predicted secondary structure elements in Gr-pel-1 is shown as connected rectangles.*

Hydrophobic cluster analysis

For further characterisation, HCA plots of Gr-pel-1 and a pectate lyase of *Bacillus* sp. KSM P-15 (GenBank accession AB011839) were compared (Fig. 2). The latter, whose three dimensional structure has been determined, was used to identify secondary structure elements constituting the parallel β-helical structure in the corresponding nematode pectate lyase, including the asparagine ladder. The overall identity between both amino acid sequences was only 15.6%, however, all 18 beta-sheets in the *Bacillus* sequence (see also black rectangles in Fig. 2) could be aligned with corresponding hydrophobic clusters in Gr-pel-1.

Phylogenetic analysis

An unrooted tree of the amino acid sequences from all pectate lyase active members of PL 3 family based on maximum parsimony is shown in Fig. 3. The topologies and the relative support for each branch inferred by maximum parsimony (Protpars) and maximum likelihood (Puzzle) using 3 models of rate heterogeneity were similar. Since no outgroup is known for the PL 3 family we were not able to infer how the tree is rooted. Irrespective of the method used, the split of the Gr-pel-1 branch was predicted to be between the pectate lyases of *Streptomyces* and *Pectinobacterium.* Therefore, the pectate lyase of *G. rostochiensis* is closer related to the pectate lyases of either of these latter two species than to the genes of any other eukaryotic species that have pectate lyases in the PL 3 family.

DNA-blot Analyses

Genomic DNA of second stage juveniles was digested with four different restriction enzymes (*Eco*RI, *Bam*HI, *Kpn*I, and *Bgl*II) in separate reactions (Fig. 4). A 637 nt single strand digoxigenin-11-dUTP probe of Gr-pel-1 hybridised to two genomic fragments on the DNA blot made of the restricted genomic DNA. In a parallel reaction genomic DNA of *Caenorhabditis elegans* was cut with the enzymes *Eco*RI and *Bam*HI to serve as negative control. No homologous genes are predicted for *C. elegans* and no hybridising bands were observed on the DNA blot of this species. None of the used restriction enzymes should cut in the gene sequence of GR-pel-1. Therefore, at least two homologous pectate lyase genes are predicted to be present in the genome of *G. rostochiensis*.

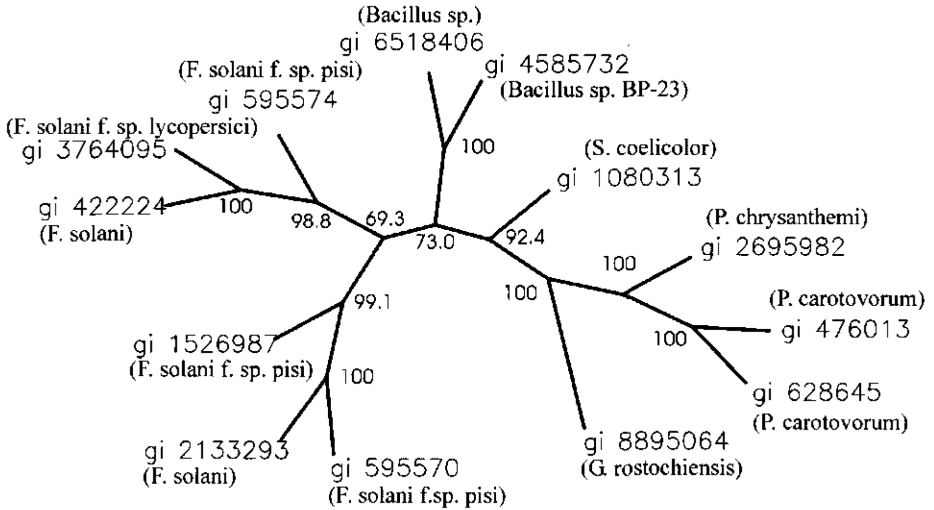

Figure 3. *Phylogenetic analysis of PL family 3 members using maximum parsimony (ProtPars in Phylip at http://bioweb.pasteur.fr/seqanal/phylogeny/phylip-uk.html). The bootstrap values are positioned at the corresponding branches of the unrooted three.*

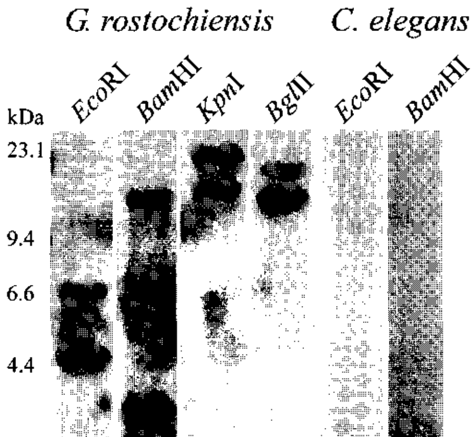

Figure 4. *Southern blot analysis revealed two homologous genes probed with Gr-pel-1 in genomic DNA of G. rostochiensis (lanes 1-4) restricted with EcoRI or BamHI, KpnI, and BglII. No hybridisation signal was observed in genomic DNA of C. elegans (lanes 5 and 6) restricted with EcoRI or BamHI.*

DISCUSSION

In this paper we describe a novel cell wall degrading enzyme – a pectate lyase – produced in the subventral esophageal gland cells of *G. rostochiensis*. The expression of the pectate lyase gene is up-regulated just prior invasion of the root system of host plants and turned off again in later developmental stages. Both the spatial and temporal expression of this pectate lyase is identical to that of the cellulases in the *G. rostochiensis*. The gene and the transcript encoding the nematode pectate lyase show typical eukaryotic features such as polyadenylation and cis-splicing. Furthermore, DNA blot hybridisation and the fact that no symbionts have ever been found in the esophageal gland cells make it most likely that the pectate lyase is of endogenous origin.

Based on similarities in the primary amino acid sequence and the predicted protein structure the nematode pectate lyase is assigned to polysaccharide lyase family 3. The PL 3 family is a heterogeneous group of proteins that only share an overall identity between 15 to 36% if evident paralogues and genes from sibling species are excluded from the analysis. Based on the direct sequence comparisons as well as the phylogenetic analysis the nematode pectate lyase seems to be the most diverged member of this family (15 to 21% in overall amino acid identity). The strongest similarity and the smallest maximum likelihood distance are found between the nematode pectate lyase and the pectate lyase of *P. chrysanthemi* that causes soft-rot diseases in plants. This ranking in sequence similarities of pectate lyase is identical to that of the cellulases of *G. rostochiensis* that also showed highest sequence similarities with *P. chrysanthemi* homologues. These observations may reflect either a conservation of a common ancestor gene or substrate driven convergence due to an overlap in host range.

The three-dimensional structure of the extracellular pectate lyases of *P. chrysanthemi*, from the two related PL families 1 and 2, revealed a motif of parallel β-strands wound into large right-handed coil (Yoder, Lietzke, & Jurnak, 1993). Within the core structure of the enzymes the side chains of the amino acids form linear stacks that include an asparagine ladder, which stabilises a rare type β bend (Yoder et al., 1993). The recent structure analysis of the *Bacillus* sp. KSM-P15 pectate lyase, which is classified as a family 3 PL, also revealed a right-handed wound helical structure of parallel β-strands (Hakamada et al., 2000). Members of the PL 3 family show no significant sequence similarity with those of PL families 1 and 2. Hence, the core topology of the enzymes seems to be more conserved than is expected from the primary protein sequence alignment. Despite only small similarities in their primary amino acid sequences HCA plots of *Bacillus* sp. KSM-P15 could be used to identify the parallel β-strands in Gr-pel-1 constituting the large helical structure unique to the pectate lyase superfamily.

In this study we have shown that primary transformants of *P. pastoris* harbouring the Gr-pel-1 open reading frame exhibited enzyme activity on polygalacturonic acid in the presence of calcium. Unfortunately the lack of stability of these transformants in liquid cultures undermined further biochemical analysis of the Gr-pel-1. Considerable effort using alternative expression system is now undertaken to acquire a conclusive substrate fingerprint.

Breaking down the plant cell wall, a composite made of complex and highly diverse polysaccharides, requires a mix of degrading enzymes. In order to gain access to the resources in their host plant pathogens secrete a variety of enzymes that cleave the polymeric chains in cell walls (Annis & Goodwin, 1997). The enzyme cocktail secreted by the bacteria and fungi comprise at least of enzymes that attack the two fundamental components in the cell walls – the scaffolding hemi(cellulose) network and the pectin matrix. In recent years ample evidence supports the hypothesis that cell wall breakdown by nematodes is the combined effect of enzymatic weakening and physical impact of the stylet. So far only enzymes that degrade the cellulose component in cell walls had been found in nematode secretions (Goellner et al., 2000; Rosso et al., 1999; Smant et al., 1998; Uehara, Kushida, & Momota, 2001; Yan, Smant, & Davis, 2001). Here we have shown that plant parasites too use a mixture of enzymes that degrade the two main structural components in plant cell walls. Furthermore, with our finding a primary role in migration is established for the subventral esophageal gland cells in sedentary plant-parasitic nematodes.

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Chapter 5:

TWO NOVEL BETA-1,4-ENDOGLUCANASES FROM THE POTATO CYST NEMATODE *GLOBODERA ROSTOCHIENSIS*

Herman E. Popeijus, Patrick Butterbach, Hein Overmars, Ling Qin, John Jones, Arjen Schots, Jaap Bakker, and Geert Smant.

Manuscript in preparation

ABSTRACT

Two novel beta-1,4-endoglucanases were identified in the cyst nematode *Globodera rostochiensis* by expressed sequence tag analysis. The two endoglucanases, Gr-eng-3 and Gr-eng-4, are 99% identical. They differ in a few nucleotides in the coding sequence resulting in four amino acids substitutions (Lys_{21}=>Leu_{21}, Asp_{56}=>Tyr_{56}, Arg_{91}=>Lys_{91}, and Thr_{316}=>Ala_{316}). Furthermore, intron I of the Gr-eng-3 gene is 83 bp larger in size than the corresponding intron I of Gr-eng-4. The other introns show marginal differences. Heterologous expressed protein of the Gr-eng-3 and Gr-eng-4 open reading frame showed hydrolytic activity towards carboxyl methyl cellulose. Transcripts of Gr-eng-3/4 were localised in the subventral glands of *Globodera rostochiensis* using *in situ* hybridisation. Sequence comparison revealed that Gr-eng-3/4 are distantly related to the endoglucanases cloned in nematodes so far. The expanding number of beta-1,4-endoglucanases indicate that the cell wall degrading enzymes play a more important role in the onset of parasitism than was previously expected.

INTRODUCTION

Cellulose is the most abundant carbohydrate polymer on earth made of linear chains of β-1-4 linked D-glucose. In plants stacked sheets of these parallel β-glucan polymers interconnect along their length with hydrogen bonds to form insoluble microfibrils. The microfibrils assemble into the cellulose network, which represents the main scaffolding structure in plant cell walls. The cellulose network is reinforced by hemicelluloses, xyloglucan (in type I cell walls) and glucuronarabinoxylans (in type II cell walls). This (hemi)cellulose network in plant cell walls lays embedded in a matrix composed of pectic polysaccharides and lignin (Carpita et al., 1996; Carpita & Gibeaut, 1993).

The complex composite nature of the cell wall provides the plant with constitutive protective armor against invading organisms. Complete cellulose breakdown alone requires the concerted action of β-1,4-endoglucanase (EC 3.2.1.4), β-1,4-cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Beguin, 1990; Beguin & Aubert, 1994). For plant pathogens, however, cleavage of the cellulose main chain by β-1,4-endoglucanases suffices to penetrate plant tissues and yield efficient colonisation. For instance, soft rot bacteria that typically produce extensive tissue maceration such as *Pectobacterium carotovora* and *P. chrysanthemi* harbour large gene families encoding β-1,4-endoglucanases. Similarly, multigene families of β-1,4-

endoglucanases are found in *Fusarium* species associated with stem and root rots in many different kinds of vegetables, flowers, and field crops.

Sedentary plant parasitic nematodes penetrate the root system of host plants close to the root tips and migrate through the root tissues until they commence the formation of a feeding site. Recently, it was shown that plant penetration and intracellular migration is facilitated by cell wall degrading enzymes secreted from the esophageal glands of the infective juvenile stage (Wang et al., 1999). Initially, β-1,4-endoglucanase genes were identified in two cyst nematodes species that thrive on potato and soybean respectively following a complex and time-consuming procedure (De Boer et al., 1996; De Boer et al., 1996; Smant et al., 1997; Smant et al., 1998). Specific antibodies directed against secretions were used to immunopurify their corresponding antigens from nematode homogenates. Following N-terminal amino acid sequencing the putative β-1,4-endoglucanases were cloned with degenerated oligonucleotide primers and PCR based cloning (Smant et al., 1998). These endoglucanase genes proved to be the first cellulases of metazoan origin.

Since this finding of endogenous cellulase in cyst nematodes homologs have been identified in root knot nematodes (*Meloidogyne* sp.) and many other plant-parasitic nematodes species (Goellner et al., 2000; Rosso et al., 1999; Uehara et al., 2001; Yan et al., 2001). In addition to gene families encoding endoglucanases, evidence is found for the endogenous production of xylanases and pectate lyases (Dautova et al., 2001; Popeijus, Overmars et al., 2000); Chapter IV). The cumulative data on the cell wall degrading capabilities of plant-parasitic nematodes indicate that these parasites have at their disposal a repertoire of enzymes with a catalytic potential similar to that of plant pathogenic fungi and bacteria. Furthermore, the nature of the genes transcribed in the two subventral oesophageal gland cells in sedentary nematodes establishes their role at the onset of parasitism – cell wall breakdown during host penetration and migration.

The monoclonal antibody (MGR48) that lead to the cloning of two β-1,4-endoglucanase genes of both potato cyst nematode and soybean cyst nematode recognised an epitope available on 6 distinct proteins in the potato cyst nematode *Globodera rostochiensis* (Smant et al., 1997). The genes that encoded two of the larger proteins (viz. molecular masses of 39 and 49 kDa) were cloned, however, the genes encoding the three smaller proteins of 30, 31a, 31b and 32 kDa respectively appeared to be refractory to cloning with straight-forward PCR based cloning (Smant et al., 1998). In earlier experiments these putative low-molecular weight cellulases were found to be the most abundant cellulases in stylet secretions of the potato cyst nematodes indicating their importance at the onset of parasitism. Here we combine N-terminal protein sequencing and 5'-end expressed sequence tags to efficiently clone the two major cellulases in the potato cyst nematode.

EXPERIMENTAL PROCEDURES

Nematodes

Dried cysts of *G. rostochiensis* pathotype Ro1 were soaked on a 100 µm sieve in potato root diffusate (PRD) to collect hatching second-stage juveniles (J2s; (De Boer et al., 1996). Freshly hatched juveniles were mixed in a centrifuge tube with an equal volume of 70% (w/v) sucrose, and covered with a layer of sterile tap water. Following centrifugation for 5 minutes at 1,000 *g* juveniles were collected from the sucrose-water interface using a Pasteur pipette and washed 3 times with sterile tap water. Subsequently, juveniles were either used for experiments directly or stored at -80°C until further use.

DNA cloning and analysis

An oligo-dT-primed cDNA library from *G. rostochiensis* second stage juveniles was prepared in the vector pcDNAII (Invitrogen, San Diego; (Popeijus, Blok et al., 2000; Smant et al., 1998)). Individual colonies plated on Luria-Bertani medium were randomly picked and grown in 1ml liquid cultures. Plasmid DNA was isolated from the cells following overnight growth at 37°C. The 5'-end expressed sequencing tags (ESTs) were subsequently generated using cycle sequencing with dye terminator chemistry.

The sequencing reactions described in this paper were analysed on an Automated Laser Fluorescent DNA sequencer (Amersham-Pharmacia) supported by the ALFwin SA2.01 software. Raw sequences were validated and assembled using the DNASTAR software program (DNASTAR, Madison). The nascent sequences were analysed using the program Basic Local Alignment Search Tool algorithm (BLAST) with default settings at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST).

Out of a total of 914 ESTs (Popeijus, Blok et al., 2000) one tag (ge1985) encoded a partial open reading that showed similarity to β-1,4-endoglucanases of various origins. The sequence similarity with other nematode β-1,4-endoglucanases, however, was only slightly higher than those found in other organisms. The sequence downstream of EST ge1985 was identified using the M13 universal sequencing primer (Invitrogen). A start codon was missing in the ge1985, therefore, a primer (Eng-r1) was designed to amplify the region upstream of the tag when combined with a plasmid derived primer (PCDNA5-f1) in a PCR using a plasmid prep of the cDNA library. To confirm the integrity of the cDNA that could be deduced from the amplified fragments two primers (Eng-f1 and Eng-r2) were combined in a similar PCR that produced a single band of 1194 bp. This band was cloned into the pCR2.1-TOPO (Invitrogen) as subjected to DNA sequencing.

An identical PCR with the primers Eng-f1 and Eng-r2 was done using genomic DNA as template, which was extracted from hatched J2s by alkaline/SDS lysis and phenol/chloroform extraction (Sulston & Hodgkin, 1988). The genomic fragment was cloned into pCR2.1-TOPO vector and subjected to DNA sequencing.

The program Signal-P was used to predict the presence of a signal peptide consensus sequence (http://www.cbs.dtu.dk/services/SignalP-2.0/). Protein patterns and domains in the predicted open reading frames were identified using the PFam analysis tool as provided on the world-wide-web (http://pfam.wustl.edu/hmmsearch.shtml).

Table 1. *Primers used to amplify and clone cDNA and genomic DNA from G. rostochiensis*

Primer	Primer sequence
Eng-f	5'-GAATGTGCGCTTTGATTTATG-3'
Eng-r1	5'-GCACAACATTGCTGTTACAGC-3'
Eng-r2	5'-CTAAGCTTTGATTTATTCACCTTTC-3'
Eng-pETf	5'-CACCGTCACAGCCCCTCCCTAT-3'
Eng-pETr	5'-ACCGCGGCAACTTACT-3'
Eng-insitu-f	5'-CCGCGGAATATGCCAAAATGAAG-3'
Eng-insitu-r	5'-CCGGCGTAAAAGGCAATGTGTATG-3'
PCDNA5-f	5'-GGTGACACTATAGAATACTCAAGCTATGCA-3'
PCDNA3-r	5'-GACGGCCAGTGAATTGTAATACGACTCACT-3'

In situ hybridisation

DNA probes were amplified from clone ge1985 using the oligonucleotide primers Eng-insitu-f and Eng-insitu-3 and digoxigen-11-dUTP. J2s were fixed overnight in 2% paraformaldehyde, cut into sections, and permeabilised as described (De Boer, Yan, Smant, Davis, & Baum, 1998; Smant et al., 1998). Fixed sections were then incubated at 50°C with sense or anti-sense DNA-probes followed by digestion with RNAse A and stringency washings. Hybridised DNA-probe was detected by alkaline phosphatase staining (Genius kid, Boehringer Mannheim). Stained J2 were examined with differential interference contrast microscopy (Leica, Dfeerfield).

Heterologous Expression in *E. coli*

The open reading frames of Gr-eng-3 and Gr-eng-4 were subcloned into the pET101/D-TOPO expression vector using the oligonucleotide primers Eng-pETf and Eng-pETr. The plasmids were introduced into the *E. coli* TOP10 for characterisation, propagation and maintenance of the constructs. Recombinant plasmid DNA was isolated from positive transformants and introduced into *E. coli* strain BL21Star (DE3) for expression. Expression of the recombinant protein was determined on SDS-PAGE with Coomassie Brilliant Blue staining and western blotting following induction of the transformants with 1 mM isopropyl β-D-thiogalactoside.

***Figure 1.** Nucleotide sequences of Gr-eng-3 and Gr-eng-4 and predicted ORFs. The introns are indicated with I, II, III, IV, V and VI at the positions in the cDNA where they are found in the genomic DNA sequences. The start codon ATG and stop codon TGA are printed in italic. Nucleotides coding for the predicted signal peptide are underlined.*

```
Gr-eng-3    AGA ATG TGC GCT TTG ATT TAT GCC GTT TTA CCC CTG CTT CTC GTG CTG ATC GCC GAT CAT TCT     63
Gr-eng-4    AGA ATG TGC GCT TTG ATT TAT GCC GTT TTA CCC CTG CTT CTC GTG CTG ATC GCC GAT CAT TCT     63
Gr-eng-3/4       M   C   A   L   I   Y   A   V   L   P   L   L   L   V   L   I   A   D   H   S      20

Gr-eng-3    AAG ATC GGC GCT GTC ACA GCC CCT CCC TAT GGC CCA TTG GCT GTC AAT GGA AAA TTC CTT GTC    126
Gr-eng-4    CTG ATC GGC GCT GTC ACA GCC CCT CCC TAT GGC CCA TTG GCT GTC AAT GGC AAA TTC CTT GTC    126
Gr-eng-3/4  K/L  I   G   A   V   T   A   P   P   Y   G   P   L   A   V   N   G   K   F   L   V      41

Gr-eng-3    CAA AAG AGT ACA AAA AAG ACG GTG AAA TTG CAC GGA CTC TCA CTG GAT TGG AGC CAG TGG CAA    189
Gr-eng-4    CAA AAG AGT ACA AAA AAG ACG GTG AAA TTG CAC GGA CTC TCA CTG TAT TGG AGC CAG TGG CAA    189
Gr-eng-3/4   Q   K   S   T   K   K   T   V   K   L   H   G   L   S   L  D/Y  W   S   Q   W   Q      62

                                                   I
Gr-eng-3    CCG CGC TTT TGG GTG GCA GAA ACG GTC AAT|CGA ATT AAA TGC GGC TGT AAC AGC AAT GTT GTG    252
Gr-eng-4    CCG CGC TTT TGG GTG GCA GAA ACG GTC AAT|CGA ATT AAA TGC GGC TGT AAC AGC AAT GTT GTG    252
Gr-eng-3/4   P   R   F   W   V   A   E   T   V   N   R   I   K   C   G   C   N   S   N   V   V      83

                                       II
Gr-eng-3    CGT GCC GCA ATG GGC ACT CAG|AGA AGC TTC GGA GGA TAT GTT GCC AAT CCG GCC GCG GAA TAT    315
Gr-eng-4    CGT GCC GCC ATG GGC ACT CAG|AAA AGC TTC GGG GGA TAT GTC GCC AAT CCG GCC GCG GAA TAT    315
Gr-eng-3/4   R   A   A   M   G   T   Q  R/K  S   F   G   G   Y   V   A   N   P   A   A   E   Y     104

Gr-eng-3    GCC AAA ATG AAG ACA ATC ATC GAA GCA GCG TTG GAC CAA GGC ATT TAT GTT ATC GTT GAT TGG    378
Gr-eng-4    GCC AAA ATG AAG ACA ATC ATC GAA GCA GCG TTG GAC CAG GGC ATT TAT GTT ATC GTT GAT TGG    378
Gr-eng-3/4   A   K   M   K   T   I   I   E   A   A   L   D   Q   G   I   Y   V   I   V   D   W     125

                                                                  III
Gr-eng-3    CAC ACC GGG GAC GAT TTG GCC ACC GAT GAA ATC AAT TCT GCT|AAA GAA TTC TTC ACA AAA ATT    441
Gr-eng-4    CAC ACC GGG GAC GAT TTG GCC ACC GAT GAA ATC AAT TCT GCT|AAA GAA TTC TTC ACA AAA ATT    441
Gr-eng-3/4   H   T   G   D   D   L   A   T   D   E   I   N   S   A   K   E   F   F   T   K   I     146

Gr-eng-3    GCT CAA ACT TAT GGC AAA CAT CCG CAC ATT ATC TAC GAA ATT TGG AAT GAG CCC CTC AAA CAG    504
Gr-eng-4    GCT CAA ACT TAT GGC AAA CAT CCG CAC ATT ATC TAC GAA ATT TGG AAT GAG CCC CTC AAA CAG    504
Gr-eng-3/4   A   Q   T   Y   G   K   H   P   H   I   I   Y   E   I   W   N   E   P   L   K   Q     167

                                                                  IV
Gr-eng-3    ACT ACT ACA TGG GAA GCT GTG ATT AAA CCA TAC TCA AAA ACG|ATG GTC GAA TTG ATT CGA AAA    567
Gr-eng-4    ACT ACT ACA TGG GAA GCT GTG ATT AAA CCA TAC TCA AAA ACG|ATG GTC GAA TTG ATT CGA AAA    567
Gr-eng-3/4   T   T   T   W   E   A   V   I   K   P   Y   S   K   T   M   V   E   L   I   R   K     188

Gr-eng-3    TAT GAC AAG AAC AAC GTG ATC ATT GTT GGC ACA CCA AAC TGG GAC CAG GAC GTG GAT ATT GTG    630
Gr-eng-4    TAT GAC AAG AAC AAC GTG ATC ATT GTT GGC ACA CCA AAC TGG GAC CAG GAC GTG GAT ATT GTG    630
Gr-eng-3/4   Y   D   K   N   N   V   I   I   V   G   T   P   N   W   D   Q   D   V   D   I   V     209

Gr-eng-3    GCC AAA AGT CCA CTG ACC GGC TAT TCA AAC ATT GCA TAC ACA TTG CAC TTT TAC GCC GGC CAG    693
Gr-eng-4    GCC AAA AGT CCA CTG ACC GGC TAT TCA AAC ATT GCA TAC ACA TTG CAC TTT TAC GCC GGC CAG    693
Gr-eng-3/4   A   K   S   P   L   T   G   Y   S   N   I   A   Y   T   L   H   F   Y   A   G   Q     230

                                                                        V
Gr-eng-3    CAC AAT GAA TGG CTT CGA ACT CGG ACA AAA ACG GCA TAC AAT TTA GGC CTG CCA ATG TTC GTG    756
Gr-eng-4    CAC AAT GAA TGG CTT CGA ACT CGG ACA AAA ACG GCA TAC AAT TTA GGC CTG CCA ATG TTC GTG    756
Gr-eng-3/4   H   N   E   W   L   R   T   R   T   K   T   A   Y   N   L   G   L   P   M   F   V     251

Gr-eng-3    ACG GAG TAT GGC ATC TAT TCG GAG CCC AAA AAT GAG GCA AAC AAT CTC AAA GAG TTG GCA CTT    819
Gr-eng-4    ACG GAG TAT GGC ATC TAT TCG GAG CCC AAA AAT GAG GCA AAC AAT CTC AAA GAG TTG GCA CTT    819
Gr-eng-3/4   T   E   Y   G   I   Y   S   E   P   K   N   E   A   N   N   L   K   E   L   A   L     272

Gr-eng-3    TGG TAC AAA TTG TTG GAC AGT CTG AGT ATG TCC TAT GCC GCT TGG CAA GTG ACT GAC ATT AAC    882
Gr-eng-4    TGG TAC AAA TTG TTG GAC AGT CTG AGT ATG TCC TAT GCC GCT TGG CAA GTG ACT GAC ATT AAC    882
Gr-eng-3/4   W   Y   K   L   L   D   S   L   S   M   S   Y   A   A   W   Q   V   T   D   I   N     293

Gr-eng-3    GAG CAG CAT GCA ATG TTT ACC CCT GGT GTG ACA ATC AAC AAC ATT TGC AAC CCA GCC TAC TTG    945
Gr-eng-4    GAG CAG CAT GCA ATG TTT ACC CCT GGT GTG ACA ATC AAC AAC ATT TGC AAC CCA GCC TAC TTG    945
Gr-eng-3/4   E   Q   H   A   M   F   T   P   G   V   T   I   N   N   I   C   N   P   A   Y   L     314

                                                                            VI
Gr-eng-3    ACC ACC TAT GGG AAG TAC ATT TAC AAC AAG CTG AAG TCT CAA AAC AAC GGA GTA AGT TGC CGC   1008
Gr-eng-4    ACC GCC TAT GGG AAG TAC ATT TAC AAC AAG CTG AAG TCT CAA AAC AAC GGA GTA AGT TGC CGC   1008
Gr-eng-3/4   T  T/A  Y   G   K   Y   I   Y   N   K   L   K   S   Q   N   N   G   V   S   C   R     335

Gr-eng-3    GGT TGA ATA GCG TTT GAT TGT AGA ACG AAC GAG CAA GAT TGT TAA CAA AGA TGC ATA ACT TTA   1071
Gr-eng-4    GGT TGA ATA GCG TTT GAT TGT AGA ACG AAC GAG CAA GAT TGT TAA CAA AGA TGC ATA ACT TTA   1071
Gr-eng-3/4   G                                                                                    336

Gr-eng-3    ATA AAA ATT TAA CCT AAA CTA GTG ACT TAT TAT TTG AAC AAT TAC TGT TAA ACC ATG AAA GGT   1134
Gr-eng-4    ATA AAA ATT TAA CCT AAA CTA GTG ACT TAT TAT TTG AAC AAT TAC TGT TAA ACC ATG AAA GGT   1134

Gr-eng-3    GAA TAA ATC AAA GCT TAG AAG AAG TCA ACT GAA AAA AAA AAA AAA AAA AAA AAA AAA AAA A     1195
Gr-eng-4    GAA TAA ATC AAA GCT TAG AAG AAG TCA ACT GAA AAA AAA AAA AAA AAA AAA AAA AAA AAA A     1195
```

The predicted molecular masses of the mature proteins of Gr-eng-3 and Gr-eng-4 are 35,388 Da and 35,378 Da respectively. The predicted isolectric points of the two proteins are 8.67 and 8.80 respectively. In 1998, Smant et al. used a monoclonal antibody to purify two β-1,4-endoglucanases of 47.9 kDa and 39.1 kDa from preparasitic J2s of *G. rostochiensis*. Previously this antibody was shown to react with four proteins on western blot following SDS-PAGE (De Boer et al., 1996) and six proteins on western blots following two-dimensional electrophoresis (Smant et al., 1997). We hypothesised that Gr-eng-3 and Gr-eng-4 encoded either of these remaining proteins recognised by the monoclonal antibody MGR48. Thus, monoclonal antibody MGR48 was used to immunopurify a 32 kDa protein band from homogenates of *G. rostochiensis* J2s. Five to ten micrograms of purified protein was used for amino acid sequencing. The straight sequence analysis of the 32 kDa protein band showed multiple N-termini including one major sequence. Additional runs using the reagent o-phtalaldehyde at the fourth cycle blocked all other N-termini except the major sequence and yielded 26 amino acids (Table 2). The identity of the Gln_{24} and Asp_{26} residues is tentative because of the weakness of the signal obtained from the HPLC. The N-terminus of the 32 kDa protein is a perfect match with the predicted N-terminus of the open reading frame of Gr-eng-3 and Gr-eng-4.

Table 2. *N-terminal sequences as determined after amino sequencing and the predicted ORF of Gr-eng-3/4. The identity of the shaded residues is tentative.*

Protein	Amino acid sequence
32 kDa band	VTAPPYGPLAVNGKFLVQKSTKQTVD
Gr-eng-3/4	VTAPPYGPLAVNGKFLVQKSTKKTVK

Sequence analysis

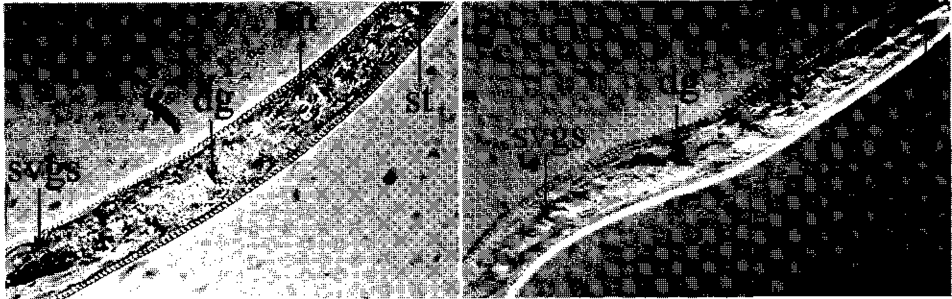
The predicted protein sequences of Gr-eng-3 and Gr-eng-4 are 99% identical (Table 3). The identity of Gr-eng-3/4 with other β-1,4-endoglucanases of the genus *Globodera* is on average 36.3% with a standard deviation of 0.36, which is in strong contrast with the average identity among all *Globodera* β-1,4-endoglucanases (84.35% with a standard deviation 7.78). Strikingly, GR-eng-3/4 showed the strongest identity with *Heterodera glycines* eng-2 and eng-3 (Genbank accession AF052734, AF044210). The average identity of Gr-eng-3/4 with all previously cloned β-1,4-endoglucanases from cyst-nematodes is 38.4% with a standard deviation of 2.20, which is contrasting with the overall identity among β-1,4-endoglucanases in cyst-nematodes (75.21% with a standard deviation of 12.25).

Table 3. *Percentage identity in amino acids between complete coding sequences following alignment in ClustalW.*

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
	98.5	36.8	36.5	35.9	36.2	38.9	41.1	41.7	39.2	40.1	32.9	27.7	37.1	37.2	28.5	**1**	*Gr-eng3*
		36.5	36.2	35.9	35.9	38.9	41.1	41.7	39.2	40.1	33.5	27.7	37.7	37.2	28.5	**2**	*Gr-eng4*
			80.1	96.2	79.0	67.2	68.7	69.3	64.8	63.0	39.8	30.7	37.8	44.5	27.0	**3**	*Gr-eng1*
				79.8	92.3	64.8	69.0	69.6	65.9	66.3	40.6	30.7	43.1	47.0	30.4	**4**	*Gr-eng2*
					78.7	66.8	67.4	68.0	63.0	62.2	40.0	31.9	37.8	45.1	27.2	**5**	*Gt-eng1*
						63.3	67.7	68.3	65.9	64.9	39.2	31.0	42.0	46.3	29.9	**6**	*Gt-eng2*
							77.4	77.7	72.2	69.6	40.3	32.2	37.8	47.6	29.1	**7**	*Hg-eng1*
								99.1	94.7	96.6	44.8	33.5	47.0	48.0	33.2	**8**	*Hg-eng2*
									95.6	97.5	45.5	33.5	48.0	48.9	33.5	**9**	*Hg-eng3*
										94.9	42.3	32.2	45.5	46.3	31.8	**10**	*Hg-eng4*
											41.6	31.9	44.9	47.6	31.0	**11**	*Hs-eng2*
												32.2	47.9	60.4	31.9	**12**	*Mi-eng1*
													33.4	31.4	22.3	**13**	*Mi-eng2*
														74.1	33.6	**14**	*Pp-eng1*
															39.6	**15**	*Pp-eng2*
																16	*P. chrysanthemi*

Given these differences between Gr-eng-3/4 and the β-1,4-endoglucanases previously cloned from cyst-nematodes it may be questioned whether they are of nematode origin and whether they encode β-1,4-endoglucanase activity. An antisense digoxigen-11-dUTP labelled cDNA probe was constructed in order to localise the expression of Gr-eng-3/4 in whole mount nematodes. The antisense probe specifically hybridised in both subventral oesophageal gland cells from preparasitic juveniles of *G. rostochiensis* (Fig. 2a). The sense probe did not result in a hybridisation signal (Fig. 2b). Therefore, it is reasonable to assume that Gr-eng-3/4 are of nematode origin. It should be note, however, that it is not possible to discriminate between two genes (99% identity) with *in situ* hybridisation.

Figure 2. *Localisation of Gr-eng-3/4 transcripts using DIG-labelled sense and anti sense probes derived from the Gr-eng-3/4 cDNA. In second stage juveniles of G. rostochiensis. The anti sense probe specifically hybridised in the anterior of the subventral glands (svgs)(**A**). No hybridisation signal could be observed with the sense probe (**B**). In both pictures A and B the dorsal gland (dg), metacorpus (m) and stylet (st) are pointed with arrows.*



Previously, it was shown that intron positions are more conserved in nematode β-1,4-endoglucanases than the primary amino acid sequence (Yan et al., 1998). Introns I, III, IV, V, and VI of Gr-eng-3/4 are conserved in Gr-eng-1, Gr-eng-2, Hg-eng-1 and Hg-eng-2 too. Intron II of Gr-eng-3/4 is not found in the other β-1,4-endoglucanases, whereas intron IV which is conserved in Gr-eng-1, Gr-eng-2, Hg-eng-1 and Hg-eng-2 is not found in Gr-eng-3/4.

Further sequence analysis using the Pfam HMM search program showed that Gr-eng-3 and Gr-eng-4 have a single domain (amino acids 37-297) which is recognised as a glycosyl hydrolase family 5 β-1,4-endoglucanase domain (significance 10^{-38}). In this domain two conserved regions, IYEIWNE$_{163}$P and FVTE$_{253}$YGI, including the active glutamic acid residues could identified in an alignment of Gr-eng-3/4 with other endoglucanases of the GH-5 family (Fig.) (Jenkins, Lo Leggio, Harris, & Pickersgill, 1995). These findings make it likely that the two genes encode a catalytic domain typical for the GH-5 family.

Figure 3. *Clustal W multiple alignment of Gr-eng-3 (Genbank accession AF408155), Gr-eng-4 (Genbank accession AF408156), H. schachtii eng-2 (Genbank accession AJ299387), H. glycines eng-3 (Genbank accession AF044210), M. incognita eng-1 (Genebank accession AF100549), P. chrysanthemi Cel5 (Genbank accession AF208495), and P. fluorescens (Genbank accession S56132) (http://www2.ebi.ac.uk/clustalw/) (Tompson, et al. 1994). The active side residues are printed bold. Boxed are the conserved core sequences of the GH-5 family (Jenkins et al., 1995).*

```
Gr-eng-3 IIYEIWNEPLKQTTTWEAVIKPYSKTMVELIRKYDKNNVIIVGTPNWDQDVDIV 209
Gr-eng-4 IIYEIWNEPLKQTTTWEAVIKPYSKTMVELIRKYDKNNVIIVGTPNWDQDVDIV 209
AF044210 LLYETFNEPLDVS--WNDVLVPYHKKVISAIRAIDKKNVIILGTPKWSQDVDVA 193
AJ299387 ILYEDFNEPLDVS--WTDVLVPYHKKVIAAIRAIDKKNVIILGTPKWSQDVDVA 147
AF100549 IIYETFNEPLQVD--WSGVKS-YHEQVVAEIRKYDTKNVIVLGTTTWSQDVDTA 201
AF208495 VIYEIYNEPLQVS--WSNTIKPYAEAVISAIRAIDPDNLIIVGTPSWSQNVDEA 218
S56132   VIYEIYNEPLQVS--WSNTIKPYAQAVIAAIRAIDPDNLIIVGTPTWSQDVDVA 215
         ::** :****.     *   .    * : ::   **   * .*:*::**..*.*:**  .

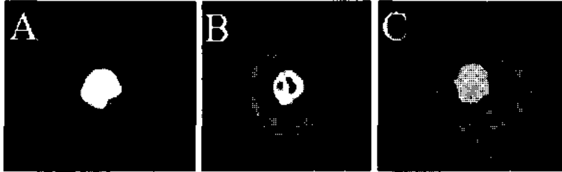
Gr-eng-3 AKSPLTGYSNIAYTLHFYAGQHNEW-LRTRTKTAYNLGLPMFVTEYGIYSEPKN 262
Gr-eng-4 AKSPLTGYSNIAYTLHFYAGQHNEW-LRTRTKTAYNLGLPMFVTEYGIYSEPKN 262
Af044210 AQNPIKGFSNLMYTLHFYASSHFVDGLGNKLKTAVNKGLPVFVTEYGTCEASGN 247
AJ299387 SQNPIKDYQNLMYTLHFYASSHFTSDLGAKLKTAVNNGLPVFVTEYGTCEASGN 201
AF100549 ANNPVSG-TNLCYTLHFYAATHKQN-IRDKAQAAMNKGACIFVTEYGTVDASGG 255
AF208495 SRDPIN-AKNIAYTLHFYAGTHGES-LRNKARQALNNGIALFVTEWGAVNADGN 272
S56132   ANDPITGYQNIAYTLHFYAGTHGQY-LRDKAQTALNRGIALFVTEWGSVNANGD 268
         :..*:.   *: *******. *     :  : : * * *  :****:*   .   .
```

Enzymatic activity in CMC assay

Cell lysates of transformed *E. coli* containing Gr-eng-3 and Gr-eng-4 constructs showed significant hydrolytic activity in a cup plate assay with 0.2% carboxymethylcellulose (Fig. 4b, 2c). The lysates of *E. coli* transformants that were not induced or that harboured the empty plasmid vector did not show activity after staining the CMC-plates with Congo red (Fig. 4a).

The constructs of Gr-eng-3 and Gr-eng-4 in the expression vector pET101 included a His-tag for detection of the recombinant protein on western blots. Subsequently, this His-tag was recognised on a several protein bands in the range of 37 to 24 kDa on a western blot with lysates of *E. coli* transformed Gr-eng-3 and Gr-en-4. In contrast these protein bands could be not detected on western blots with a mixture of monoclonal antibodies including MGR48, which was used to immunopurify the 32 kDa protein band from nematode homogenates.

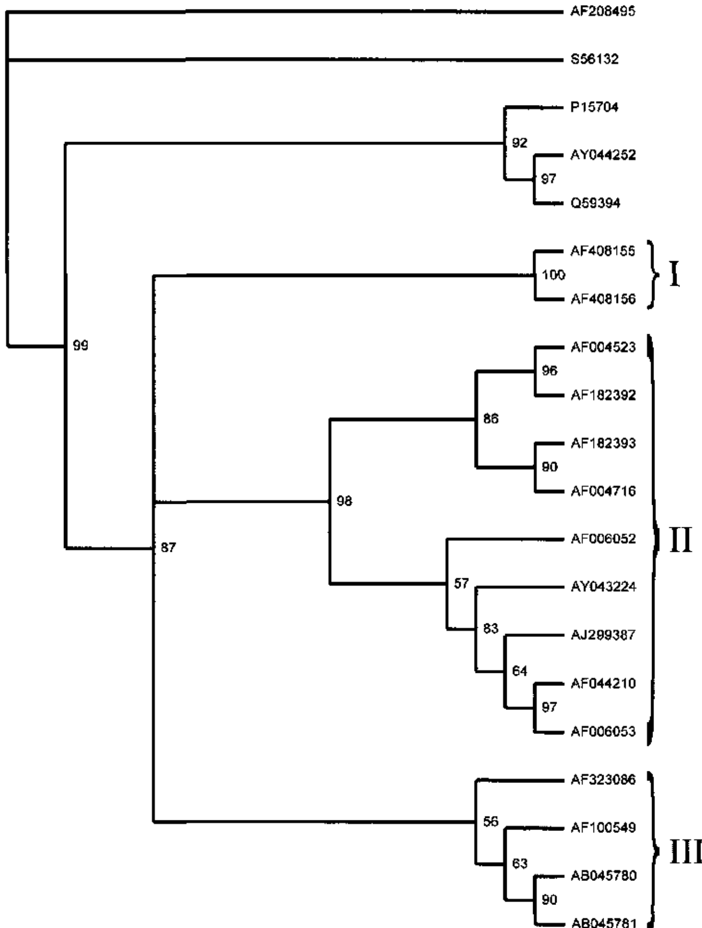
Figure 4. *Hydrolytic activity by Gr-eng-3 (B) and Gr-eng-4 (C) of CMC visualised by the presence of a halo. No halo could be detected while applying lysates harbouring the empty vector (A).*



Phylogenetic analysis

In the phylogenetic analysis the β-1,4-endoglucanases from *Meloidogyne incognita* appear in a monophyletic cluster (III) with the β-1,4-endoglucanases from *Pratylenchus penetrans* (Fig. 5). The β-1,4-endoglucanases from *Heterodera* sp. and β-1,4-endoglucanases previously cloned from *Globodera sp.* appear in two separate monophyletic clusters too (II). Gr-eng-3 and Gr-eng-4 cluster (I) monophyletically distinct from β-1,4-endoglucanases from all other cyst nematode species as well the β-1,4-endoglucanases in the cluster of *Meloidygne incognita* and *Pratylenchus penetrans.* The exact tree topology at the split of cluster I, II, and III could not be resolved using this data set.

Figure 5. *Phylogenetic analysis using maximum likelihood of endoglucanases from Erwinia chrysanthemi (GenBank accession AF208495), Pseudomonas fluorescens (GenBank accession S56132), Clostridium acetobutylicum (GenBank accession P15704), Bacillus subtilis (GenBank accession AY044252), Pectobacterium carotovorum (GenBank accession Q59394), Globodera rostochiensis (GenBank accession AF408155, AF408156, AF004523, and AF004716), Globodera tabacum solanacearum (GenBank accession AF182392 and AF182393), Heterodera glycines (GenBank accession AF006052, AF006053, AF044210, and AY043224), Heterodera schachtii (GenBank accession AJ299387), Meloidogyne incognita (GenBank accession AF100549 and AF323086), and Pratylenchus penetrans (GenBank accession AB045780 and AB045781). The roman numbers I, II and III indicate three monophyletic clusters containing nematode β-1,4-endoglucanases.*



Discussion

In this paper we describe two novel but highly similar β-1,4-endoglucanase genes in *G. rostochiensis*. Previously, two other β-1,4-endoglucanases were cloned from *G. rostochiensis* which were found to be closely related to β-1,4-endoglucanases of other cyst nematode species. In contrast, the cumulative data of the direct comparison of the amino acid sequences, the phylogenetic analysis and the analysis of the intron positions of Gr-eng-3 and Gr-eng-4 show that they are only distantly related to the β-1,4-endoglucanases identified in cyst nematodes so far. Based on the partially conserved intron positions it is concluded that Gr-eng-3 and Gr-eng-4 share an ancient evolutionary history with the other β-1,4-endoglucanases of cyst nematodes. However, the finding that Gr-eng-3 and Gr-eng-4 cluster separately from the β-1,4-endoglucanases previously cloned from *G. rostochiensis*, *G. solanum* sp. *tuberosum*, *H. glycines*, and *H. schachtii* suggest that they arise from a gene duplication prior to the speciation of cyst nematodes.

This study was initiated to clone the gene that encodes a secretory protein of approximately 32 kDa. This protein previously known as svp32 is the most abundant antigen of four proteins on one-dimensional western-blots of stylet secretions of *G. rostochiensis* J2s recognised by monoclonal antibody MGR48. Several results favour the hypothesis that svp32 is encoded either by Gr-eng-3 or Gr-eng-4. First, the predicted amino termini of Gr-eng-3 and Gr-eng-4 are identical to the amino terminal sequences obtained from the protein fraction purified from nematode homogenates with MGR48. The amino termini are not strongly conserved among β-1,4-endoglucanases of cyst nematodes. Secondly, in immunofluorescence microscopy MGR48 specifically binds to the secretory granules of the two subventral oesophageal gland cells. cDNA probes designed from Gr-eng-3 and Gr-eng-4 specifically hybridise to the subventral oesophageal gland cells *in situ* too. Thirdly, recombinant protein produced in *E. coli* from the two previously cloned antigens of MGR48 exhibited catalytic activity on carboxymethylcellulose. Recombinant protein produced from Gr-eng-3 and Gr-eng-4 in *E. coli* showed activity on the carboxymethylcellulose substrate.

One observation, however, contradict with the hypothesis. Which is the lack of binding of MGR48, which was used to immunopurify svp32, to the proteins recombinantly produced from Gr-eng-3 and Gr-eng-4. The epitope of MGR48 may not be properly exposed in the recombinant protein of Gr-eng-3 and Gr-eng-4. This scenario, may be solved by selecting a different host for heterologous expression (*e.g. Pichia pastoris*).

Plant pathogenic bacteria (e.g. *Pseudomonas fluorescens*) typically have a considerable number of cellulolytic enzymes at their disposal in order to deal with complete degradation of plant cell walls at various sites in a host plant. Degradation of cell walls during plant invasion by infective juveniles of plant parasitic nematodes is not

purely chemical. It is rather the combined effect of the physical impact of the stylet and cell wall softening by cellulolytic enzymes that facilitates migration of nematodes *in planta*. With rising numbers of β-1,4-endoglucanase genes in *G. rostochiensis* (at least four genes) the contribution of enzymatic cell wall softening in migration appears to a factor of higher importance than was previously expected.

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Chapter 6:

General discussion and Summary

Introduction

Plant-parasitic nematodes remain a threat to the global food production. Host plant resistance constitutes an environmentally sound method to limit crop losses by plant parasitic nematodes. To date, several natural resistance genes against plant-parasitic nematodes are cloned or are in the process of being cloned from all major crops species. These natural resistance genes are studied for their potential to control nematodes in crops in which no resistance genes are known. In addition, various more artificial strategies are currently explored in order to achieve resistance via so-called tailor-made transgenes.

Expanding the use of resistance genes, either natural or artificial, in crops implicates knowledge of nematode genes that act at the interface of nematode and host plant. These parasitism genes may represent a wide range of functions ranging from basic-compatibility factors to avirulence gene products. The objective of this thesis is to identify parasitism genes in cyst nematodes using expressed sequence tags.

The potato cyst nematode species, *Globodera pallida* and *G. rostochiensis*, are used as model organisms for other cyst nematode species for two reasons. First, potato cyst nematodes are relatively easy to handle for experimental purposes because of their long-term viability in a desiccated state. This biological trait enables laboratories to have large quantities of infective juveniles available from the shelf. Secondly, as opposed to other cyst nematode species the oesophageal gland cells are already fully activated in pre-parasitic juveniles of potato cyst nematodes. The oesophageal gland cells are a major gateway through which nematodes deliver the products of the parasitism genes to the plant. Therefore, it is reasonable to assume that the bulk of the parasitism genes are expressed in the preparasitic juveniles of potato cyst nematodes.

Monoclonal antibodies (mAbs)

At the start of this study only a small number of parasitism genes were identified from sedentary plant-parasitic nematodes. The identity of these genes was revealed via PCR based cloning using degenerate primers, which were designed from short stretches of amino acid sequences. The amino acid sequences derived from proteins that were immunopurified from homogenates of preparasitic juveniles with monoclonal antibodies (mAbs) directed to oesophageal gland secretions. The mAbs were generated in mice following immunisation with a diverse set of protein extracts (De Boer et al., 1996a; De Boer et al., 1996b).

In spite of its success this strategy has a number of serious drawbacks. Huge numbers of nematodes are required to obtain sufficient starting material for protein purification. Moreover, the production of antibodies in vertebrates is quantitatively

biased toward immunodominant proteins. Antibodies against less immunodominant proteins are likely to be missed in the screening procedures of antibody producing hybridoma cell lines. Furthermore, the PCR primers in this strategy are designed from short protein sequences usually with high degeneracy, which makes it difficult to specifically amplify DNA fragments encoding the protein sequence. But above all it is the time that it takes to proceed according to the strategy from mAb to gene. The discovery of the first parasitism gene from plant-parasitic nematodes took more than 5 years. The inefficiency makes this strategy not suitable for a comprehensive analysis of gene expression in nematodes.

MAb MGR48 was used to immunopurify the svp32 protein from homogenates of *G. rostochiensis* (chapter 5). Previously, immunopurification of two other antigens recognised by MGR48 successfully lead to the identification of two β-1,4-endoglucanase genes in *G. rostochiensis* following PCR based cloning (Smant et al., 1998). The same strategy, however, failed in the identification of the svp32 protein (Chapter 5; De Boer et al., 1996). The degeneracy of the PCR primers proved to be an insurmountable obstacle for the specific amplification of the corresponding cDNA. However, data mining an EST database of *G. rostochiensis* second stage juveniles (Chapter 2) revealed a 5'-end sequence with an open reading frame that was identical to the N-terminal protein sequence of svp32, which offered an way out of this impasse. The full-length cDNA of the EST encoded a β-1,4-endoglucanase that was only distantly related to the β-1,4-endoglucanases previously found in cyst nematodes. Further analysis of a cDNA library of the same juvenile stage resulted in the identification of two highly similar β-1,4-endoglucanase genes that have an identical N-terminal sequence. The data in chapter 5 that was generated to provide evidence for svp32 being the product of the either of the two novel β-1,4-endoglucanase genes is unfortunately still inconclusive.

EXPRESSED SEQUENCE TAGS (ESTs)

ESTs are short DNA sequences between 250 and 600 bp of the ends of complementary DNA (cDNA) derived from single pass sequencing of individual clones from a cDNA library. The cDNA is obtained after reverse transcription of mRNA and reflect a pool of coding sequences of genes after splicing. The mRNA used for library construction primarily determines, which genes will be tagged. During parasitism the expression pattern of mRNAs changes in tissues at different events and time points (Davis, Allen, & Hussey, 1994; De Boer et al., 1999; Gregory, Blaxter, & Maizels, 1997). It is assumed that a majority of the parasitism genes are expressed in the oesophageal glands of cyst nematodes, which are active in the preparasitic juveniles of potato cyst nematodes. In this thesis pre-parasitic second stage juveniles of the potato

cyst nematode, *G. rostochiensis* and *G. pallida*, served as the source for mRNA to select for genes that are transcribed during the onset of parasitism and are potentially involved in penetration and migration into the plant root.

The main advantage of ESTs is the relative ease with which large amounts of DNA sequence data can be generated from parasites. As compared to the immunodominancy in the strategy mentioned above there is no bias in EST projects towards a specific subset of genes. Nor is there a demand for extraordinary large numbers of nematodes for the preparation of cDNA libraries in EST projects. Moreover, with current DNA sequencing capacity in the scientific community large numbers of ESTs can be generated almost instantly. To date, rate limiting are the production of high-quality cDNA libraries and the analysis of the nascent DNA sequences.

Obviously the efficacy of an EST project is depending on the quality of the cDNA library that is used for sequencing. In theory, the library should be a complete representation of the full-length sequences of all the genes expressed in the tissue that is used for the preparation of the library. Practical limitations (e.g. relative abundance, transcript size) always lead to compromises in the library construction. In chapter 2, a classical method for cDNA library preparation was chosen, which makes use of non-amplified template of oligo-dT primed cDNA. The consequences of this choice are a bias towards highly abundant transcripts and a high percentage of cDNA inserts that are not full-length. Variations in amplification efficiencies in PCR templates yield an overrepresentation of shorter inserts in libraries that are made from amplified templates. As opposed to PCR amplified cDNA libraries the size distribution of the original template will still be preserved in oligo-dT primed cDNA library.

A key feature of parasitism genes is the encoded signal peptide for secretion at the amino terminus of the open reading frame. An efficient selection of expressed sequence tags for candidate parasitism genes depends on the presence of full-length sequences in the cDNA library, including the predicted amino terminus of the open reading frame. Two PCR based methods have been used to improve the proportion of full-length sequences in a cDNA library of parasitic nematodes. One method uses a so-called spliced leader sequence, which is trans-spliced from a separate locus in the genome to the 5'-end of mRNA molecules. Specific amplification of cDNA using the spliced leader sequence produces a high abundance of 5'-ends in the library template, which enables a fast selection of signal-peptide in the encoded open reading frames. Unfortunately, the number of genes in nematodes that are trans-spliced varies per species. Furthermore, it is not clear whether there is a bias in trans splicing towards specific classes of genes. For instance, the vast majority of parasitism genes that have been identified to-date are not trans-spliced. A second method to improve the prevalence of full-length transcripts in a cDNA library uses a SMART-oligonucleotide system, which essentially yields the same effect as spliced-leader amplifications without any possible bias towards certain mRNA species.

IDENTIFICATION OF PARASITISM GENES

Parasitism genes are defined as genes that have evolved specifically in a parasite to promote parasitism in a host (Davis et al., 2000). This is a broad definition, but we limit our focus to the genes whose products are secreted by the nematode, and which are involved in migration, feeding site formation or self-defence in plants. The challenge of EST projects is not to produce large quantities of sequence data but the recognition of candidate parasitism genes in large sequence databases. The initial analysis of DNA sequences occurs via one-to-one comparison of nascent sequences with databases of previously annotated sequences of various origins (e.g. WU-BLAST, BLAST, FASTA, BLITZ). For a large percentage of the EST in chapter 2 homologues could be found following this procedure.

Typically, the DNA sequences of genes that are recognised as pathogenicity factor in other plant-pathogens matched with homologues in potato cyst nematodes, *e.g.* pectate lyase (chapters 3 and 4) and β-1,4-endoglucanases (chapter 5). A number of ESTs, classified as PCN specific genes in chapter 2, produce no significant match in any database. These latter genes are perhaps the most interesting ones as they may reflect specific adaptations to plant-parasitism. Despite extensive searching of many databases using a variety of search types no clue as to the function of these genes could be found. Hence, the efficacy of ESTs projects in identifying parasitism genes relies on the presence of properly annotated homologues in databases.

Additional bioinformatics may further help to select for candidate parasitism genes among the pioneers that have no match in any database. Automated programs that predict the subcellular destination of the gene product may reveal if the encoded proteins are secreted. However, additional experimental data is required for a more conclusive identification of parasitism genes. *In situ* hybridisation microscopy may reveal the tissues in which the pioneers are expressed. In plant-parasitic nematodes the oesophageal glands, genital glands, and amphidial glands as well as the cuticle are the primary structures that secrete proteins. Hybridisations of cDNA probes with either of these structures favour the identification of a parasitism gene. In addition to spatial expression patterns temporal expression analyses may strongly enhance the identification of parasitism genes. Genes whose expression pattern correlates with specific events during parasitism, e.g. migration or feeding site induction, are more likely to be involved in these processes than other genes. cDNA-AFLP and the micro-array technology are two high-throughput methods that allow to make an efficient link between expression patterns and ESTs.

FUNCTIONAL ANALYSES

The primary focus in ESTs projects lies at the identification of structural homology of the candidate parasitism genes in sequence databases. Based on the findings of these searches genes are more or less likely to be involved if not crucial in plant parasitism. These searches, however, do not clarify the function of a gene in plant parasitism nor its relative importance. With a given homology, for some genes it is relatively easy to design assays that give insight in the function of a gene. Via heterologous expression in bacteria or yeast sufficient recombinant protein can be isolated to perform activity assays or binding studies. The recombinant protein of Gr-pel-1 and Gr-eng-3/4 were tested on their appropriate substrates and found to be active enzymes. Hence, it could be concluded that plant-parasitic nematodes use mixtures of pectinolytic and cellulolytic enzyme during migration in plants. Pectate lyases had never been discovered in animals before and now it seems that pathogens and parasites of plants have adopted similar strategies for invasion.

Designing functional assays for pioneering genes is more of challenge. First, the production of specific antisera against the encoded proteins may reveal the localisation of their target *in planta*. An antiserum against a cellulase of *H. glycines* localised the enzyme in the migratory tract inside the plant root thereby firmly establishing its role in cell wall degradation during penetration and migration in the plant root (Wang et al., 1999). Similarly, antisera may localise proteins of pioneering parasitism genes at specific subcellular positions in the cells of a feeding site. Alternatively, yeast-two-hybrid systems may be used to find the target proteins of parasitism genes in plants. In some instances clues for the function of parasitism genes may even be found in phenotypic changes in plant cells following ectopic expression of parasitism genes. It is evident, that the initial selection of the candidate parasitism genes has to be stringent because the functional assays mentioned above are far from being high-throughput at the moment.

A key test to establish whether candidate parasitism genes are of any importance to the plant-nematode interaction is to eliminate their effect by gene-inactivation. In *C. elegans* gene-inactivation is routinely done by genetic transformation via microinjection of DNA into the gonads of hermaphrodites. *C. elegans* is such a good genetic model for its short generation time (~3 days), its ability to reproduce on petri-dishes seeded with bacteria, and the presence of self-fertilising hermaphrodites. In contrast, sedentary plant-parasitic nematodes have a generation time of at least several weeks, engage in a biotrophic interaction with host plants, and have complex modes of reproduction. As compared to *C. elegans* the thick and non-transparent cuticle of adult female plant-parasites makes it difficult to inject the gonads. Moreover, there is no selective marker available for easy identification of transformed plant-parasites. In short, currently no

routine transformation system with significant efficiency is available for plant-parasitic nematodes.

Introduction of double-stranded RNA (dsRNA) is able to specifically disrupt the activity of genes containing homologous sequences in *C. elegans.* dsRNA can be delivered in several ways to achieve this gene silencing effect: (a) it can be micro-injected into the body cavity of the nematode (Fire et al., 1998); (b) bacteria transformed with a dsRNA-encoding construct can be fed to the nematode (Timmons & Fire, 1998); (c) the nematode can be soaked in solutions containing dsRNA (Tabara, Grishok et al. 1998). Because of the difficulties in microinjecting plant-parasitic nematodes, the first method is obviously not suitable. The second option is also not feasible because obligate plant-parasitic nematodes do not feed on bacteria. To overcome these difficulties, host plants could be transformed with constructs encoding short complementary RNA molecules that produce dsRNA with a hairpin (reviewed by (Bosher and Labouesse 2000)). When nematodes are feeding on the transgenic plants, they may automatically take up dsRNA. It was shown that the 21-23 nucleotide fragments of dsRNA are guiding targeted mRNA degradation (Elbashir, Lendeckel et al., 2001; reviewed by (Bosher & Labouesse, 2000)). Such small dsRNA molecules should easily pass the narrow stylet opening of the nematode. It is also worthwhile testing whether soaking the plant-parasitic nematodes in dsRNA solution will have the same effect as for *C. elegans.*

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Samenvatting

De volgende samenvatting is bedoeld voor de belangstellende die niet bekend is met of op het gebied van de nematologie. Geprobeerd is in eenvoudige bewoordingen de inhoud van het proefschrift kort samen te vatten. Collega's en anderen die geïnteresseerd zijn in achtergrondinformatie kunnen als verdieping de abstracts raadplegen die vooraf gaan aan de afzonderlijke hoofdstukken en naar de "General discussion and summary".

Aardappelcystenaaltjes zijn kleine rondwormen of nematoden die parasiteren op aardappelwortels en op wortels van planten die nauw verwant zijn aan de aardappel (de zogenaamde Solanacea). De eieren van het aaltje bevinden zich in een soort zakje dat bestaat uit de verharde huid van het gestorven vrouwtje (de cyste). Deze cyste kan ongeveer 300-600 eieren bevatten die, indien er een geschikt wortelstelsel aanwezig uitkomen. De jonge nematode, verplaatst zich vervolgens door de grond op zoek naar een adequate plantenwortel. Als de nematode bij zo'n wortel is aangekomen dan dringt ze deze binnen. In de wortel verplaatst de jonge nematode zich in de richting van de xyleemvaten van de plant op zoek naar een geschikte plantencel waar ze straks haar voedingsstoffen uit kan onttrekken. Deze plantencel wordt vervolgens op ingenieuze wijze omgebouwd tot een volwaardige voedingsfabriek.

Deze voedingsfabriek bestaat uit de oorspronkelijke cel die door het gericht oplossen van aangrenzende celwanden versmelt met meerdere omliggende cellen. Gedurende enkele weken groeit de jonge nematode door middel van deze voedingsstructuur uit tot een volwassen diertje. In de laatste fase van haar bestaan wordt een vrouwtje bevrucht door een mannetje waarna de eitjes zich in het afstervende vrouwtje verder ontwikkelen. Na een rustfase die loopt tot het volgende seizoen komen de nieuwe jonge nematoden weer uit hun ei, waarmee de cyclus zich herhaalt.

Omdat de plant-nematode-interactie veel energie vraagt van de plant zal de opbrengst van een geïnfecteerd landbouwgewas drastisch lager zijn. Hierdoor neemt de voedselproduktie wereldwijd af en beloopt de economische schade aangericht door nematoden (waaronder cystenaaltjes) in de vele miljarden guldens per jaar.

De boer, die direct hierdoor in zijn inkomsten is getroffen, wil dat niet en zal de schade door aaltjes willen beperken. De aaltjes zijn echter heel moeilijk te bestrijden. Slechts weinig middelen blijken effectief en economisch toepasbaar. Effectief en economisch toepasbaar betekent veelal dat de boer op de eerste plaats naar chemische bestrijdingsmiddelen zal grijpen. De effectieve middelen zijn helaas nogal giftig en dat betekent weer dat er sprake is van schadelijke neveneffecten voor de mens en de natuur. Vele van de toepasbare bestrijdingsmiddelen zijn om deze reden inmiddels verboden of het gebruik ervan is aan strenge voorwaarden gebonden.

Dit betekent dat het nodig is om te zoeken naar alternatieve bestrijdingsmethodes. Om deze te kunnen ontwikkelen is elementaire kennis nodig van de nematode-plant-interactie. In deze dissertatie is onderzocht op welke manier de nematode kans ziet de plant binnen te dringen en zich in de plant te verplaatsen.

Uit eerder onderzoek is gebleken dat de nematode gebruik maakt van celwand afbrekende enzymen om de plantenwortel binnen te kunnen dringen. Deze enzymen kunnen worden gezien als een soort biologische schaar waarmee de nematode de plantencelwand op speciale plaatsen open kan knippen. Deze enzymen bestaan onder meer uit zogenaamde cellulases (endoglucanases) en pectinases (waaronder pectate lyases). Deze enzymen worden geproduceerd in de twee eencellige speekselklieren van de nematode. De nematode injecteert ze in de plantencelwand via een stylet, een soort steekmond net als bij een mug, op de kop van de nematode.

De eerste van deze celwand afbrekende enzymen zijn ontdekt met behulp van antilichamen. Antilichamen zijn eiwitten die deel uit maken van het dierlijke afweersysteem en gebruikt kunnen worden voor onderzoek. Daarna werd een aantal zuiverings- en kloneringstechnieken toegepast, voordat het soort enzym dat de nematode gebruikt, kon worden bepaald. Een van de grote nadelen is de immuno-dominantie van bepaalde eiwitten bij gebruik van het dierlijke immuunsysteem. Dit houdt in dat het immuunsysteem een voorkeur heeft om te reageren op een selectie eiwitten uit het totaal van aangeboden eiwitten. Het gevolg hiervan is dat vele interessante eiwitten niet worden gevonden.

In het hier uitgevoerde onderzoek is een nieuwe techniek toegepast welke dit nadeel niet kent. Deze techniek bestaat uit het bepalen van ongeveer duizend zogenaamde korte cDNA base volgorden (in het Engels expressed sequence tags) afkomstig van de nematode *Globodera rostochiensis* en *G. pallida* (dit is beschreven in Hoofdstuk 2 van het proefschrift). Elke van deze expressed sequence tags is een soort vingerafdruk van een RNA molecule die op zijn beurt weer codeert voor een eiwit dat afkomstig is van de pre-parasitaire aaltjes. Dat wil zeggen uit de nematode die op het punt staat in de plantenwortel binnen te dringen. Door al deze tags of vingerafdrukken te vergelijken met miljoenen tags of vingerafdrukken van eiwitten uit een openbare data base is het mogelijk gebleken om enkele van die eiwitten aan te wijzen die de nematoden kunnen gebruiken om de plantenwortel binnen te dringen.

Een van de resultaten was de ontdekking van het eerste pectine afbrekende enzym, genaamd pectate lyase, van het diertje *Globodera rostochiensis* (beschreven in hoofdstuk 3 en 4 van dit proefschrift). Buiten het pectate lyase enzym zijn er nog twee cellulase afbrekende enzymen, cellulases, gevonden van de nematode *Globodera rostochiensis* (beschreven in hoofdstuk 5 van het proefschrift).

Al deze drie enzymen produceert de nematode in zijn slokdarmklieren. Deze bevindingen ondersteunen het belang van de twee slokdarmklieren aan het begin van de nematode-plant-interactie. Door vervolgonderzoek uit te voeren, bijvoorbeeld naar

mogelijkheden om de productie van deze enzymen door de nematode, te blokkeren, lukt het hopelijk op niet al te lange termijn alternatieve "bestrijdingsmiddelen" te vinden waarmee de grote schade die deze kleine diertjes aanrichten is te verminderen. En dat komt weer ten goede aan de wereldvoedselproduktie.

Nawoord

Na een studie biologische gezondheidskunde in Maastricht vier jaar geleden, zit ik in de trein naar Wageningen op weg naar de vakgroep nematologie. Nematoden, zijn dat niet van die kleine "wormpjes"?

In een goed gesprek maken prof. dr. Jaap Bakker, dr. Arjen Schots en dr. Hans Helder me direct enthousiast om de secretie eiwitten van dit diertje te gaan ontrafelen.

De gedachte om "even" wat secretie eiwitten te vinden blijkt in de praktijk toch wel enkele jaren te omvatten, maar het eindeloze enthousiasme van Jaap voor zelfs de kleinste resultaten zijn zo aanstekelijk dat je blijft doorgaan. Ook de collegiale en vriendschappelijke sfeer op het lab dragen daaraan bij. Graag wil ik Rikus bedanken voor zijn altijd aanwezige hulp en steun als je iets zoekt of nodig hebt. De ervaringen die ik opgedaan heb zijn veelzijdig geweest en niet alleen wetenschappelijk. De eerste voordracht in Dundee is een hele belevenis evenals het onderzoeks overleg een jaar later in Italië waar ze je meteen proberen te beroven!

Het uitvoeren van een dissertatie is niet een prestatie die je alleen doet. Vele mensen dragen daaraan bij. Zonder mensen die ik niet noem tekort te willen doen, noem ik er enkele van, in wie anderen zijn vertegenwoordigd. Allereerst Jaap en Arjen voor het in stand houden van een goed gestructureerde en fijne vakgroep Nematologie en het Laboratorium voor Moleculaire herkenning en Antilichaam technologie. Geert bedankt voor je kritische en steeds opbouwende bijdragen aan het onderzoek en in het bijzonder de teksten van de artikelen en de dissertatie.

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Curriculum Vitae

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Herman Egbertus Popeijus, geboren op 26 december 1973 te Heerjansdam, studeerde in 1991 af aan het St. Joris college te Eindhoven. Na deze middelbare school studeerde hij Medische Microbiologie, een van de twee richtingen van het Hoger Technisch Laboratoriumonderwijs aan de Hogeschool Eindhoven. Na een stage bij de vakgroep Gezondheidsrisicoanalyse en toxicologie (Prof. dr. J.C.S. Kleinjans) rondde hij deze opleiding in 1995 af met het behalen van het diploma. Hierna werd de studie vervolgd met een studie Biologische Gezondheidskunde aan de Universiteit van Maastricht. Deze werd afgerond in 1997 met het behalen van de bul na een stage en afstudeerscriptie: "NSP-ruticulons" aan de vakgroep Molecular Cell Biology and Genetics (Prof. dr. F.C. Ramaekers). Direct volgend op de afronding van deze studie werd hij aangenomen als Assistent In Opleiding bij de vakgroep Nematologie (Prof. dr. ir. J. Bakker en dr. ir. G. Smant) en bij het Laboratorium voor Monoclonale Antilichamen van de Universiteit Wageningen (dr. ir. A. Schots). Aldaar werd vier jaar onderzoek verricht aan secretie eiwitten van de cyst nematode *Globodera rostochiensis*. De relevante resultaten voortvloeiend uit het verrichte onderzoek staan beschreven in dit proefschrift. Thans is hij verbonden als post-doc bij het departement Moleculaire celbiologie van het Leids Universitair Medisch Centrum. Hier verricht hij onderzoek naar de rol van AP-1 transcriptie factoren in de inhibitie van celproliferatie door gentoxische agentia gefinancierd door het Koningin Wilhelmina Fonds.

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