The role of cell wall-modifying proteins in plant penetration and feeding site proliferation by the potato cyst nematode *Globodera rostochiensis*

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Dit onderzoek is uitgevoerd binnen de onderzoekschool Experimental Plant Sciences

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Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. M.J. Kropff
in het openbaar te verdedigen
op dinsdag 17 mei 2006
des namiddags te 16.00 uur in de Aula

Kudla, U. (2006).

The role of cell wall-modifying proteins in plant penetration and feeding site proliferation by the potato cyst nematode *Globodera rostochiensis*

PhD Thesis Wageningen University, The Netherlands
with summaries in Polish and Dutch.

ISBN: 90-8504-414-6

Dla H.H.H.H.

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Chapter I

General introduction

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1. Parasitism as a way of life

1.1 Parasitism

Parasitism is the most harmful form of symbiosis, which is a general term describing situation where two dissimilar organisms live together in close physical association (*OED2* 1998). The classical definition of parasitism states that it is an intimate relationship between two organisms in which one (the parasite) lives on, off or at the expense of the other (the host) (Henderson 1997). However, in many cases, there are no sharp boundaries between parasitism and other forms of symbiosis like commensalism, or even mutualism. For example, rats infected with the intermediate stages of a tapeworm (*Spirometra spp.*) grow larger than uninfected rats. The tapeworm larva produces an analogue of the vertebrate growth hormone, which causes the rat to grow. It is obviously debatable if the growth boost is harming the host or if it is beneficial (Salem and Phares 1986). The concept of parasitism is continuously being developed, and new more specific definitions are being added such as 1) the parasite is metabolically or physiologically dependent on the host and 2) there is a genetic complementation between the parasite and its host in which the parasite has lost the genetic ability to make certain vital metabolic intermediates (Crofton 1971). In extreme cases, part of the parasite's genome may have been lost and become integrated into that of the host (Teixeira et al. 1994).

1.2 Adaptation to parasitism as a result of co-evolution with host

All adaptations of parasites are responses to specific features of the host. Therefore, any change of the host requires adequate response from parasites. On the other hand, as the parasite evolves to be better adjusted to a particular host and to be more efficient in avoiding host's defences, the host species is also evolving to counteract those changes in parasite. In this dynamic equilibrium there is a very strong selective pressure on both counterparts, as the anti-parasitic adaptations in the host as well as adaptations in the parasite, are in constant obsolescence. An intriguing hypothesis referring to the evolutionary pressure on the host is the so called Red Queen theory (Van Valen 1973) based on observations on Alice by the Red Queen in Lewis Carroll's "Through the Looking Glass" that "in this place it takes all the running you can do, to keep in the same place".

2. Parasitism in the phylum Nematoda

2.1 Nematodes

Nematodes are the most numerous multicellular animals on earth, outnumbering even insects. The estimated number of species in the phylum Nematoda varies from 40 000 to 100 million (Dorris et al. 1999; Lambshead 1993). The wide range in estimations is a result of a lack of morphological diversity of these round, unsegmented worms. The apparently successful morphological design of nematodes has not changed much throughout millions of years. The nematodes preserved in amber, which are estimated to be 120 – 135 million years old, are much like the contemporary members of this phylum (Poinar 2002). Despite their very similar body design, the genetic diversity among nematodes is high, which is reflected in their physiological versatility. Various physiological adaptations allowed them to inhabit virtually every conceivable environment, from the bottoms of the oceans to the frozen desserts of Antarctic (Ditlevsen 1926; Platonova and Gal'tsova 1976). Apart from many free-living organisms such as saprophytic or predatory nematodes, there is also a large number of parasitic species (approximately 25%) living on plants and animals (Poulin and Morand 2000). Some of the parasitic species have negligible effects on host survival while others are lethal. It is estimated, that approximately one quarter of the world's human population suffers from various nematode infections (Anderson 1992). Approximately, 10% of the nematodes are plant parasites. Plant parasitic nematodes are one of the world's major agricultural pests, causing an estimated $80 billion worldwide crop loss annually (Sasser and Freckman 1987).

2.2 Reconstruction of evolution

There is only scarce fossil evidence of nematode evolution due to the lack of hard structures in their bodies. Exceptional findings such as a mermithid nematode emerging from a male ant petrified in the amber (Poinar 2002) are not sufficient to unravel the evolutionary framework of the phylum. For a long time, phylogenetic analysis of the phylum was based on morphological traits. However, the minute size, number of species and the lack of morphological diversity were hindering the classification. The recent development of molecular markers allows much more detailed and incisive study of evolutionary relationships in the phylum. For the analysis of early events in the evolution like emerging of orders and families, slowly evolving genes such as cytochrome C (Vanfleteren et al. 1994), globin (Blaxter et al. 1994), RNA polymarase II (Baldwin et al. 1997), and heat shock protein 70 (Snutch and Baillie 1984) are used. Whereas, mitochondrial (Hyman and Slater 1990) and ribosomal spacer genes (Aleshin et al. 1998; Blaxter et al. 1998; Dorris et al. 1999) are more informative when it comes to the analysis within genus or species.

2.3 Parasitism in the evolutionary framework of Nematoda

Based on the small subunit ribosomal DNA sequence analysis the phylum was divided into seven clades: I, II and C&S (Chromadorida and Secernentea), III, IVa, IVb and V (Blaxter et al. 1998; Holterman et al. 2005). It has been hypothesized that all animal or plant parasitic nematodes are closely related and evolved from one common free-living ancestor (Lorenzen 1994). Based on the SSU rDNA analysis, animal parasitism is present in clades I, III, IVa, IVb and V and seems to have evolved independently at least six times (Dorris, De Ley, and Blaxter 1999). Whereas plant parasitic nematodes are located in clades: I (order Dorylaimida), II (order Triplonchida) and IVb (orders Tylenchida and Aphelenchida), indicating that plant parasitism may have evolved on three independent occasions.

2.4 Origins of plant parasitism in Nematoda

The fact that plant and animal parasitic nematodes are positioned in separate clades suggests that there may be different scenarios through which parasitism have evolved. Considering possible different interactions between two organisms, which have resulted in evolution of parasitism, one can envisage that animal parasitism among nematodes have evolved from phoretic ancestors that were attaching themselves to larger animals for a limited time to disperse into new areas (Anderson 1984; Athias-Binche and Morand 1993). Alternatively, parasitic ancestors could have been lumenal dwellers that fed on intestinal microflora (Anderson 1984), or like *Rhabditis strongyloides* could invade small mammals to stay within awaiting their death to feed on bacteria in the decaying carcass. Plant parasitism may have originated from endo-symbiosis of free-living nematodes with bacteria (Davis et al. 2000), or bacteriovorous, fungivorous nematodes dwelling on the plant's surface or in their vicinity. Although the precise mechanism of parasitism evolution will almost certainly be different for various groups of nematodes the underlying principles, like the necessity of the close contact between the two adversaries, will probably be the same.

3. Plant parasitic nematode: potato cyst nematode

3.1 Plant parasitic nematodes' feeding strategies

Plant parasitic nematodes have evolved various feeding strategies, which can be divided into migratory or sedentary, based on the length of contact between nematode and a host. The first group includes migratory ectoparasitic and endoparasitic nematodes. The duration of their contact with the host ranges from several seconds to several days. After this time, the nematode will move to another part of the root or even another plant. The second group is formed by sedentary ectoparasties (e.g. the

ring nematode *Criconemella xenoplax*) and endoparasitic nematodes, which have developed complex relationships with their hosts and complete the whole life cycle by extracting nutrients solely from a fixed feeding site inside the host plant root (Sijmons et al 1994).

3.2 Globodera rostochiensis - host range and history

Globodera rostochiensis - potato cyst nematode (family *Heteroderidae*, order Tylenchida) is a sedentary endoparasite, positioned in the clade IVb. Potato cyst nematodes have limited host range including Solanaceous plants, such as potato, tomato, and eggplant (Evans and Stone 1977). Potato cyst nematode was introduced in Europe probably in the 19th century together with potato breeding material from Andes in South America. Since then it has become a major pest of potato, especially in parts of Europe with temperate climate.

3.3 The life cycle

The name "cyst nematode" derives from the swollen body of an adult female, which after the female dies forms tough protective container for several hundreds of fertilized eggs. Inside a cyst, eggs may remain viable for as long as 15 years (Dropkin 1989). Juveniles molt once inside the eggs and remain dormant until they are exposed to exudates of a host plant. Upon this exposure, larvae hatch by cutting a slit in the eggshell, and migrate toward the roots of a host plant. Subsequently, they penetrate the root and move through the cortex towards the stele. There they select a single cell that becomes a starting point of a feeding site called syncytium formed by a group of cells in which cytoplasmic continuity is maintained (Dropkin 1989; Jones 1981). The syncytium is the only source of nutrients throughout the nematode's life. The quality of feeding site, its size, protein content and amino acids composition determines the sex of nematode (Grundler et al. 1991). While feeding, the body wall muscles degenerate and the nematodes become sedentary. The second stage juveniles molt three times until they reach adulthood. The females swell extensively while plant tissue surrounding them disintegrates exposing the posterior end of the nematode on the surface of the root. The males become slender again and regain their motility in time to mate with females at the surface of the root. After fertilization, females die and their bodies turn into cysts that remain in soil.

3.4 Penetration of roots

Two main events in the potato cyst nematode's life cycle are the direct reason for their pathological effect on the infested crops: 1) the physical damage inflicted during penetration of and migration through the root, 2) induction and maintenance of

syncytium that allows nutrient extraction from a plant. Freshly hatched second stage juveniles exposed to the root diffusate of a host show intensified stylet activity. Using this hollow extensible spear the nematode probes the selected area of the epidermal cells of the root. At the same time nematodes enhance the effect of the stylet by producing and releasing cell wall degrading enzymes (Smant et al. 1998). It takes approximately 20 minutes of intensive stylet thrusting to penetrate the first cell wall (Steinbach 1972). After approximately one hour of repeated stylet penetration the slit created in the cell wall is sufficiently large for the juvenile to wedge its head through it. It has been observed that *G. rostochiensis* juveniles preferentially invade the root in the differentiation zone (Steinbach 1972), while other closely related cyst nematodes like *Heterodera schachtii* (Wyss and Zunke 1986), *H. cruciferae* (Doncaster and Seymour 1973) or *H. glycines* (Atkinson and Harris 1989) prefer the root elongation zone or the base of emerging lateral roots. Once inside the root the juvenile migrates intracellularly using the intensive stylet thrusting and enzymatic softening to penetrate the cell walls. The nematode's way through the cortical cells toward the vascular cylinder is marked by necrosis of the cells aligning the track. Upon arrival in the inner cortex the nematode starts gently probing the neighbouring cells in search for an appropriate initial feeding cell. However, the exact criteria upon which the nematode decides to select particular cell remain unclear.

3.5 Induction and maintenance of syncytium

Once the initial cell of syncytium has been selected, the nematode becomes immobile. The nematode inserts the stylet into the cell cytoplasm for several hours, this is called the preparation period. The fully differentiated cortex cell changes into a metabolically highly active cell with dense cytoplasm that contains small secondary vacuoles, numerous organelles and enlarged nucleus with prominent nucleoli (Rice et al. 1985). Only after this preparation period, the nematode starts to feed while the initial syncytial cell expands towards the vascular bundle through the progressive local cell wall dissolution. To breakdown cell walls during formation of the syncytium, nematode recruits the plant enzymes. Cells are fused to the initial feeding cell and become similar in their structure. This multinucleate syncytium extends alongside the root axis in the vicinity of xylem vessels. During the course of a week the syncytium becomes increasingly hypertrophied and acts as metabolic sink from which nematode uptakes large amounts of nutrients.

4. Nematode secretions

4.1 Oesophageal glands

Compared to bacteriovorous nematodes plant parasitic nematodes have relatively large oesophageal glands. Each gland is a large single secretory cell with a wider basal

region and a long narrow extension at the apical region, which terminates in an ampullae. The valve of a single dorsal gland is located near the base of the stylet whereas the two subventral glands empty into the oesophagus just posterior to the metacorporal pump chamber (Hussey and Mims 1991). The products of oesophageal glands can be released into the environment through the hollow stylet. The volume, shape and metabolical activity of the gland changes during the course of time. The two subventral glands are at the peak of their activity prior to host invasion. The onset of the parasitism is marked by a reduction in size and activity of subventral oesophageal glands and an increased activity of the dorsal oesophageal gland. This correlation between oesophageal gland activity and key events in the parasitic interactions with a host suggests that gland secretory proteins play different roles during the two stages of parasitism.

4.2 Oesophageal glands proteins

4.2.1 Proteins produced in the subventral oesophageal glands

Not only the temporal activation pattern but also the nature of proteins produced by the two subventral oesophageal glands, advocates their involvement in nematode penetration and migration through the root tissue (Hussey and Mims 1990). Most of the identified subventral oesophageal gland proteins can be classified as cell wall modifying proteins that enable the migrating nematode to degrade all major types of polysaccharides in the cell wall of a host plant.

The first parasitism genes cloned from plant parasitic nematodes were β-1,4 –endoglucanases (cellulases) expressed in the subventral oesophageal glands of *Globodera rostochiensis* and *Heterodera glycines* (Smant et al. 1998). Cloning of these endoglucanases provided the first evidence that animals are capable of producing cell wall degrading enzymes. *G. rostochiensis* is producing four different cellulases, whereas *H. glycines* was reported to produce even six different enzymes (Gao et al. 2004). These cellulases belong to family 5 of β-1,4 –endoglucanases (E.C. number 3.2.1.4) and represent two main categories: with and without cellulose binding domain. On the other hand, *Meloidogyne* species are producing only one type of cellulase, which also belongs to family 5 and is composed of catalytic domain and cellulose binding domain (Rosso et al. 1999). This difference in the repertoire of cellulases produced by cyst nematodes and root knot nematodes may reflect the different ways of their migration. Cyst nematodes migrate intracellularly, whereas the root knot nematodes migrate intercellularly through the middle lamellae that form the interface between neighbouring cells and contain predominantly pectic polysaccharides. To successfully depolymerise this type of cell wall polymers, root knot nematodes are equipped with pectate lyases (Doyle and Lambert 2002; Huang et al. 2003; Rosso et al. 1999) and polygalacturonase (Jaubert et al. 2002). Cyst

nematodes are also producing pectate lyases (de Boer et al. 2002; Gao et al. 2001; Popeijus et al. 2002), however, there are so far no reports of polygalacturonase. In addition to cellulose and pectin degrading enzymes, plant parasitic nematodes are also equipped with endoxylanase (Dautova et al. 2001) and chitinase, the latter one is however probably related to hatching (Gao et al. 2002). Furthermore, cell wall binding proteins produced in the subventral oesophageal glands of various plant parasitic nematodes (Ding et al. 1998; Gao et al. 2003) may also be indirectly involved in the cell wall degradation.

Apart from cell wall degrading enzymes, plant parasitic nematodes also produce in their subventral glands a number of proteins with more elusive role in parasitism. Several extracellular proteins expressed in subventral oesophageal glands had strong similarity to secretory venom allergen AG5-like produced by hymenopteran insects (Ding et al. 2000; Gao et al. 2001). Their potential function in parasitism remains obscure.

Chorismate mutase gene, expressed both in subventral and dorsal glands, was identified in cyst nematodes (Bekal et al. 2003; Jones et al. 2003) and root knot nematodes (Lambert et al. 1999). Chorismate mutase is an enzyme in the shikimate pathway, an important metabolic route in plants. It has been shown that over expression of chorizmate mutase of nematode origin in plant suppresses production of salicylic acid and phenolic phytoalexins. Therefore this protein may be involved in reducing the plant defence response to the invading nematode (Doyle and Lambert 2003).

Another interesting protein expressed in subventral oesophageal glands of root knot nematode is the calcium-binding protein – calreticulin (Jaubert et al. 2002). Calreticulin has multiple functions from regulation of cell cycle in animals to cell-to-cell trafficking and pressure support in plants.

Parasitism genes encoding novel proteins are possibly a very interesting group. There are seven pioneering sequences expressed in the subventral glands of root knot nematode (Huang et al. 2003), four in subventral glands *of H. glycines* (Gao et al. 2003) and three in subventral glands of *G. rostochiensis* (Qin et al. 2000). The pioneering sequences were identified in comprehensive analysis of a parasitome, using gland cell-specific libraries (Gao et al. 2003) and cDNA-AFLP (Qin et al. 2000) and still need to be cloned. These genes, without a doubt, create the biggest challenge for functional analysis and give us a glimpse how complex and unique the nematode-plant relationship is.

4.2.2 Proteins produced in the dorsal oesophageal gland

The dorsal oesophageal gland is activated at the onset of parasitism during feeding site initiation, therefore the dorsal gland proteins are thought to be responsible for induction and maintenance of the syncytium. Removal of the nematode from an

infected plant at any point in time results in degeneration of the feeding cells indicating the necessity of constant stimuli from the nematode to maintain the syncytium. On the other hand, if the feeding site for whatever reason degenerates, the nematode dies, as at this stage it lost its ability to induce new feeding site. The process of feeding by the nematode can be divided into three succeeding phases that form a recurring pattern. In the first phase the nutrients are ingested, during the second phase the stylet is retracted and reinserted, once the stylet is reinserted the nematode starts the third phase – salivation, during which the dorsal gland secretions are presumably injected into the plant cell providing stimuli for development and maintenance of the syncytium (Wyss 1992).

Little is still known about the mechanism that allows nematodes to manipulate the host cell metabolism to nematode's own benefit. The secretions from dorsal oesophageal gland may directly or indirectly alter the gene expression in the recipient cells, either by binding to the plant cell receptors in order to elicit the required signal transduction pathways, or by entering the nucleus and direct modification of the gene expression. The latter seems to be confirmed by the presence of nuclear localization sequence at the N terminal part of some of the dorsal secretions (Huang et al. 2003).

One of the hallmarks of syncytial cells is the endoreduplication of the DNA through the repeated S-phase of a cell cycle without subsequent cellular or nuclear mitotic division. Indeed, nematode secretions were shown to induce cell proliferation and this activity was attributed to the presence of mitogenic peptide(s). These mitogenic peptide(s) might stimulate the reactivation of a cell cycle in syncytial cells (Goverse et al. 1999). On the other hand, recently cloned RanBPM like proteins expressed in dorsal oesophageal gland may be responsible for plant microtubule stabilization in the syncytium and therefore for shunting of the M-phase (Qin 2001). Simultaneous secretion of mitogenic peptide(s) and RanBPM like proteins could consequently result in endoreduplication of the genome.

There is also some evidence that plant parasitic nematodes could interfere with plant peptide signalling, which is involved in a variety of physiological processes. A domain search revealed that *H. glycines* is producing a protein similar to CLAVATA3 plant signal peptide, which is involved in differentiation of stem cells in shoot meristems (Davis et al. 2004; Olsen and Skriver 2003). Short peptides that might act as signalling molecules have been also identified in *M. incognita* (Huang et al. 2003) and *G. rostochiensis* (Goverse et al. 1999; Qin 2001; Qin et al. 2000), however only CLAVATA3 like protein has been cloned so far.

An ubiquitin extension protein was identified by comparing the gene expression profile between anterior and posterior part of *H. glycines* (Tytgat et al. 2004). The authors speculate that it plays a regulatory role in feeding cell formation and is possibly involved in protein turnover in highly active cells. In addition, RING-H2 zinc finger protein identified in *H. glycines* (Gao et al. 2003) was shown to facilitate transfer of ubiquitin in plants to target proteins destined for degradation (Estelle et al.

2001). Finally, SKP1 – like protein produced by *H. glycines* and possibly targeted to the nucleus of a plant cell, seems to add to the spectrum of secretions regulating feeding site development by selective degradation of host proteins (Gao et al. 2003). Both RING-H2 zinc finger protein and SKP1-like protein still need to be cloned.
The largest subset of genes expressed in dorsal gland of root knot nematodes as well as cyst nematodes does not have significant similarity to any of the reported genes. Thirty seven pioneering sequences are expressed in dorsal gland of *H. glycines* (Gao et al. 2003), sixteen in dorsal gland of *M. incognita* (Huang et al. 2003) and eight in *G. rostochiensis* (Qin et al. 2000). This large numbers suggests the complexity of the mechanism employed by plant parasitic nematodes in initiation and maintenance of feeding site.

4.3 Functional analysis

A considerable number of genes with apparent function (e.g. cell wall modifying proteins), enabling the nematodes to parasitize the plant is already identified. However, a substantial part of gene set potentially involved in parasitism is encoding novel proteins that have no homology with functionally annotated genes. This could simply mean that their homologs are not identified yet, or they could represent a very unique set of genes that has evolved only in plant parasitic nematodes. The pioneering genes pose a big challenge when it comes to their functional analysis. Several molecular techniques could be utilized to elucidate the role of such genes in plant nematode interaction. The RNA-mediated gene silencing is currently under scrutiny and there are some promising results indicating that indeed this method is applicable to plant parasitic nematodes (Jones 2003; Urwin et al. 2002). Alternatively, over-expression of nematode genes in the whole plant (Goverse and Karczmarek 1999) or transient expression in plant protoplast (Qin 1999) may be used to asses the changes in host in response to nematode protein accumulation. To study the interaction between parasite and a host on the protein level a two yeast hybrid system can be employed (Fields and Song 1989). Regardless of the method, understanding how the parasitism factors interact with each other and with host molecules to lead to the successful parasitism is a major challenge for the upcoming years.

5. Nematode responsive plant genes

The plant genes either up or down regulated during formation of the nematode feeding site, can be divided into several classes related to certain metabolical and developmental pathways (Gheysen and Fenoll 2002). There is a set of wound and defence responses genes, the expression of which is likely being triggered by the damage to the plant tissue afflicted by the invading nematode. Most of those genes are induced both in compatible and incompatible interaction between nematode and the

host plant and include peroxidase, chitinase, lipoxygenase, proteinase inhibitor, enzymes involved in phytoalexins' biosynthesis and deposition of callose or lignin (Gheysen and Fenoll 2002).
More than 50 genes involved in cell cycle regulation were shown to be induced in early stages of feeding site development (Gheysen and Fenoll 2002; Goverse et al. 2000). Surprisingly, despite major differences between formation of syncytia (i.e. endoreduplication) and giant cells (i.e. acytokinetic mitosis) there is no clear difference in the expression patterns of cell cycle related genes between both forms of feeding site (Gheysen and Fenoll 2002).
Genes involved in general metabolism, stress-response and water status of the plant cell are often up regulated, assumingly to keep up with a high metabolic activity within the feeding site (Gheysen and Fenoll 2002; Potenza et al. 2001). Also a set of transcription factors is being induced (Barthels et al. 1997; Gheysen and Fenoll 2002) whereas others are down regulated (Van Poucke et al. 2001).
The link between plant hormones and nematode parasitism of plants is well established (Bird and Koltai 2000). Ethylene overproducing plants attract more invasive larvae of the cyst nematodes and the developed syncytia are larger with increased number of females comparing to control plants (Goverse et al. 2000; Wubben et al. 2001). *G. rostochiensis* is unable to develop on auxin-insensitive tomato mutant *dgt* (Goverse et al. 2000) and *Heterodera schachtii* shows similar difficulties in the parasitism on *Arabidopsis* mutants with an altered auxin household. These observations point at a major role of auxin in feeding cell formation. Plant hormone associated genes (auxin-regulated cell cycle genes) are among the earliest genes induced during the formation of syncytium (Niebel et al. 1996). Finally some of the cell wall proteins such as cellulases (Goellner et al. 2001), polygalacturonase (Mahalingam et al. 1999) and pectinacetylesterases (Vercauteren et al. 2002) are also up regulated and are likely responsible for progressive cell wall dissolution that allows feeding site to expand.

6. Cell wall modifications in nematode parasitism

Plant cell wall modifications in the course of infection of a host by nematodes occur during the two main phases of parasitism – the migration and the establishment and maintenance of a feeding site. First, to facilitate migration plant parasitic nematodes secrete a cocktail of cell wall degrading enzymes to depolymerise essentially all major structural cell wall polymers. Typically the intracellularly migrating cyst nematodes cause extensive necrosis of plant cells while penetrating the apoplast as well as the symplast of successive layers of cells (Golinowski et al. 1996), whereas intercellular migration of root knot nematodes within the apoplast causes very little damage to the host tissue (Wyss et all. 1992). The composition of enzymes in the cell wall degrading cocktail secreted by the nematodes seems to differ depending on the type of migration.

Middle lamella rich in pectic polymers require presence of different pectinolitic enzymes i.e. polygalacturonase (Jaubert et al. 2002), pectate lyase (Doyle and Lambert 2002), whereas the intracellular migration through primary cell walls calls for more diverse cellulose degrading enzymes (e.g. cellulases in *H. glycines*) (Gao et al. 2003).
The nematode induces more controlled changes in the cell walls during the formation of feeding sites. These modifications are more likely triggered by manipulation of plant proteins at transcriptional or even translational level rather than directly by nematode secretions. The complex pattern of cell wall modification in the developing syncytium and giant cells includes cell wall dissolution, cell expansion, elongation and biosynthesis (Bird and Koltai 2000; Grundler et al. 1998). During formation of a syncytium, partial cell wall dissolution results in fusion of protoplasts of up to two hundred cells within the stele along the xylem vessels. The syncytial cell walls adjoining xylem elements develop elaborate finger-like ingrowths. These changes in cell wall architecture start by deposition of cell wall material around the stylet tip in the initial syncytial cell and in the neighbouring cells. The first openings in the cell walls are formed by gradual widening of the plasmodesmata, which results in fusion of protoplasts. Gradually the outer syncytial cells slightly thicken and the openings are formed without the involvement of plasmodesmata. The cell walls are usually degraded simultaneously from both sides in adjoining cells (Grundler et al. 1998). This pattern of cell wall modifications suggests predominant role for cell wall degrading enzymes (Golinowski et al. 1996). Contrary to syncytium induction, which develops from single cell, formation of giant cells starts synchronically in five to seven procambial cells adjacent to the head of the root knot nematode. These cells have thickened cell walls, which develop extensive ingrowths similar to that present in syncytium. In both syncytium and giant cells, cell wall ingrowths are possibly involved in increasing of a solute uptake from the vascular system (Jones 1981). Syncytia as well as giant cells formed in very different host species are remarkably similar suggesting that nematodes interfere with the fundamental processes involved in cell development and differentiation.

7. Outline of the thesis

In this thesis, we undertake an incisive analysis of cell wall modifying proteins involved in plant parasitism of *Globodera rostochiensis*. The study includes the role of cell wall modifying proteins of nematode as well as of plant origin.
The second chapter describes the cloning and the characterization of a novel pectate lyase produced by *Globodera rostochiensis*. It also proposes a novel approach to study the effects that cell wall modifying enzymes have on plant tissue *in vivo*. This approach combines three-dimensional modelling, site directed mutagenesis and agro-infiltration in leaves.

The third chapter presents evidence for the first expansin produced outside of a plant kingdom. The primary goal was to confirm that this protein is secreted by the invading nematode and that it has functional characteristic of plant β-expansins.
The fourth chapter provides detailed information about the cloning, the localization and the activity of β-expansin produced by *G. rostochiensis*. Moreover, it presents a three-dimensional model of Gr-EXPB1 and confirms its eukaryotic origin.
Chapter five shows evidence that *Globodera rostochiensis* is also able to manipulate the β–expansins and expansin-like proteins of plant origin, for the cell wall modifications in syncytium formation. This is the first report that links morphological changes in the cell walls of dicotes plants to the expression of β- expansin genes.
Finally, in chapter six we summarize the results and discuss the role of pectinolitic enzymes and expansins in parasitism of *Globodera rostochiensis*.

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Chapter II

Structural and functional characterization of a novel, host penetration-related pectate lyase from the potato cyst nematode *Globodera rostochiensis*

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Manuscript in preparation

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Abstract

The cell wall, a strong extraprotoplasmic layer surrounding plant cells that mainly consists of a variety of polysaccharides, constitutes a major barrier for potential parasites. Plant parasitic nematodes are well equipped to overcome this barrier as they produce and secrete cell wall degrading and modifying enzymes. Expression profiling of various life stages of the potato cyst nematode *Globodera rostochiensis* revealed a pectate lyase (*Gr-Pel2*, 759 bp) that shows only remote relatedness to *Gr-Pel1*. A profound effect on plant morphology of the recombinant protein was shown by an *Agrobacterium*-mediated leaf infiltration assay. Gr-PEL2 showed highest similarity to pectate lyases from two soil-inhabiting saprophytic *Streptomyces* species (Actinobacteria) and from two species of the facultative plant parasitic nematode *Bursaphelenchus*. Ca^{2+} is essential for pectate lyases activity, and the Gr-PEL2 calcium binding site was identified by a combination of homology modeling of the 3D structure and site directed mutagenesis. 3D modeling pointed at a highly charged catalytic cleft in Gr-PEL2, whereas its counterpart in Gr-PEL1 was significantly more hydrophobic. This result points at different substrates preferences. Our results underline the broadness of the spectrum of cell wall modifying proteins that is needed for successful plant parasitic lifestyle and it underpins the hypothesis that at least part of the cell wall modifying proteins in nematodes might have an actinobacterial origin.

Keywords: Plant-nematode interactions, cell wall degradation, homology modeling, cDNA-AFLP, infiltration, site directed mutagenesis

1. Introduction

Sedentary plant-parasitic nematodes are estimated to be responsible for nearly one third of the overall damage to crops caused by parasites and diseases (Whitehead 1998). Potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*) are obligatory parasites of a small range of S*olanaceous* plants including economically relevant crops such as potato, tomato and eggplant. Contrary to majority of other nematode species, the life cycle of *G. rostochiensis* involves only two migratory developmental stages, namely the pre-parasitic second stage juvenile (J2) and the adult male. The

pre-parasitic J2 penetrates the plant root at the elongation or differentiation zone (Steinbach 1972) and migrates intracellularly through the cortex in search for appropriate place to induce a feeding site. During migration, the nematode weakens the plant cell wall by mechanical thrusts of its stylet. A stylet is a hollow spear with which the nematode pierces cell walls in a highly coordinated manner. The stylet is also used to release secretions from the esophageal glands (Steinbach 1972; Wang et al. 1999), and to take up cytoplasmic contents from plant cells. The potato cyst nematode has three single-celled esophageal glands, two subventral ones and a single dorsal gland in a more anterior position. The subventral glands are active during intracellular migration, whereas the dorsal gland is most active from the moment that the infective juvenile has selected an initial feeding cell onwards. The content of the feeding site - a nematode-induced conglomerate of fused and redifferentiated plant cells - is the sole food source of the nematode.

For penetration and migration in the root, the nematode has to overcome a major physical barrier, namely the plant cell wall. The plant cell wall consists of a network of polysaccharides, proteins and aromatic compounds. In current models of the plant cell wall, cellulose and cross-linking glycans are embedded in the matrix of pectins (Carpita and McCann 2000). The backbone of pectic polysaccharides consists of blocks of α-1,4 linked polygalacturonic acid residues ("smooth regions") interspersed with regions of alternating galacturonic acid and rhamnose residues ("hairy regions"), rhamnose residues might be decorated with short galactans and arabinans (Willats et al. 2001). Due to the complexity of this highly branched polysaccharide, pectin degradation requires a variety of enzymes; usually acetyl- and methylesterases (*e.g.* pectinesterase) de-esterify pectin to make it more accessible to lytic enzymes such as pectin lyase, pectate disaccharide-lyase, and pectate lyase. Pectate lyases cleave the glycosidic bond between α-1,4-polygalacturonic acid units in demethylated or low methylesterified homogalacturonan (pectate) via a β-elimination reaction. Plants use mixtures of pectolytic enzymes in processes such as seed germination and fruit ripening, whereas a number of plant pathogenic microorganisms secrete such mixtures for host plant invasion.

The relatively important role of pectate lyases in plant pathogenicity can be illustrated by *Nectria hematococca* - a fungal pathogen of pea plants - and *Erwinia chrysanthemi,* a bacterium that causes soft rot in a wide variety of crop plants. Disruption of *pelA* and *pelD* drastically reduced virulence of the fungus (Rogers et al. 2000). In case of the host-promiscuous bacterium *E. chrysanthemi*, the contribution of each of the at least eight different pectate lyases to bacterial virulence was shown to be host plant dependent (Beaulieu, Boccara, and Van Gijsegem 1993; Hugouvieux-Cotte-Pattat et al. 1996)

Only recently, Popeijus *et al.* (2000) showed that a potato cyst nematode produces and secretes a pectate lyase during plant invasion (Popeijus et al. 2000). Subsequently, homologs were found in other, related endoparasitic nematodes such as the soybean

cyst nematode *Heterodera glycines* (de Boer et al. 2002), and the root knot nematodes *Meloidogyne incognita* (Huang et al. 2005) and *M. javanica* (Doyle and Lambert 2002). Here, we present a novel pectate lyase, Gr-PEL2 from the potato cyst nematode *G. rostochiensis* that (1) shows only remote relatedness to Gr-PEL1, (2) is - based on the charge distribution within the catalytic cleft - predicted to have a different substrate specificity as compared to Gr-PEL1, and (3) shows closest homology to actinobacterial and nematode pectate lyases.

2. RESULTS

2.1 cDNA-AFLP and the full length cDNA of *Gr-Pel2*

Comparison of gene expression patterns of five distinct developmental stages of *G. rostochiensis* using cDNA-AFLP with the enzyme combination of *Kas*I and *Taq*I resulted in 53 transcript-derived fragments (TDF) solely or predominantly expressed in potato root diffusate-exposed J2s (H-stage) and water re-hydrated J2s (S-stage). One of these TDFs (387 nucleotides, primer extensions: K+T and T+AA), which was strongly and specifically up-regulated in pre-parasitic juveniles exposed to potato root diffusate (Fig. 1), was excised from gel, cloned and sequenced. RACE was performed to clone the 5' and 3' ends of this TDF, and the complete open reading frame spanned 759 bp, coding for 252 amino acids. At its N-terminal end the encoded protein was predicted to have a signal peptide for secretion encompassing 20 amino acid residues (IFA ↓ NW). The molecular mass of the mature protein was 24.7 kDa and its isoelectric point was calculated to be 8.36. Protein motif searches identified putative phosphorylation and myristylation sites, but no glycosylation site was found [data not shown].

2.2 Tissue localization of *Gr-Pel2* transcripts

To determine the localization of the *Gr-Pel2* transcript in the pre-parasitic J2s, an antisense DNA probe was synthesized using the corresponding TDF. The probe specifically labeled the posterior ends of two subventral esophageal gland cells, whereas no signal was detected in the extensions and the ampullae of the glands (Fig. 2A). No hybridization signal was observed in sections with the corresponding sense probe (Fig. 2B).

2.3 Sequence analysis

The predicted protein sequence of GR-PEL2 was compared with protein sequences from Genbank using the BLASTP program at NCBI (Altschul et al. 1997). The amino acid sequence revealed significant similarities (*E*-values between e^{-49} and e^{-8}, identity

between 44% and 29%; similarity between 57% and 43%) with members of the polysaccharide lyase family three (PL3) (Shevchik, Robert-Baudouy, and Hugouvieux-Cotte-Pattat 1997). The PL3 family is a rather diverse group that includes proteins from bacteria and fungi, and also all known nematode pectate lyases. Gr-PEL2 shares the highest similarity (E-values < e^{-40}) with three putatively secreted pectates lyases from the facultative plant parasitic nematodes *Bursaphelenchus mucronatus* (Genbank accessions BAE48374 and BAE48374; E-value $6e^{-49}$ and $2e^{-44}$) and *B. xylophilus* (BAE48372; E-value $1e^{-43}$), and with pectate lyases from *Streptomyces avermitilis* (BAC74094; $2e^{-46}$) and *S. coelicolor* (CAC13062; $3e^{-43}$).
Site-directed mutagenesis of the Pel-15 gene from a *Bacillus* sp. (a PL3 family member) resulted in the identification of seven amino acid residues involved in the catalytic activity and/or calcium binding (Hatada et al. 2000). Interestingly, Gr-PEL2 contains all seven amino acids (E_{62}, D_{87}, E_{107}, D_{135}, K_{136}, K_{158}, R_{161} – numbers refer to amino acids positions in the precursor Gr-PEL2), whereas in Gr-PEL1 only 3 of these putative catalytic residues could be identified. Another typical feature of family PL3 is the presence of four conserved cysteine residues (C_{91}, C_{95}, C_{163}, C_{165} - numbers refer to position in precursor Gr-PEL2), these residues are also present in Gr-PEL1.

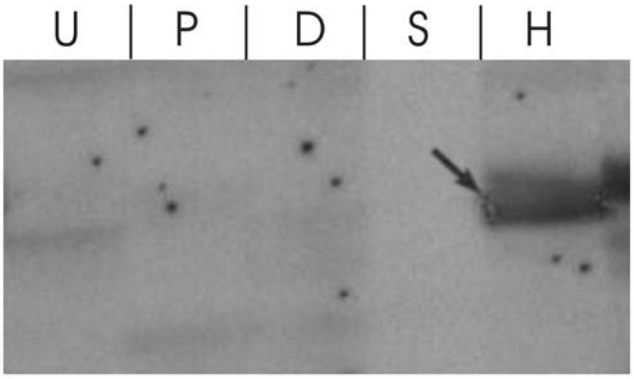
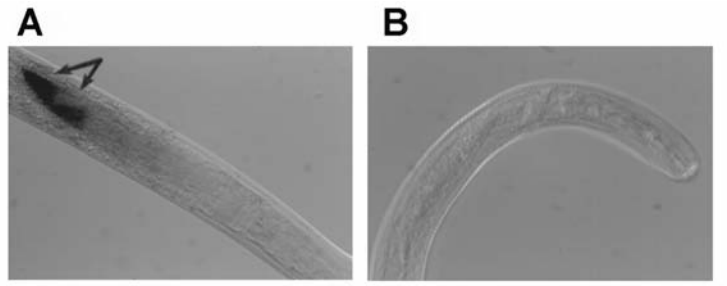


Fig. 1 Expression pattern of *Gr-Pel2* from the potato cyst nematode *G. rostochiensis* as displayed by cDNA-AFLP. Developmental stages are indicated by capitals: U - developing nematodes (J1) in gravid females two months post-inoculation; P - developing nematodes (J2) in gravid females three months post-inoculation; D - unhatched J2 in diapause; S - unhatched J2 after diapause, rehydrated for 2 days in water; H - freshly hatched J2 in potato root diffusate. The arrow-pointed band was cloned and sequenced.

Fig. 2 Tissue localization of *Gr-Pel2* transcript – in situ hybridization using TDF-dervied probe on preparasitic J2. A: labeling pattern using *Gr-Pel2* antisense probe, B: sense probe labeling. Arrows point at the subventral glands. Scale bar = 20 µm



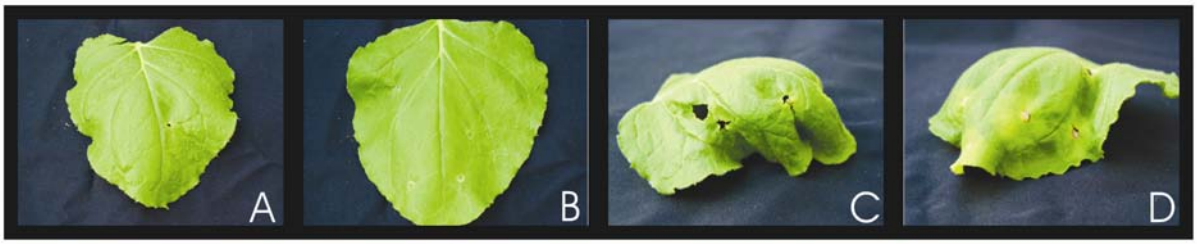


Fig. 3 *Nicotiana benthamiana* leaves infiltrated with *A. tumefaciens* carrying: A: empty vector, B: Gr-PEL2 no SP; C: Gr-PEL2 + SP young tobacco leaf, D: Gr-PEL2 + SP old tobacco leaf

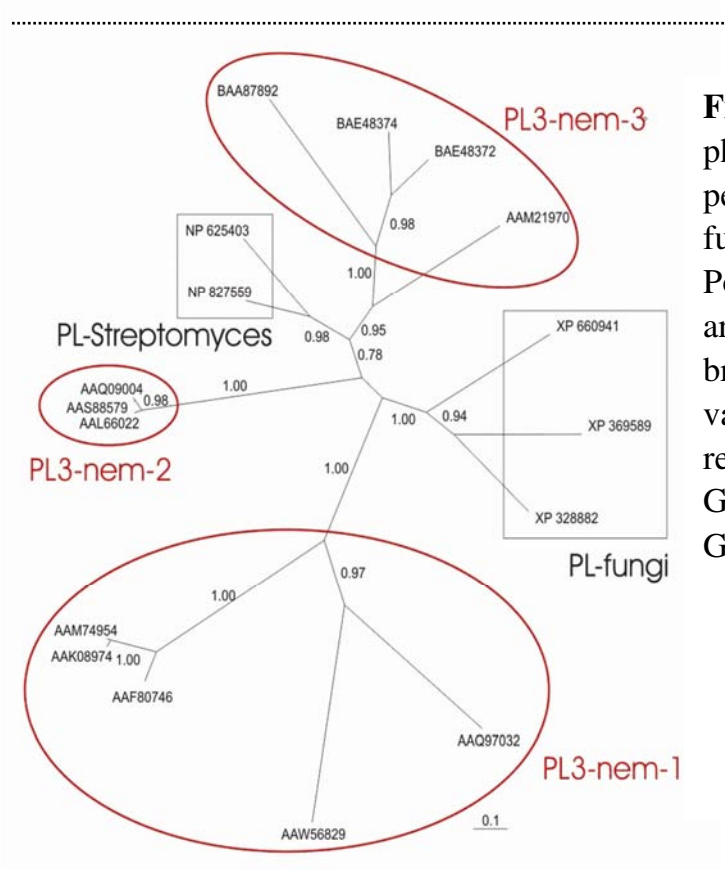


Fig. 4 Bayesian inference-based phylogenetic tree of family 3 pectate lyases of nematode (#11), fungal (#3) or bacterial (#3) origin. Posterior probability (PP) values are positioned at the corresponding branches of the unrooted tree (PP values > 0.95 point at a robust resolution). Gr-PEL2: AAM21970, Gr-PEL1: AAF80746. For other Genbank numbers see text

2.4 Expression of *Gr-Pel2* in *N. benthamiana*

To demonstrate the effect of Gr-PEL2 on plant tissues, *N. benthamiana* leaves were infiltrated with *Agrobacterium* harboring *Gr-Pel2* +SP. Four days post infiltration *N. benthamiana* plants started to develop a phenotype at the site of application, whereas plants infiltrated with *Agrobacterium* containing either an empty binary vector (Fig. 3A) or a *Gr-Pel2*noSP (Fig. 3B) construct showed only minor tissue damage due to the infiltration procedure. In case of *Gr-Pel2*+SP construct the infiltrated leaves showed bleaching around the infiltration area and necrotic spots within the infiltration place itself. Moreover, leaves showed severe folding and wrinkling, and affected tissues became rigid and very fragile. Relatively young leaves showed severe phenotypic changes due to *Gr-Pel2* expression (Fig. 3C), whereas in older leaves the most obvious symptom was bleaching (and slight folding) (Fig. 3D).

2.5 Phylogenetic analysis of PL3 family members

Bayesian inference was used to establish the relationships between Gr-PEL2 and several other PL3 family members (Fig. 4). Whereas fungal and actinobacterial pectate lyases cluster in distinct clades, pectate lyases of nematode origin appear in at least three clusters (Fig. 4). One clade (PL3-nem-1) includes PLs from the soybean cyst nematode *Heterodera glycines*, pectate lyase 2 and 3 from the root knot nematode *Meloidogyne incognita* and Gr-PEL1 from the potato cyst nematode *G. rostochiensis*. A second distinct clade (PL3-nem-2) consists of pectate lyases (Mi-PEL, Mi-PEL1 and Mj-PEL1) from *M. incognita* and *M. javanica*. Gr-PEL2 belongs to a third cluster (PL3-nem-3) that also includes pectate lyases from *Bursaphelenchus mucronatus* and *B. xylophilus*. The relatively high divergence between Gr-PEL1 and Gr-PEL2 makes it unlikely that both PLs are derived from a shared common ancestor.

2.6 3D protein structure modeling

To gain insight into the structure-function relation in Gr-PEL2 at atomic level and to further pinpoint differences between Gr-PEL2 and Gr-PEL1, 3D structure models were built. Fold recognition techniques identified Pectate lyase 15 from *Bacillus sp.* strain Ksm-P15 (Pel15: PDB accession # 1EE6) as the best template for both Gr-PEL2 and Gr-PEL1 with E-values of $1.2e^{-16}$ and $4.9e^{-09}$ respectively, corresponding to over 95% prediction confidence. Alignment of the two potato cyst nematode pectate lyases to Pel15 gives a segment spanning 216 aa, including insertions (Fig.5). Secondary structure prediction was further used to refine the alignment and place insertions within loops interconnecting the β-strands. This allows to conserve the overall right-handed β-helix structure of the pectate lyase superfamily (Fig.6 and 7) which consists of three β-strands per turn giving rise to three parallel β-sheets, named PB1, PB2 and PB3 connected by turns referred to as T1, T2 and T3 respectively (Jurnak et al. 1996).

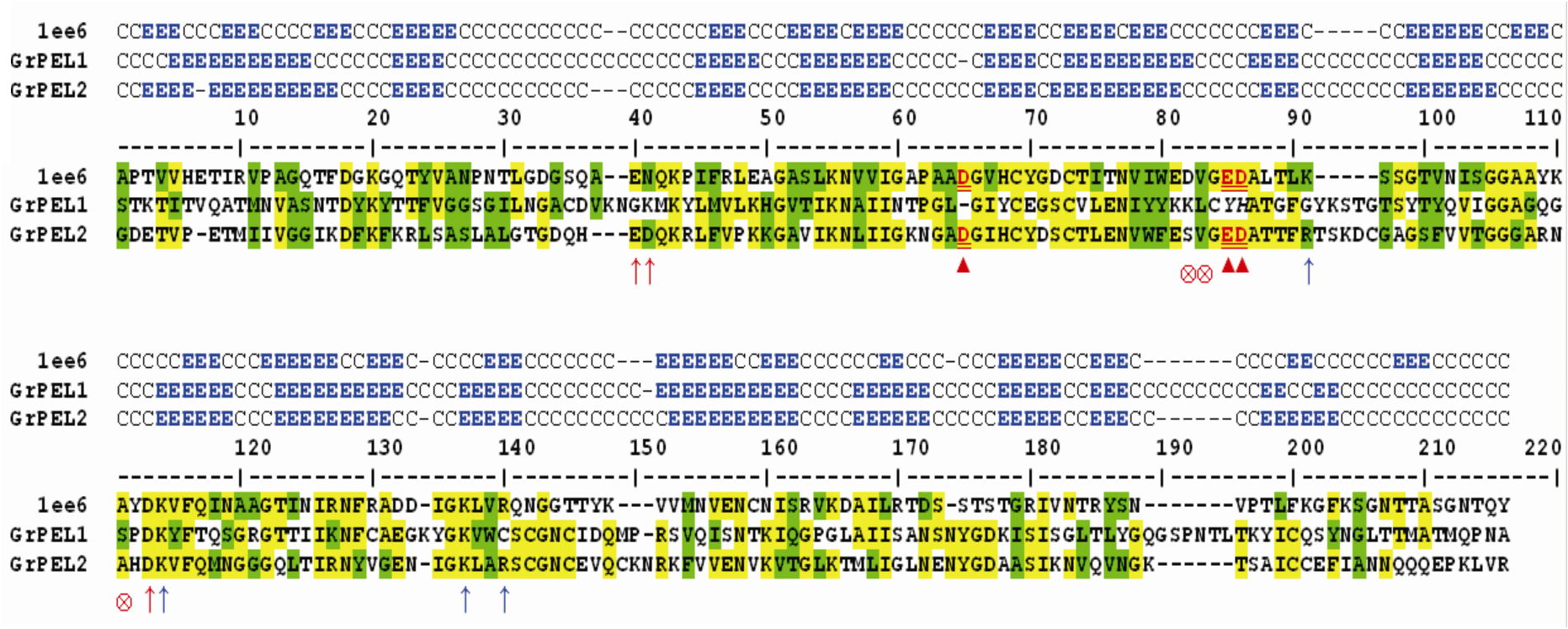


Fig. 5 Sequence to structure alignment of Pel15 (1ee6), Gr-PEL1 and Gr-PEL2. The conserved residues are highlighted in yellow and similar residues are highlighted in green. Location of Ca^{2+} sites and charged aa putatively involved in substrate binding is marked as follows: crossed circles - the Ca^{2+} site experimentally observed in the template (D_{82}, V_{83}, K_{110}); filled triangles - the proposed Ca^{2+} site (D_{85}, E_{105}, D_{106}); red arrows - the negatively charged residues in the T3-PB1-T1 region (E_{62}, D_{63}, D_{135}); blue arrows - the positively charged residues in the T3-PB1-T1 region (R_{113}, K_{136}, K_{158}, R_{161}).
Abbreviations for secondary structure elements are: H = helices, E = extended β-strands; C = coil. For 1ee6, the secondary structure elements are based on its determined 3D structure, whereas the usual 7 state structural assignment is reduced to H, E, C. Here, C also comprises R (random), B (bends), T (turns) and B (isolated β-bridges), *viz.* states that can not be discriminated by prediction methods. For the Gr-PEL1&2 sequences, H, E, C are the result of a consensus three state prediction.

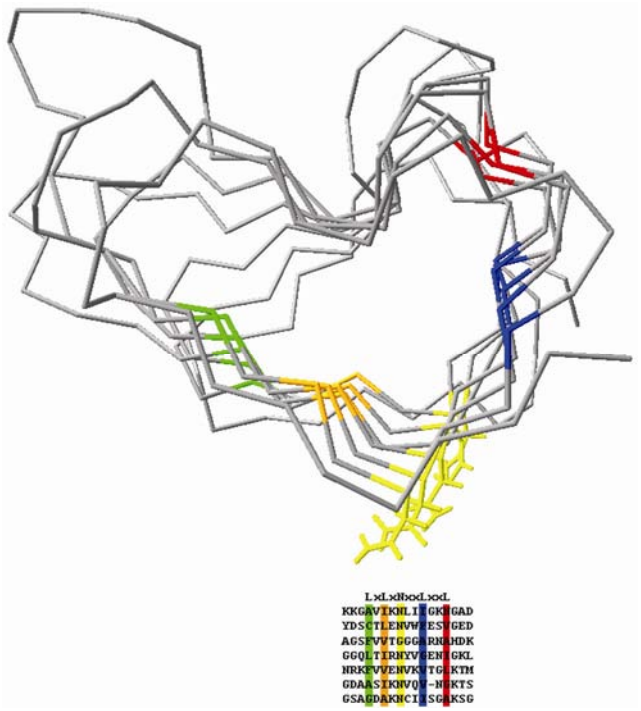


Fig. 6 Asparagine Ladder and the LxLxNxxLxxL motif in Gr-PEL2 model. Colors are used to highlight the spatial distribution of this motif

These insertions in the loops interconnecting the β–strands, are also consistent with the observation that variable loops interconnecting the three β-strands are commonly observed within this fold (Akita et al. 2001; Jurnak et al. 1996).

Calcium (Ca^{2+}) is essential for pectate lyase activity, and in Pel15 the Ca^{2+} binding site consists of three oxygen atoms - OD1 in D_{82}, and O in V_{83} and K_{110} (numbers in templates refer to the position of aa in the 3D model, whereas numbers in Gr-PEL2 always to the position in the precursor, Genebank accession # AY094613) An equivalent site may exist in Gr-PEL2, where three equivalent oxygen atoms can be found - OG1 in S_{104}, and O in V_{105} and N_{132}. However this site is located on a convex, highly exposed region of the protein surface at the edge of PB3-T3, which suggests a rather weak binding of the calcium ion. In other pectate lyases such as PelC from *E. chrysanthemi*, the Ca^{2+} binding site is located in a more protected area, found in the cleft formed by T3, PB1 and T1 (Herron et al. 2000; Herron et al. 2003; Pickersgill et al. 1994). Structural analysis of the cleft formed by T3-PB1-T1 in the Gr-PEL2 model shows the existence in this region of three acidic amino acid residues (D_{87}, E_{107} and D_{108}) that are in the right configuration to bind Ca^{2+}. This site is placed in a larger highly charged cluster containing both negatively (E_{62}, D_{63}, D_{135}) and positively charged amino acids (R_{113}, K_{136}, K_{158}, R_{161}). The nature of this cluster would imply a significant role of electrostatic forces in ligand binding in Gr-PEL2. In contrast to Gr-PEL2, Gr-PEL1 does not have acidic amino acids in the T3-PB1-T1 region and from the aforementioned cluster of 7 charged amino acids only three equivalent residues are present: D_{135}, K_{136}, K_{158}. These differences make this part of the Gr-PEL1 surface significantly more hydrophobic than its counterpart in Gr-PEL2

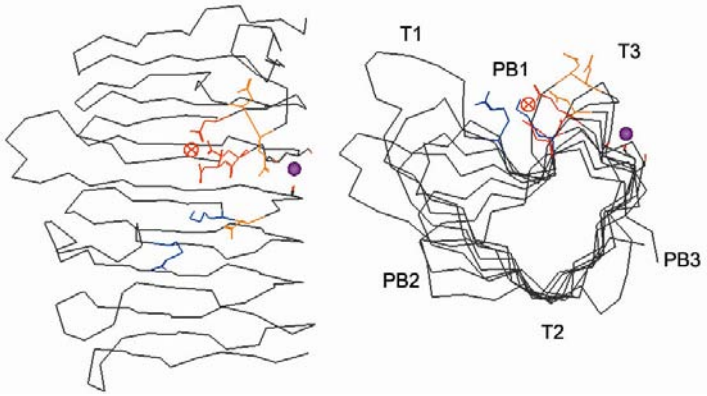


Fig. 7 The Ca^{2+} binding site in the Gr-PEL2 modeled according to the template. The Ca atom is figured in magenta, and the co-ordination oxygens (S_{104}:OG1, V_{105}:O, N_{132}:O) in red. These oxygen atoms are equivalent to D $_{82}$:OD1, V_{83}:O & K_{110}:O that coodinate calcium in Ksm-p15 (1ee6). A Ca^{2+} binding site is predicted in the T3-PB1-T1 region of the protein. This is formed by the side chains of three acidic aminoacids, D_{87}, E_{107} and D_{108} (represented in red) that are members of a larger patch that contain in addition E_{62}, D_{63} and D_{135} (represented in orange) and K_{136}, R_{161} (represented in blue). The Ca ion in this putative site is depicted with an crossed-circle. This geometry is very similar to that of the Ca^{2+} binding site of *E. chrysanthemi (pdb 1o88)*.

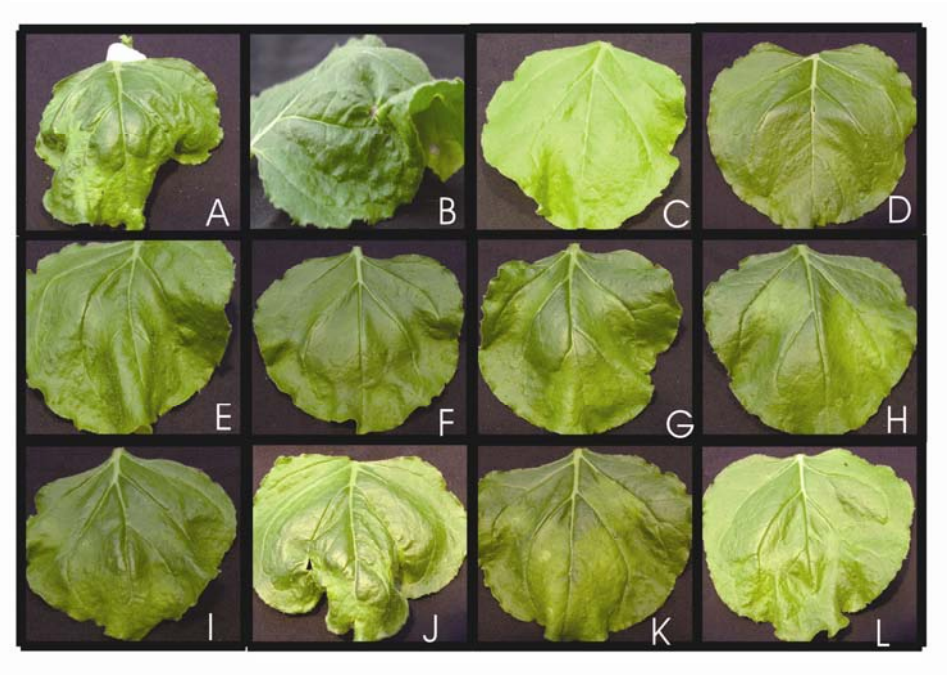


Fig. 8 Three week old *Nicotiana benthamiana* leaves Infiltrated with mutated *Gr-pel2* A: Gr-PEL2 with serine84 substituted with alanine and valine85 with alanine (S_{104}A_V_{105}A); B: N_{132}A, C: D_{87}A, D: E_{107}A_D_{108}A; E: E_{107}A_D_{108}A_D_{87}A; F: E_{62}A_D_{63}A, G: D_{135}A; H: E_{62}A_D_{63}A_D_{135}A; I: K_{136}A, J: K_{158}A; K: R_{161}A; L: K_{135}A_K_{158}A

2.7 Determination of the Ca^{2+} binding site in Gr-PEL2 by single amino acid substitutions

Based on our structure model of Gr-PEL2 there are two potential Ca^{2+} binding sites. To identify the correct site two sets of mutants were analyzed: (1) with substitutions in amino acids residues potentially binding Ca^{2+} in a similar mode as those in 1EE6 (template selected for 3 D modeling), namely $S_{104}A$, $V_{105}A$, $N_{132}A$; (2) with substitutions in highly charged amino acids equivalent to those binding Ca^{2+} in pectate lyases from *B. subtilis* (PDB accession # 1BN8) and *E. chrysanthemi* (PDB accession # 1O88) ($D_{87}A$, $E_{107}A$, $D_{108}A$, $E_{62}A$, $D_{63}A$, $D_{135}A$). Mutants set (1) caused very similar response of the infiltrated tissue as compared to the positive control, whereas infiltration with mutants set (2) severely hampered enzyme activity (no significant change in leaf structure or coloration) (Fig. 8). These results indicate that acidic residues in the cleft formed by T3-PB1-T1 are responsible for Ca^{2+} binding in Gr-PEL2.

Positively charged residues are thought to play a role in the neutralization of the negative charge on the substrate and initialize proton abstraction during catalysis. Infiltration with Gr-PEL2 containing alanin substitution in K_{136} and R_{161} residues was not followed by triggering of a phenotype specific for a wild type protein, whereas infiltration with mutant K_{158} exhibited a mild phenotype, suggesting that K_{136} and R_{161} are indispensable and are likely to take part in substrate neutralization and proton abstraction respectively, K_{158} seems to be of a lesser importance (Fig. 8).

3. DISCUSSION

Plant-parasitic cyst nematodes recruit cell wall degrading enzymes and expansins to penetrate epidermal cell layers and to migrate in tissues underneath these layers in host plants (Davis et al. 2000; Qin et al. 2004). Once inside a plant the migratory juveniles breakdown the cell walls ahead and induce the collapse of protoplasts, until they settle down to establish a feeding site (Endo 1986; Wyss and Zunke 1986). In this paper, we report the finding and the characterization of the novel pectate lyase gene *Gr-Pel2* from the potato cyst nematode *G. rostochiensis*, which likely assists the nematode in its migratory phase. The expression of *Gr-Pel2* is specifically localized in the subventral oesophageal glands of juveniles that have been exposed to potato root exudates. This is remarkable since other cell wall modifying proteins from subventral oesophageal gland including Gr-PEL1 were transcribed merely upon rehydration in tap water (exposure to root diffusate from the host was simply boosting expression) (Popeijus et al. 2002).

The pectic polysaccharides in plant cell walls are characterized by an immense structural diversity due to extensive decorations of the backbone polymer (Willats et al. 2001). Our finding of *Gr-Pel2* suggest that *G. rostochiensis* uses for the breakdown of these pectic polysaccharides at least two pectate lyase genes. Phylogenetic

inference with a representative set of members from the polysaccharide lyase family 3 revealed that the two pectate lyases from *G. rostochiensis* reside in distinct clades. The genomes of plant parasitic nematodes have not been exhaustively searched for pectate lyase genes and further research will most probably resolve other separate clades. However, at present time it is striking that *Gr-Pel2* appears to be most related to PLs from two facultative plant parasitic *Bursaphelenchus* species and to actinobacterial members of the PL3 family, rather than to pectate lyases from other cyst or root knot nematodes including *Gr-Pel1*.

The β-expansin gene *Gr-EXPB1* from *G. rostochiensis* recently identified in our laboratory also shows such a remarkable similarity with genes from actinobacteria (Kudla et al. 2005; Qin et al. 2004). These soil-dwelling bacteria partly occupy the same habitat as cyst nematodes. However, unlike the obligate plant-parasitic cyst nematodes they are mainly associated with the conversion of biomass (not specifically from potato or related plant species). It is therefore questionable whether these similarities are the result of converging evolution due to a substrate shared by cyst-nematodes and actinobacteria. We are currently studying the genomic loci flanking the pectate lyase and the expansins genes to see if the similarities with actinobacteria extend into larger sections of the nematode genome. If nematodes have indeed acquired these genes by lateral gene transfer from these bacteria, we expect to find physical linkage between nematode genes that have their best matching homologs in Actinobacteria.

The structures of Gr-PEL1 and Gr-PEL2 proteins were modeled to see how the moderate similarity in primary amino acid sequences translates into different protein folds, structures, and consequent functions. The overall protein structures for Gr-PEL1 and Gr-PEL2 predicted from the models resembled a right-handed β-helical turn, which is considered as characteristic for the pectate lyase superfamily (Herron et al. 2000; Jurnak et al. 1996). The overall topology of the proteins is stabilized by a typical asparagine-ladder (Kamen et al. 2000) in the predicted core of the protein of both Gr-PEL1 and Gr-PEL2 (Fig. 6). Significant structural differences between Gr-PEL1 and Gr-PEL2 were found in the putative Ca^{2+} binding sites, and in the substrate-binding cleft. The Ca^{2+} binding is likely to take place at different sites in Gr-PEL1 and Gr-PEL2. The calcium-binding site of Gr-PEL2 is positioned in a cleft, which is believed to condition hydrostatic interactions between enzyme and substrate (Pickersgill et al. 1994). The overall charge distribution in this cleft is also significantly different between Gr-PEL1 and Gr-PEL2 suggesting that both enzymes target dissimilar pectic polysaccharides as substrate (Mayans et al. 1997).

We have explored a novel system to study the activity of cell wall modifying enzymes using transient expression *in planta*. This system has several advantages over assays based on artificial model substrates especially when the focus is at plant-microbe interactions. First, the rich structural diversity in cell wall polymers as it is present in cell walls of plants is not represented in artificial model substrates (Hagerman et

al.1985). Moreover, the cell wall constitutes a complex mixture of interacting cell wall polymers. These interactions between different types of cell wall polymers further increase the structural diversity present in the cell wall. This implicates that enzymes showing little activity on model substrates may have a profound effect on the cell wall polymers *in vivo*, and *vice versa*. Consequently, the steady state parameters of substrates such as polygalacturonic acid may be poor predictors for disease development. Second, nematodes co-secrete a range of cell wall modifying enzymes to collectively breakdown the plant cell wall. *In planta* assay enables to test enzymes from several classes simultaneously by transient co-expression to study their collective activity, including potential synergetic effects.
The complexity of plant cell wall is matched by the wide array of cell wall degrading enzymes produced by various plant pathogenic organisms (Warren 1996). Likewise, this newly discovered pectate lyase adds to the known arsenal of the cell wall degrading machinery of the plant parasitic nematodes, which combined with the thrusting of the nematode's stylet facilitates the penetration of host cells. *Agrobacterium* mediated infiltration proved to be quick and sensitive method not only to test the effect that Gr-PEL2 had on the plant tissue *in vivo*, but also to identify amino acids responsible for catalysis and therefore to confirm our 3D model of a protein. This method can be applied to test other parasitism factors, which will ultimately lead us to a better understanding of the characteristic that render plant cell walls susceptible to nematode attack.

4. MATERIALS AND METHODS

4.1 cDNA-AFLP analysis

Messenger RNA was extracted from five developmental stages of *G. rostochiensis* pathotype Ro1-Mierenbos, and cDNA-AFLP analysis was performed essentially as described previously (Qin et al. 2000). The primary cDNA templates synthesized from each of the five mRNA pools were digested using the restriction enzymes *Kas*I and *Taq*I. For the specific amplification reactions, oligonucleotide primers (K+0: and T+0: see Table 1) annealing to the *Kas*I and *Taq*I adapter sequences (K-adapter-upper, K-adapter-lower, T-adapter-upper, T-adapter-lower; Table 1) (Vos et al 1995) were used in standard protocols. Differentially expressed transcript-derived-fragments (TDFs) were excised from acrylamide gels. After re-amplification using the original primers, TDFs were cloned into TOPO-pCR4 (Invitrogen, Breda, The Netherlands) and transferred into *E. coli* TOP10 chemically competent cells (Invitrogen). After purification, inserts were sequenced using standard procedures.

4.2 Cloning of the full-length cDNA

Total RNA was isolated from the pre-parasitic second stage juveniles of *G. rostochiensis* incubated in potato root difusate using the TRIzol RNA isolation method (Invitrogen). The full-length cDNA of *Gr-Pel2* was obtained in a 5' and 3'-RACE (rapid amplification of cDNA ends) reaction with The Gene Racer^{tm} Kit with SuperScript II (Invitrogen) using gene specific primers (Pel2forA and Pel2revB, Table 1:) in combination with GeneRacer5' and GeneRacer3' primers. The complete longest open reading frame of Gr-Pel2 was cloned using primers annealing to the 5' and 3'end of the sequence (Pel2orfFor and Pel2orfRev; Table 1).

Table 1 – Names of primers as used in the text, and their sequence. In case of primers used for amino acid substitutions, the modified base pairs are listed in bold.

Primer name	Sequences (5' → 3')
K-adapter-upper	CTC GTA GAC TGC GTA TA
K-adapter-lower	GCG CTA TAC GCA GTC TAC
T-adapter-upper	GAC GAT GAG TCC TGA C
T-adapter-lower	CGG TCA GGA CTC AT
K+0	GAC TGC GTA TAG CGC C
T+0	GAC GAT GAG TCC TGA CCG A
Pel2forA	GCG CAC TAA TTT CGG CTC CTG TTG CTG GTT G
Pel2orfFor	ATG CTG CAC CAT TTA TTC GTT CTT TGC
Pel2revB	CGG CG TCT ATC AAA ACG TGC AGG TCA ATG GG
Pel2orfRev	TCA GCC GCT TTT GGC GCC TGA AAT G
ForGr- Pel2att	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGCTGCACCATTTATTCGTTC
ForGr-Pel2-SPatt	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTATGAATTGGCCCACCGCGACGG
RevGr-Pel2att	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGCCGCTTTTGGCGCCTGA
F_S104A_V105A	GGA GAA TGT TTG GTT CGA G**G**C CG**C** TGG CGA AGA TG
R_S104A_V105A	CTC GAA CCA AAC ATT CTC CAA AGT ACA ACT ATC G
F_N132A	CGG GTG GTG GCG CTC GA**G C**TG CTC ACG ACA A
R_N132A	TCGAGC GCC ACC ACC CGT GAC
F_D87A	GAT TAT CGG CAA AAA TGG TGC CG**C** CGG CAT TCA TTG
R_D87A	CGG CAC CAT TTT TGC CGA TAA TCA AAT T
F_E107A_D108A	TTT GGT TCG AGT CCG TTG GCG **C**AG **C**TG CGA CAA CGT TT
R_E107A_D108A	CGC CAA CGG ACT CGA ACC AAA CAT T
F_E62A_D63A	GGG CAC AGG CGA CCA ACA TG***C*** GG**C** CCA AAA GCG ATT A
R_E62A_D63A	ATG TTG GTC GCC TGT GCC CAA TGC TAA T
F_D135A	GGT GGC GCT CGA AAT GCT CAC G**C**C AAA GTG TTT CAG
R_D135A	CGT GAG CAT TTC GAG CGC CAC CA
F_K136A	GGC GCT CGA AAT GCT CAC GAC **GC**A GTG TTT CAG ATG
R_K136A	GTC GTG AGC ATT TCG AGC GCC ACC A
F_K158A	GTG GGC GAA AAC ATT GGT **GC**A TTG GCG CGC TCG
R_K158A	ACC AAT GTT TTC GCC CAC ATA ATT TCT AAT TGT
F_R161A	CAT TGG TAA ATT GGC G**GC** CTC GTG CGG TAA TTG C
R_R161A	CGC CAA TTT ACC AAT GTT TTC GCC CA

4.3 Sequence analysis

DNA sequences were analyzed using the DNASTAR program (Laser Gene, Madison, WI, USA). Database searches were done using the BLAST program *blastp* at the National Center for Biotechnology Information (NCBI). SignalP version 2 (14) was used to predict the presence of a signal peptide for secretion and the cleavage site (Nielsen et al. 1997). PROSITE (ExPASy Molecular Biology Server) was used to find biologically significant sites, patterns, profiles and potential post-translational modifications of the Gr-PEL2 protein (Appel, Bairoch, and Hochstrasser 1994).

4.4 *In situ* hybridization

Fixation of pre-parasitic second stage juveniles, hybridization and signal detection were essentially performed as described by de Boer *et al.* (1999). A *Gr-pel2* fragment (nt 360 – 746) was cloned, re-amplified and checked on a 1% agarose gel. Specific sense and antisense ssDNA probes were synthesized in separate reactions by an asymmetric PCR in the presence of digoxigenin (DIG) labeled dUTP (Roche Diagnostics, Mannheim, Germany) using the primers K+T and T+AA primers. Alkaline phosphatase activity was detected after the addition of X-phosphate and NBT (Roche Diagnostics). The labeling was observed using a Leica inverted microscope (Leica, Deerfield, IL, USA).

4.5 Homology modeling of Gr-PEL2

The optimal template for 3D homology modeling was identified by fold recognition using the 3D-PSSM program (Kelley et al. 2000). The target and template sequences were aligned using MULTALIN (Corpet 1988). The alignment was further optimized manually, and tuned to fit information on the secondary structure. The prediction of the target secondary structure was performed using four methods: PHD (Rost and Sander 1993), PsiPred (McGuffin et al. 2000), Prof (Rost and Sander 1993) and SSPRO (Baldi et al. 1999). The optimized sequence alignment was used to build a 3D model using the Homology module of Insight II package Modeller (Accelrys Inc., San Diego, CA, USA). This model was further subjected to simulated annealing in the region of non-conserved loops and finally repeated rounds of energy minimization in order to relieve steric conflicts. Structure modeling, refinement and analysis was performed using the programs Insight II, Discover, Homology Modeller (Sali and Blundell 1993), Delphy and Affinity (Accelrys) on a Silicon Graphics Octane 2 station.

4.6 Phylogenetic analysis

Seventeen protein sequences showing significant similarity to Gr-PEL2 were aligned using the ClustalW algorithm in BioEdit version 6.0.7 (Isis Pharmaceuticals, CA, USA). The alignment included three bacterial (putative) pectate lyases (Genbank accessions NP_827559 and NP_625403 from *Streptomyces avermitilis* and *S. coelicolor* respectively, and BAA87892 from *Bacillus* sp.), three hypothetical proteins of fungal origin (Genbank accessions XP_369589, XP_660941, XP_328882 from *Magnaporthe grisae*, *Aspergillus nidulans* and *Neurospora crassa*, respectively) and eleven pectate lyases from nematodes (Genbank accessions BAE48372 and BAE48374 from *Bursaphelenchus xylophilus* and *B. mucronatus*, respectively; AAS88579, AAQ09004, AAQ97032, and AAW56829 from *Meloidogyne incognita*; AAL66022 from *M. javanica*; AAK08974 and AAM74954 from *Heterodera glycines*, and AAF80746 and AAM21970 (this paper) from *Globodera rostochiensis*). The putative signal peptide sequences as predicted by SignalP-2.0 were removed and the initial alignment was further optimized manually. Due to high variability of the C-terminal ends, these parts of the proteins could not be aligned. Therefore aa 178 - 232 (AAM21970) and equivalent parts of other pectate lyases mentioned above were removed. Four Bayesian runs (MrBayes, version 3.1) were performed with random trees as starting point using the BLOSUM substitution model. Each run spanned 1 million generations, sampling took place every 100th generation, and the first 70,000 generations were discarded as burn-in. In the resulting unrooted phylogenetic tree, nodes with a posterior probability lower than 0.95 are considered to be unresolved.

4.7 Expression of *Gr-Pel2 in planta*

Gene specific primers were designed (ForGr-Pel2att and RevGr-Pel2att; Table 1) to allow directional cloning of the largest open reading frame of *Gr-Pel2* into a Gateway entry vector (pDONR207) (Invitrogen, Breda, The Netherlands). To generate a similar construct without the predicted signal peptide for secretion the forward primer was replaced by ForGr-Pel2-SPatt (Table 1). The cycling parameters of the PCR were as follows: 5 min at 94^{o}C, 25 cycles of 1 min at 94^{o}C, 1 min at 50ºC and 1.5 min at 72ºC, and finally 10 min at 72^{o}C. In the following recombination reactions, inserts were transferred from pDONR207 to pK2WG7 (Karimi, Inze, and Depicker 2002) for expression in plants. Subsequently, the pK2WG7 vectors containing the *Gr-Pel2* sequence with or without signal peptide for secretion were introduced into *Agrobacterium tumefaciens* (strain LBA4404) by electroporation (Shen and Forde 1989). Cultures of transformed *A. tumefaciens* (5ml) were grown for 12 h at 28°C in YEB medium containing 0.5% beef extract (Difco, Detroit, USA), 0.1% yeast extract (GibcoBRL, Peisley, Scotland), 0.5% peptone (GibcoBRL), 0.5% sucrose (Merck, Darmstad, Germany), and 50µg ml^{-1} of kanamycin and rifampicine (Duchefa,

Haarlem, The Netherlands). One ml of this culture, was transferred to 50 ml of an induction medium containing 20 µM 5'-dimethoxy-4'-hydroxyacetophenone (Aldrich, Zwijndrecht, Netherlands). The induction medium was similar to the YEB medium described above except that beef extract was replaced by 0.5% nutrient broth (Braunschwig Chemie, Amsterdam, the Netherlands). and bacterial cultures were grown overnight at 28°C. Subsequently, cells were pelleted and resuspended in infiltration media (10mM $MgCl_2$, 10mM MES [pH 5.7], 200µM 5'-dimethoxy-4'-hydroxyacetophenone (Aldrich)) to a final OD_{600} of 1.0 and incubated overnight at 25 C. Prior to the infiltration the OD_{600} was adjusted to 0.5 and cultures were used to infiltrate 3 week old *Nicotiana benthamiana* leaves with a syringe (without the needle) (Hoekema et al. 1983). The plants were kept in the greenhouse at light and at 24°C.

4.8 Site directed mutagenesis

To identify amino acids involved in catalysis and calcium binding, site directed oligonucleotide mutagenesis of *Gr-Pel2* was done by PCR using an overlapping extension method (Ho et al. 1989) and the GeneTailor Site-Directed Mutagenesis System (Invitrogen, Breda, The Netherlands). Primers were designed to substitute the amino acids of interest with an alanine (Table 1). The pDONR207 vector carrying *Gr-Pel2* with signal peptide sequence was used as a template in the mutagenesis reactions using the primer combinations as indicated in Table 2 (the substituted base pairs are in bold). The following PCR conditions were used: 3 min 94ºC, 25 cycles of 1 min 94°C, 1 min 50°C and 6.5 min of 68°C, and finally 10 min of 68°C. After transformation into the *E.coli* DH5α (Invitrogen), the genes were checked for proper mutation by sequencing. The mutants of *Gr-Pel2* were transferred to the destination vector pK2WG7 and over-expressed in *N. benthamiana* as described above.

ACKNOWLEDGEMENTS

This work was supported by funding from the European Union: QLK5-1999-01501 (UK, AM, LQ) and BIO4-CT96-0318 (LQ).

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Chapter III

A nematode expansin acting on plants

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Published
Nature 2004. 427:30

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Expansin proteins, which have so far been identified only in plants, rapidly induce extension of plant cell walls by weakening the non-covalent interactions that help to maintain their integrity (Cosgrove 2000). Here we show that an animal, the plant-parasitic roundworm *Globodera rostochiensis*, can also produce a functional expansin, which it uses to loosen cell walls when invading its host plant. As this nematode is known to be able to disrupt covalent bonds in plant cell walls (Popeijus et al. 2002; Smant et al. 1998), its accompanying ability to loosen non-covalent bonds challenges the prevailing view that animals are genetically poorly equipped to degrade plant cell walls.

The plant cell wall is a rigid network of interwoven polymers, and many organisms that use plants as a food source use a variety of glycanase enzymes to break covalent bonds in this polysaccharide-based structure. We used complementary DNA–AFLP (for amplified fragment-length polymorphism)- based transcript profiling of synchronized life stages as a starting point to identify the proteins that are used by *G. rostochiensis* to degrade cell walls. A cDNA fragment, KT21 (137 nucleotides), was found to be predominantly expressed in infective second- stage juveniles (J2) and the corresponding full-length cDNA (*Gr-EXPB1*; accession number AJ311901; length, 1,061 base pairs) encoded a protein of 271 amino acids that has a predicted amino-terminal signal peptide for secretion.

Similarity searches (BLASTP) indicated that two distinct regions are present in the predicted mature protein. Domain 1 (residues 26–118) shows significant similarity to the carbohydrate-binding module family II of endoglucanases (AF056110, BAB68522 and AF323087; 39–43% identity and expectation values (E-value from $2.0x10^{-12}$ to 0.0008) from various nematode species. Domain 2 (residues 150–271) showed significant similarity to a β-expansin-like protein (PPAL) from *Nicotiana tabacum* (AAG52887; Evalue, 2.221015) and a putative β-expansin from *Arabidopsis thaliana* (O04484; E-value, $6x10^{-14}$).A local alignment of domain 2 with these β-expansins indicated the presence of a series of conserved cysteine residues, the HFD motif (although Gr-EXP1 harbours a conservative substitution (F→V)) and other conserved motifs (Cosgrove 2000).

Whole-mount in situ hybridization was carried out on pre-parasitic infective secondstage juveniles of *G. rostochiensis* (Qin et al. 2000). Antisense cDNA probes amplified from the *Gr-EXPB1* cDNA (nucleotides 54–427) hybridized specifically to the subventral oesophageal glands (Fig. 1a).

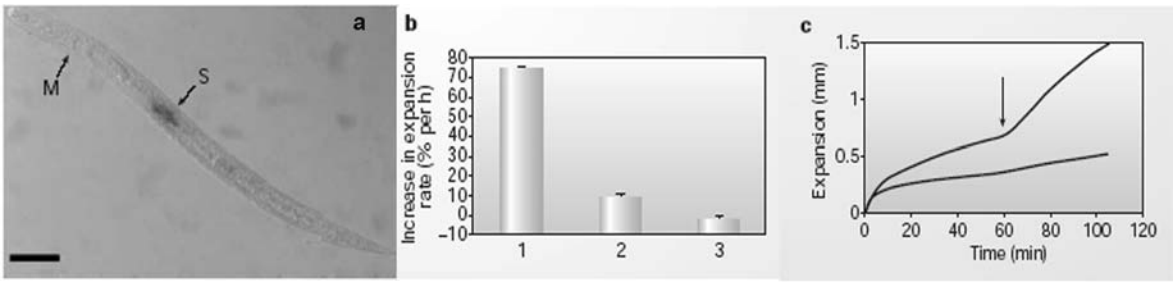

Fig 1 Localization of the nematode *Gr-EXPB1* transcript and extension activity of Gr-EXPB1 on plant cell walls.
1a, In situ hybridization labelling pattern in infective second-stage juveniles (J2) using a *Gr-EXPB1* antisense probe. Arrows indicate the subventral glands (S) and the metacorpus (M), respectively. Scale bar, 20 mm.
1b, Effects of nematode homogenate on the extension rate of heat-inactivated wheat coleoptiles and cucumber hypocotyls. Bar 1, J2 homogenate on wheat coleoptiles; bar 2, J2 homogenate on cucumber hypocotyls; bar 3, young female homogenate on wheat coleoptiles.
1c, Extension curves for a wheat coleoptile treated with mature-leaf extracts from *Gr-EXPB1*-harbouring (upper curve) and empty-vector-harbouring (lower curve) tobacco plants. Arrow indicates the point at which the control buffer was replaced by leaf extract

Gr-EXPB1 antiserum reacted strongly with nematode secretions, induced by potato-root diffusate, on dot blots (results not shown). We conclude that Gr-EXPB1 is produced in the subventral oesophageal glands of infective juveniles, and that Gr-EXPB1 and cell-wall-degrading enzymes are secreted simultaneously (Davis et al. 2000).

Cell-wall-extension activity was demonstrated in homogenates of infective secondstage juveniles, and was much stronger on wheat than on cucumber (Fig. 1b) (McQueen-Mason et al. 1992). Homogenates of adult females showed no such activity (Fig. 1b). Protein extracts from mature leaves of *Gr-EXPB1*-transformed tobacco produced significantly more expansin activity on wheat coleoptiles than did empty-vector controls (Fig. 1c). On the basis of the significant similarity of Gr-EXPB1 to putative β-expansins from *N. tabacum* and *A. thaliana*, the presence of several amino-acid motifs that are characteristic of expansins, and the potent expansin activity of both recombinant Gr-EXPB1 and nematode homogenates on plant-cell walls, we conclude that *Gr-EXPB1* encodes a functional expansin.

Sequences that remotely resemble expansins have been found in various taxa outside the plant kingdom (Laine et al. 2000; Saloheimo et al. 2002; Xu et al. 2000), but cell-wall-loosening activity has not been demonstrated for any of the corresponding proteins. To our knowledge, *Gr-EXPB1* is the first non-plant gene found to have the structural and functional characteristics that define the expansin superfamily.

This finding undermines the previously accepted view that animals are poorly equipped for degrading plant cell walls. When cell-wall-degrading enzymes and expansin are simultaneously secreted by the cyst nematode, the activity of expansin may increase the accessibility of cell-wall components to glycanases. This might account for the remarkably high rate (about 2 min per cell layer) at which cyst nematodes can penetrate the host plant.

Acknowledgements.

Funding from the European Union project QLK5-1999-01501 (LQ and UK) is gratefully acknowledged

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Chapter IV

Origin, distribution and 3D-modelling of Gr-EXPB1, an expansin from the potato cyst nematode *Globodera rostochiensis*

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Published
FEBS Letters 2005. 579: 2451-7

Origin, distribution and 3D-modelling of Gr-EXPB1, an expansin from the potato cyst nematode *Globodera rostochiensis*

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Abstract

Southern analysis showed that Gr-EXPB1, a functional expansin from the potato cyst nematode *Globodera rostochiensis*, is member of a multigene family, and EST data suggest expansins to be present in other plant parasitic nematodes as well. Homology modeling predicted that Gr-EXPB1 domain 1 (D1) has a flat β-barrel structure with surface-exposed aromatic rings, whereas the 3D structure of Gr-EXPB1-D2 was remarkably similar to plant expansins. Gr-EXPB1 shows highest sequence similarity to two extracellular proteins from saprophytic soil-inhabiting Actinobacteria, and includes a bacterial type II carbohydrate-binding module. These results support the hypothesis that a number of pathogenicity factors of cyst nematodes is of procaryotic origin and were acquired by horizontal gene transfer.

Keywords: Expansin; Plant parasitic nematode; Horizontal gene transfer, Protein modelling; Cell wall modifying proteins

1. Introduction

The plant cell wall is a complex composite of cellulose microfibrils and cross-linking hemicelluloses, which are embedded in a matrix of pectic polymers and structural glycoproteins. To overcome this structural barrier, a wide range of saprophytic and plant pathogenic bacteria, oomycetes, fungi and nematodes secrete various types of cell wall-degrading enzymes (CWDEs). The compactness of plant cell walls limits the efficiency of CWDEs. Expansins, a relatively diverse protein superfamily that is widespread within the plant kingdom, directly modulate the mechanical properties of the cell wall by weakening non-covalent interactions. These proteins are suggested to open up this compact structure, making it accessible to enzymatic attack (Cosgrove 2000). As such, expansins could substantially increase the fitness of CWDE-harboring plant pathogens.

So far, only a few proteins have been identified among plant pathogens and saprophytes that remotely resemble expansins, and for none of these proteins expansin activity was shown (e.g. (Laine et al. 2000) and (Saloheimo et al. 2002)). Recently, we showed the presence of a functional expansin in secretions of infective juveniles of the potato cyst nematode *Globodera rostochiensis* (Qin et al. 2004). Here we show that *Gr-EXPB1* is member of a multigene family, and present in other cyst nematode

species as well. Remote homology modeling revealed that the predicted 3D structures of Gr-EXPB1 D2 and plant expansins are similar, even though their primary amino acid sequences show a relatively low level of homology. The striking similarity of Gr-EXPB1 to two proteins from saprophytic soil bacteria and its bacterial type carbohydrate binding module (CBM) constitute support for the hypothesis that a number of nematode pathogenicity factors has a procaryotic origin. The information given in this paper could facilitate the identification of more expansins outside the plant kingdom, and – more specifically - among other cell wall-degrading pro- and eukaryotes.

2. Materials and methods

2.1 Genomic analysis

Primers designed to anneal to the most extreme 5'- and 3'-ends of the cDNA sequence of Gr-EXPB1, gDNA-GrEXPf (GAGCTCCTCTGAAGCAATTC) and gDNA-GrEXPr (AACACTGTATAAACCTTTATGCAATT), were used to amplify the corresponding sequence from genomic DNA isolated from pre-parasitic J2-s as described (Yan et al. 2001). For Southern blotting, 5 µg of genomic DNA was isolated from pre-parasitic J2-s as described (Curran et al. 1985), digested with the restriction enzyme EcoRI, separated on an agarose gel and blotted. A DIG-labeled dUTP cDNA probe was synthesized from the Gr-EXPB1 genomic DNA clone (nt 1524 to 1932) using the primers D2f (ATGGTTTATTGAAAAATTCGTTG) and D2r (CCTGTCTCGACAAAAGAGTCC).

2.2 Antibody production and immuno-assays

Primers GrExp-pBAD-SPf (AGCTCCTCTGAAGCAATTCTGTGTTTGTTGTGCC) and GrExppBADr (AATAGGTGAGCGTACGCCCGTCGCTTTGCC) were used to amplify the coding region of the predicted mature protein (nt 39 – 848 in *Gr-EXPB1*). The amplified fragment was cloned into the pBAD/Thio-TOPO vector (Invitrogen, Leek, The Netherlands). Hens were immunized with purified recombinant protein, and the resulting chicken IgY antibody was isolated from the eggs as described by (Polson et al. 1980) and further purified (Harlow and Lane 1997). Western blots of homogenates and immuno-fluorescence microscopy of pre-parasitic J2-s were performed as described previously (de Boer et al. 1996).

2.3 Cell wall extension assay

Recombinant carbohydrate-binding module (CBM; residues 26 to 118) was produced in tobacco using the Gateway-compatible pK2GW7 vector (VIB, Ghent, Belgium)

whereas transcription was driven by the 35S CaMV promoter. Both CBM and Gr-EXPB1 were preceded by the original (nematode) signal peptide for secretion. Proteins were extracted from mature leaves by grinding in liquid nitrogen and resuspending in 50 mM sodium acetate pH 4.5. After centrifugation, the protein concentration of the supernatant was adjusted to approximately 150 µg/ml and used in an extensometer assay (Cosgrove 1997).

2.4 3D structure of Gr-Exp1 EXPB1 based on homology modeling

Template identification was performed using 3D-PSSM (Kelley et al. 2000). Target and template sequences were aligned by using MULTALIN (Corpet 1988), and the output was optimized manually. Based on the CASP4 assessment (Venclovas et al. 2001), PHD, PsiPred, Prof and SSPRO (Baldi et al. 1999; McGuffin et al. 2000; Rost and Sander 1993, 1994) were used to produce the secondary structure profile of the target. Model building, refinement and analysis were performed using the following Accelrys (Accelrys, San Diego, CA) programs: Insight II, Discover, Homology, Delphy and Affinity. 3.

3. Results

3.1 Analysis of cDNA and genomic sequences of Gr-EXPB1

The nematode expansin Gr-EXPB1 contained two distinct domains: domain 1 (D1: residues 26 to 118) showed significant similarity with carbohydrate-binding module family II (CBM2), and domain 2 (D2: residues 150 to 271), the actual expansin (Qin et al. 2004). Besides being similar to plant expansins and expansin-like proteins, D2 was even more similar to a hypothetical protein from the *Amycolatopsis mediterranei* (AJ319869 – *orf*D (Wang et al. 2002); 35% identity, E-value = $8.2e^{-12}$) and a predicted open reading frame ORF11 from *Streptomyces lavendulae* (AF127374 (Mao et al 1999); 36% identity, E-value = 1.2 e^{-10}). Both are aerial mycelium-forming soil saprophytes belonging to the order Actinomycetales (phylum Actinobacteria). Another remarkable homology was found between Gr-EXPB1 and MAP-1 (Meloidogyne avirulence protein) from the root knot nematode *M. incognita* (AJ278663 (Semblat et al. 2001); 35% identity, E-value = 2.1 e^{-10}). *orf*D, ORF-11 and map-1 are predicted to encode extracellular proteins with unknown functions.
To confirm the eukaryotic origin of *Gr-EXPB1*, we investigated its genomic sequence. A single fragment of 2.4 kb (AJ556781) was amplified from genomic DNA of *G. rostochiensis*. Six introns (with length ranging from 57 – 412 bp) were present in the *Gr-EXPB1*. The interspersion of the expansin gene by introns and the presence of a poly-A tail in the corresponding mRNA (AJ311901) exclude a procaryotic origin of *Gr-EXPB1*.

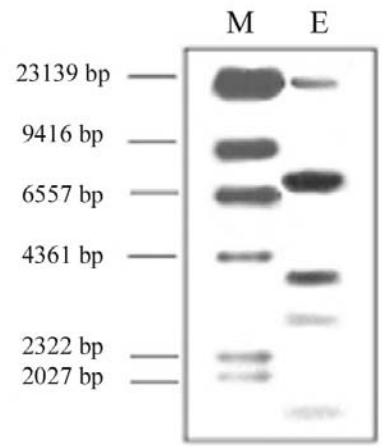


Fig. 1. Southern blot hybridized with a 409-bp genomic probe derived from *Gr-EXPB1*. Lane M is molecular weight marker; Lane E is loaded with 2.5 µg *G. rostochiensis* genomic DNA digested by EcoR I.

3.2 Multiple expansin-like sequences in cyst nematodes

A Southern blot analysis of *G. rostochiensis* genomic DNA probed with a D2 fragment showed five bands, suggesting the presence of several expansin-related sequences in the genome of *G. rostochiensis* (Fig. 1).
The presence of D2-like sequences in *G. rostochiensis* EST databases confirmed this finding as exemplified by CAC84564 - a hypothetical protein translated from AJ311902 - showing 75% identity with Gr-EXPB1-D2. CAC84564 is preceded by a signal peptide for secretion and lacks a carbohydratebinding module. Searching soybean cyst nematode EST databases revealed BF014507 (519 bp) with 82% identity to *Gr-EXPB1*. A local alignment of the three nematode sequences with a plant β-expansin and a plant β-expansin-like protein indicated the presence of a series of conserved cysteine residues and an additional number of conserved motifs previously described as signature motifs for α- and β-expansins (Fig. 2) (Cosgrove 2000).

3.3 Localization of Gr-EXPB1 protein

The polyclonal antibody against Gr-EXPB1 specifically bound to a band of about 28 kDa on western blots of homogenates of *G. rostochiensis* J2-s (Fig. 3A). This size is in broad agreement with the calculated molecular mass of the predicted protein once post-translational modifications have been allowed for. In a dot-blot experiment, Gr-EXPB1 was shown to be present in concentrated secretions of potato root diffusate-induced secretions from pre-parasitic J2-s (Fig. 3B). Immuno-fluorescence microscopy revealed distinct binding of this antibody to the subventral esophageal glands of preparasitic J2-s. Gr-EXPB1 protein was detected in the subventral gland lobe and in the extensions (Fig. 3C). Unlike MAP-1 (Semblat et al. 2001), Gr-EXPB1 was not detected in the amphidial region of infective J2-s.

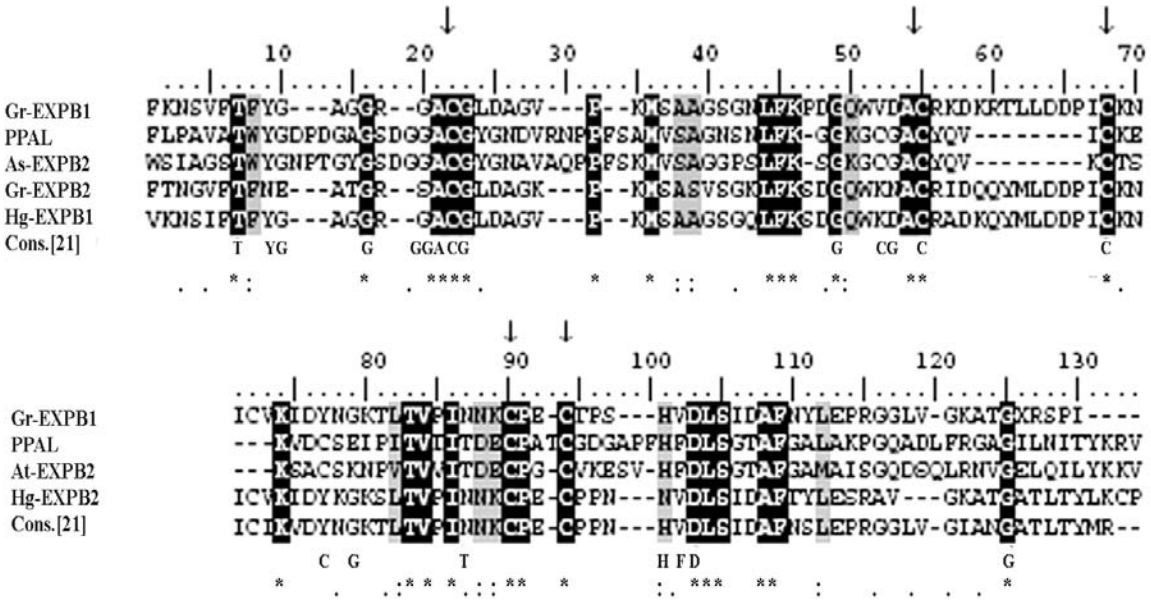
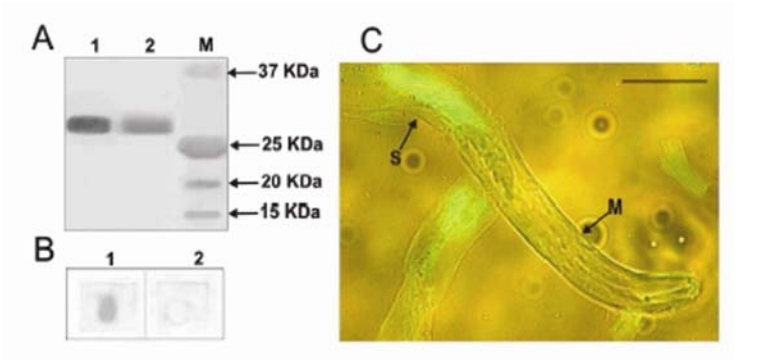


Fig. 2. Alignment of the expansin domain (D2) of Gr-EXPB1 (CAC83611) with domain 1 of PPAL: a pistil-specific β-expansin-like protein from *Nicotiana tabacum* (AAG52887), At-EXPB2: a putative β-expansin from *Arabidopsis thaliana* (Q9SHY6), GrEXPB2: a hypothetical protein translated from *G.rostochiensis* (AJ311902, CAC84564) and Hg-EXPB1: a conceptually translated mRNA sequence from *Heterodera glycines* (BF014507). Conserved residues between α- and β-expansins as proposed in (Cosgrove 2000). *- identical residues in all five proteins (in black boxes); :- conserved substitutions (in grey boxes); . - semi-conserved substitutions; ¯ - conserved cysteine residues.

Fig. 3. **A**. Detection of Gr-EXPB1 in J2 homogenate on western blot probed with anti-Gr-EXPB1 IgY antibody (diluted 100 times). 1: Potato root diffusate (PRD)-exposed pre-parasitic J2-s, 2: pre-parasitic J2s, not exposed to PRD; M: molecular weight marker. **B**. Dot blot of concentrated natural secretions from nematode J2-s incubated in PRD. Left panel (1) is probed with anti-GR-EXP1 IgY antibody; right panel (2) is probed with pre-immune IgY. **C**. Immuno-fluorescence labeling of the subventral gland with IgY antibody against GR-EXPB1. Arrows point at the subventral glands (S) and the metacarpus (M) respectively. Scale bar = 20 μm



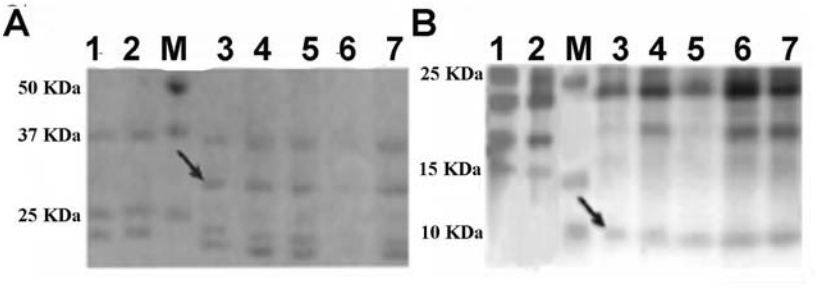


Fig. 4. A. Coomassie Brilliant Blue-stained SDS-PAGE gel showing expression of Gr-EXPB1 in tobacco plants (lanes 3-7) and two empty vector control lines (lane 1-2). **B**. Expression of CBM in tobacco (lanes 3–7) and two empty vector control lines (lanes 1-2); M, marker lane; Relevant products are indicated with an arrow.

3.4 Expansin activity assay of CBM

Protein samples extracted from plants transformed with empty vector, CBM or Gr-EXPB1 were analyzed on a SDS-PAGE gel. As compared with samples from empty vector controls, an additional band corresponding to the molecular weight of either CBM or Gr-EXPB1 was detected (Fig. 4). Proteins extracted from transgenic tobacco harboring *Gr-EXPB1* showed a significant increase in expansin activity as compared to the empty vector control (Fig. 5). Gr-EXPB1 consists of an expansin domain and a carbohydrate binding domain (CBM). CBMs may also induce disruptions of non-covalent interactions between plant cell wall carbohydrate polymers (Din et al. 1994). Therefore, it could be argued that the CBM domain in Gr-EXPB1 is responsible for the cell wall-loosening activity observed in the extensometer assays. However, the activity in transgenic tobacco solely expressing the CBM was not significantly different from the empty vector controls (Fig. 5).

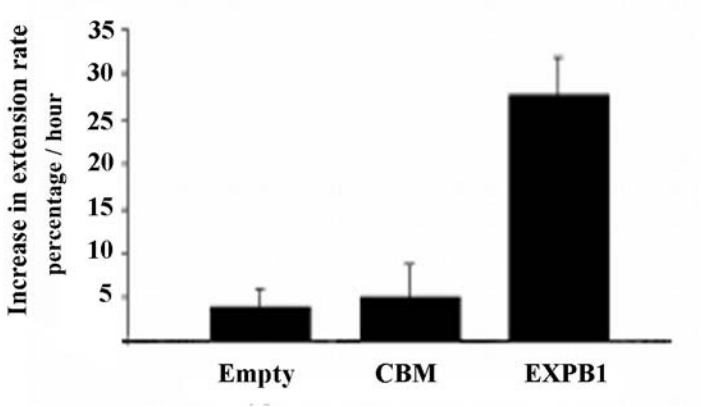
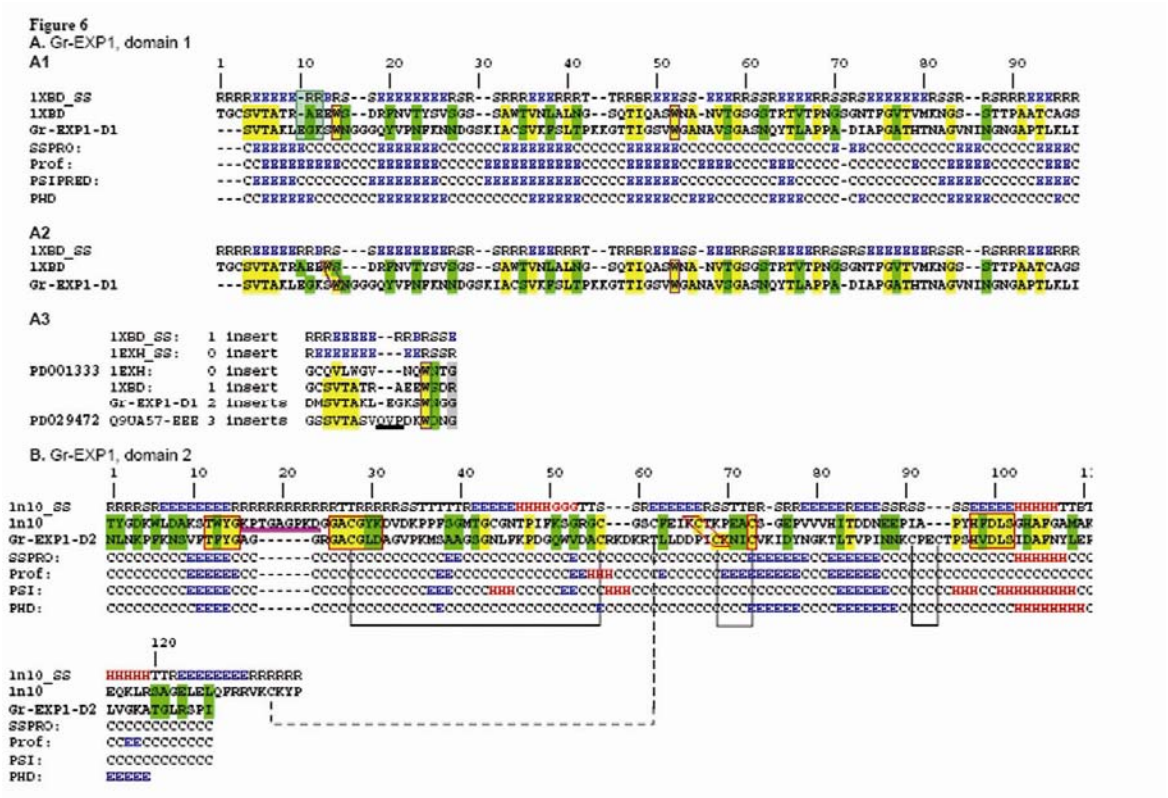


Fig. 5. Effect of tobacco-produced recombinant CBM on the extension rate of wheat coleoptiles. Mature leaf material was collected from two empty vector control lines (Empty), from 3 independent CBM-harboring lines and from 4 independent Gr-EXPB1-lines. For each of the lines, expansin activity was calculated as the difference in extension rates before and after addition of the extract (% increase in length per hour), and activity measurements were done in quadruplicate. Results are given as means with standard errors.

Fig. 6. A. Target-template sequence alignment of Gr-EXPB1 domain 1 and 1XBD, the xylan-binding domain of *Cellulomonas fimi* exo-1,4-β-glycanase (AAB34464). The 1XBD_SS line shows the secondary structure elements derived from the determined 3D structure of 1XBD. The alignment was refined by incorporation of the secondary structure information of Gr-EXPB1 - domain 1 as predicted by four different methods: SSpro, Prof, PSIPRED and PHD. **A1**. Alignment preserving the position of Trp residues (Model 1); **A2**. Alignment preserving the length of the loop between the first β-strand and the first β-bridge (Model 2). **A3**. Alignment of Gr-EXPB1 - domain 1 with various carbohydratebinding domains with different insertion lengths between the first β-strand and the first Trp. PD# PRODOM entries. 1EXH: cellulose-binding domain from *Cellulomonas fimi* (P07986; aa 375-484). Q9UA57: β-1,4-endoglucanase from *Meloidogyne incognita* (aa 406-422). B. Target-template sequence alignment of Gr-EXPB1 - domain 2 and the N-terminal domain of 1n10, pollen allergen phl p 1 from *Phleum pratense*. The 1n10_SS line shows the secondary structure elements derived from 1n10. Straight brackets represent predicted disulphide bridges. The template disulphide bridge not conserved in the model is indicated by a dotted line. Sequence motifs in expansin family (conserved also in Gr-EXPB1 - domain 2) are outlined in red. Conserved residues are highlighted in yellow, similar residues are highlighted in green. Residues affecting the orientation of Trp side-chain (Arg in 1xbd) are highlighted grey. Trp-s binding the ligand are outlined in purple box. B = β bridge; C = coil; E = β strand; G = 310 helix; H = α helix; S = bend; T = turn; R= random.



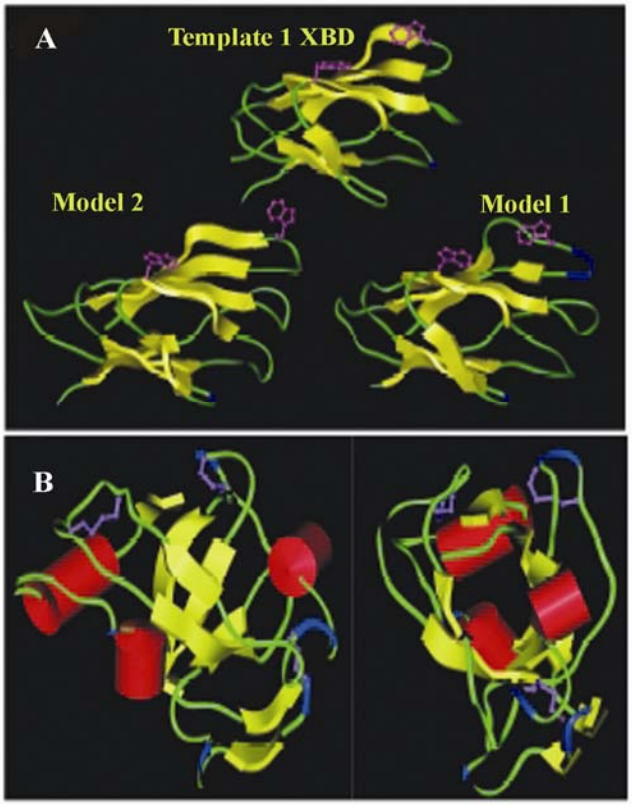


Fig. 7. **A**. Ribbon diagrams of the overall fold for template viz. 1XBD (up), Gr-EXPB1 - domain 1, Model 1 (right) and Model 2 (left). The β-strands are colored yellow; the turns are colored blue and random regions are colored green. Two Trp residues involved in glycan binding are shown in magenta, all atoms. **B.** Overall fold of Gr-EXPB1 - domain 2. The helices are red cylinders; Cys residues forming disulphide bridges are shown in magenta, all atoms

3.5 The 3D structure of Gr-EXPB1 based on remote homology modeling

Domain 1.

The xylan-binding domain from *Cellulomonas fimi* xylanase D (PDB code 1xbd) was identified by 3D-PSSM as the closest template for Gr-EXPB1 - D1 (E-value < 0.005; 18% identity). The target-template alignment was optimized by incorporating secondary structure information and locking the sequence motifs emerged from a PRODOM database analysis. The consensus secondary structure prediction indicated an all-β pattern. Uncertainties in the alignment due to a 2 aa insertion between the first extended β-strand and the first Trp involved in ligand binding prompted us to generate two alternative models (Fig. 6-A1, A2). Both models have the β-barrel structure with aromatic amino acids displayed on the flat surface of CBM2s (Levy et al 2002). Model 1 (Fig. 7, right) preserves a 18.7 Å distance between the two Trp-s involved in ligand-binding – typical for the two crystallized members of PD001333 – by introducing a 3aa loop between the first β-strand and the following β-bridge. This causes an increase in the local flexibility as compared to the other PD001333 structures that show either continuous β-strand or 1 β-bulge in this region (see Fig. 6-A3). It is interesting to note that even longer insertions in the region are present in CBMs from the PD029472 family, suggesting that sequence variability in this region does not affect ligand binding. However, no CBM 3D-structures from the PD029472 family are available. Model 2 (Fig. 7, right) eliminates the 3aa loop by allowing the first β-strand to extend all along to the first Trp involved in ligand binding. Thus, a more stable local structure is formed, but the distance between the two Trp-s involved in recognition increases by ~5-6Å, which may result in a different carbohydrate polymer binding specificity.

Domain 2.

The N-terminal domain of pollen allergen phl p1 from *Phleum pratense* (PDB 1N10) ranked first in searching for Gr-EXPB1-D2 templates (E-value = 3.18 e^{-6} and 20% identity). Other putative, lower ranked templates such as Barwin lectin (E-value = 1.57e^{-1} and 14% identity) significantly differ in the arrangement of local patterns. The conserved motifs between plant expansin domain 1 and Gr-EXPB1-D2 are presented in Fig. 6-B, shaded in red boxes. Two out of the three disulphide bridges of the template are conserved in the target; the third is not present in Gr-EXPB1-D2. It is replaced by another disulphide bridge that emerges naturally from the sequence to 3D structure alignment confirming that the model is presumably close to the real structure. The core structure of Gr-EXPB1-D2 is very similar to the first domain of plant expansins, and differences are located only in three loops of the protein.

4. Discussion

Gr-EXPB1, a functional expansin from the potato cyst nematode *Globodera rostochiensis* (Qin, et al. 2004), consists of a bacterial type (II) CBM with an expansin domain that is more similar to two hypothetical proteins from the aerial mycelium-forming soil bacteria *A. mediterranei* and *S. lavendulae* than to plant expansins. It remains to be shown whether these members of the Actinomycetales produce functional expansins, but this finding could point at expansins or expansin-like proteins among procaryotes. Previously, nematode cell wall-degrading enzymes such as β-1,4-endoglucanases (Smant et al. 1998) and polygalacturonases (Jaubert et al. 2002) were shown to be remarkably similar to their procaryotic and not to their eukaryotic equivalents. Here, the eukaryotic origin of *Gr-EXPB1* is confirmed by the presence of a polyA tail and introns, and our results support the hypothesis that nematode pathogenicity factors related to plant invasion were acquired from bacteria as a result of horizontal gene transfer (Keen and Roberts 1998). Southern blot analysis revealed that *Gr-EXPB1* is presumably member of a small gene family, and this result was confirmed by the finding of another expansin-like cDNA fragment in a potato cyst nematode EST database. Searches in EST databases from other cyst nematode species suggest that the presence of functional expansins is unlikely to be exceptional.

The nematode expansin Gr-EXPB1 showed higher similarity with β-expansins than with α-expansins, and displayed higher cell wall expansion activity on type II primary cell walls (Qin et al. 2004). As compared to α-expansins, β-expansins are known to be less effective on type I primary cell walls (typical for dicots and non-grass monocots). Nevertheless, β-expansins have been identified in a range of dicotyledons (Cosgrove 2000). So far, the function of β-expansins in dicotyledons is not known. Hosts of potato cyst nematodes invariably have type I primary cell walls, and it is at first sight surprising that potato cyst nematodes produce an expansin with apparently a limited impact on type I cell walls.

Remote homology modeling was used to further characterize the two Gr-EXPB1 domains. D1 belongs to the mainly procaryotic CBM 2 family containing members that bind either crystalline cellulose (CBM2a) or xylan (CBM2b). Carbohydrate binding consists of face-to-face hydrophobic stacking interactions between the surface-exposed aromatic rings in the CBM and the non-polar faces of sugar rings in the polysaccharides. The closest template of Gr-EXP1-D1 is an active xylan-binding domain from *Cellulomonas fimi* xylanase D (Millward-Sadler et al. 1994) and the presence of only two (and not three) Trp-s at the binding site (Trp11 and Trp49) points at a high affinity for xylan (Simpson et al. 2000). However, the presence of Gly (instead of Arg) after the first Trp (Fig. 5-A3) was shown to favor cellulose binding (Simpson et al. 2000). Further studies are therefore needed to determine the natural ligand and to discriminate between the two models proposed for D1. The similarities between Gr-EXPB1-D2 and the GH45-like domain (SCOP 50685) of plant expansins

indicate a comparable mechanism for loosening hydrogen bonds. More study is needed to reveal which part of the structure is crucial for this unique activity and why the two domains of Gr-EXPB1 are swapped as compared to plant expansins.
It is concluded that the presence of a functional expansin in the potato cyst nematode *G. rostochiensis* is unlikely to be exceptional among plant parasitic nematodes. Moreover, the remarkable similarity of two putatively extracellular (both ORFD and ORF11 include a predicted signal peptide for secretion) bacterial proteins with Gr-EXPB1 suggests that production of expansin is not necessarily bound to eukaryotes. It would be interesting to test whether saprophytic soil-bound Actinomycetales use expansins for the degradation of plant material.

Acknowledgements.

Funding from the European Union project QLK5-1999-01501 (LQ and UK) is gratefully acknowledged.

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Chapter V

Feeding site development by the potato cyst nematode *Globodera rostochiensis* includes the recruitment of plant β-expansins and expansin-like genes

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Abstract

The expansin superfamily comprises four families: α- and β-expansins (EXPs) and the expansin-like families A and B (EXLs). α- and β-Expansins are extracellular proteins that loosen plant cell walls by weakening the non-covalent bonds between cell wall polymers. So far, genome sequencing projects suggest that β-expansins have relatively more family members in monocotyledons, and - as compared to α-expansins - their capacity to promote cell wall extension in Type I cell walls (mainly dicots) is limited. At present, little is known about the biological functions of β-expansins in dicots. EXLs are identified on the basis of a loosely defined set of conserved amino acid residues, and – virtually without exception – their cell wall-loosening capacity has not been investigated. Here we present evidence that host plant β-expansin and expansin-like genes are up-regulated in the feeding site induced by the potato cyst nematode *Globodera rostochiensis* within roots of dicotyledonous host plants. Both in tomato (*Solanum lycopersicum*) and in potato (*Solanum tubersoum*) novel β-expansins and expansin-like sequences were demonstrated to be induced during early phases of syncytium development. *In situ* mRNA hybridization on infected tomato roots showed expression of the investigated genes in cells newly incorporated into the syncytium. Our data suggest that local cell wall dissolution during syncytium formation requires the concerted action of cell wall-loosening proteins such as β-expansins, and cell wall-degrading enzymes. Moreover, the high expression level of an expansin-like protein in the developing syncytium indicates that EXLs may play important role in the feeding site proliferation as well.

Keywords: β-expansins, expansin-like proteins, plant parasitic nematodes, syncytium

1. Introduction

Expansins are a relatively novel class of proteins that have a unique rheological effect on the plant cell walls. Discovered over a decade ago, they were first associated with a phenomenon called “acid induced growth” which is a rapid increase in extension of plant cell walls at a low pH (McQueen-Mason et al. 1992). Since their first discovery, expansin activity is associated with a whole range of different developmental and physiological processes in plants (see e.g.(Cosgrove 2000). It is hypothesized that expansins weaken non-covalent interactions between cellulose microfibrils and matrix

glycans. As a consequence, the structural network of cell wall polymers becomes more susceptible to turgor-driven extension and to cell wall-degrading enzymes (McQueen-Mason and Cosgrove 1994). Initially, expansins were thought to have evolved exclusively in the land plant lineage. However, the recent finding of a functional expansin in the microscopic worm *Globodera rostochiensis* showed that expansins are also produced outside of the plant kingdom (Qin et al. 2004).

Expansins are subdivided into four families designated α-expansins (EXPA), β-expansins (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB) (Cosgrove et al. 2002). EXPA and EXPB are known to have cell wall loosening activity. α-Expansins are usually dominant in dicotyledones, whereas in monocotyledons the number of EXPB family members is relatively high (Li et al. 2003). This can be illustrated by *Arabidopsis,* poplar and rice that harbor 26, 27 and 34 EXPAs, and 6, 2 and 19 EXPBs, respectively (Sampedro and Cosgrove 2005). In extensometer assays, α-expansins have more effect on cells walls from dicotyledons whereas β-expansins show most activity in cell walls from monocotyledons. Dicots and non-commelinoid monocotyledons have Type I cell walls, whereas the cells walls of monocotyledons are indicated as Type II (Lee et al. 2001). In Type I cell walls the cellulose microfibrils are interlocked by xyloglycans, whereas in Type II cell walls the principal cross-linking polymers are glucoarabinoxylans. Despite the fact that their impact on the extensibility of Type I cell walls is relatively low, the presence of EXPB genes in dicotyledons suggest they have an essential function. Recently, the potato cyst nematode (*Globodera rostochiensis*) was shown to secrete a β-expansin during host plant penetration and it was suggested that this protein could increase the effectiveness of the cell wall-degrading enzymes towards the Type I cell walls (Kudla et al. 2005; Qin el al. 2004).

Cyst nematodes, members of the genera *Heterodera* and *Globodera*, induce the formation of a feeding structure inside the plant root. This feeding structure, commonly indicated as syncytium, is the result of highly coordinated local cell wall dissolution and subsequent protoplast fusion. The resulting syncytium is the exclusive and sole food source for the developing nematode. The developmental pattern of the expanding syncytium virtually excludes the possibility that this progressive cell wall dissolution is the result of the activity of proteins of nematode origin. More likely, the infective second stage juvenile is able to recruit locally plant cell wall-modifying proteins. This was confirmed for several cyst nematode-plant interactions. Infection of tobacco with the tobacco cyst nematode *Globodera tabacum* ssp *solanacearum* was shown to induce the local expression of cellulases (Goellner et al. 2001). *Arabidopsis thaliana* roots infected with *Heterodera schachtii* showed a local up-regulation of pectin acetylesterases (Vercauteren et al. 2002). In another system – soybean cyst nematodes (*Heterodera glycines*) infecting soybean - a polygalacturonase gene was shown to be specifically expressed from 1 to 5 dpi (Mahalingam et al. 1999). Apart from cell wall-degrading enzymes, feeding site induction by nematodes is also

accompanied by the local expression of expansins. Infection of tomato roots with potato cyst nematodes resulted in the up-regulation of specific α-expansins (Golecki et al. 2002). Gal *et al.* (2006) showed recently that the recruitment of specific α-expansins in tomato is not a unique consequence of cyst nematode infection as *LeEXPA5* was also up-regulated as a result of *Meloidogyne javanica* infection (Gal et al. 2006).
In this paper, we demonstrate that tomato and potato harbor β-expansins and expansin-like genes, relatively poorly characterized families in dicotyledones in general and unknown so far in tomato and potato. Moreover, we show that expression of these genes is up-regulated during the first stages of potato cyst nematode-induced syncytium development. Furthermore we discuss why there is apparently a necessity for cyst nematodes to induce α- and β-expansins and expansin-like proteins in the process of feeding site development.

2. Materials and methods

2.1 Plant material

Tomato seeds (*Lycopersicum esculentum* cv Money Maker) were surface sterilized with 0.8% bleach for 25 minutes and rinsed three times with sterile water. Subsequently, surface-sterile seeds were transferred to 1.5% Gelrite plastic plates containing Gamborg B5 medium, including vitamins and minerals, and 2% sucrose (pH6.2); (Duchefa, Haarlem, The Netherlands). For optimal root development, plants were kept in the dark at 24°C. The agar plates were tilted to stimulate root growth along the agar surface.

2.2 Nematode cultures, inoculation and tissue harvesting

In vitro cultures of tomato were inoculated with pre-parasitic second stage juveniles (J2) of the potato cyst nematode *Globodera rostochiensis* pathotype Ro1 Mierenbos. J2 were obtained by soaking cysts in filter-sterile potato root diffusate for 5 days (de Boer et al. 1992). J2 were superficially sterilized using 0.5% (w/v) streptomycin-sulphate and penicillin for 20 min (Duchefa), 0.1% (w/v) ampicillin-gentamicin for 20 minutes (Sigma, Breda, The Netherlands), and 0.1% (v/v) chlorhexidine-digluconate for 3 minutes (Sigma). After washing nematodes three times in sterile tap water, approximately 300 individuals were then transferred to 14 to 18-day-old root tips using a sterile glass Pasteur pipette. Inoculated plants were kept at 18°C in the dark. Small root segments (~ 10 mm in length) were collected from infected and non-infected plants at 3 and 7 day post inoculation (dpi). Root segments were immediately frozen in liquid nitrogen, and kept at -80°C until further processing.

2.3 Identification of tomato β-expansins and expansin like sequences

The SOL (Solanaceae) Genomics Network (http://www.sgn.cornell.edu/) was used to search for expressed sequence tags (ESTs) from tomato (*Solanum lycopersicum*) using β–expansins and expansin-like sequences from *A. thaliana* as query (ATEXPB1-5; GenBank accessions NM_127640, NM_105241, NM_118965, NM_130074, and NM_115921, and ATEXLA1-3; GenBank accessions NM_114466, AF378855, and NM_114465). ESTs showing significant similarity to β-expansin or to expansin-like sequences (Expect (E) value < e^{-20}) were assembled into contigs using the VectorNTI 9.10 software package (Invitrogen, Breda, The Netherlands), and the resulting contigs were checked for the presence of complete open reading frames.

2.4 Semi-quantitative RT-PCR

Total RNA was isolated from 100 mg of (N_2(l))-homogenized tomato root segments using TRIzol Reagent (Life Technologies, Breda The Netherlands). The amount of total RNA was estimated using a spectrophotometer (Amersham, Roosendal, The Netherlands) and the quality was checked by visual assessment on a 1% agarose gel containing Gelstar (Cambrex, Verviers, Belgium). RNA samples were incubated for 1 hour at 37°C in DNAse (0.1U/µl of total volume) (Life Technologies, The Netherlands). First strand cDNA was transcribed from total RNA using the Superscript III kit according to the manufacturer's protocol (Invitrogen). Following first strand cDNA synthesis samples were incubated in 2 units of RNase H in 37°C for 20 minutes (Life Technologies). Controls without reverse transcriptase were included to check for contaminating genomic DNA. Gene specific primers designed for each contig and for a tomato ubiquitin (*Ubi3*; Genbank accession X58253) were used in the following combinations for PCR: F_Contig 1 and R_Contig 1; F_Contig 2 and R_Contig 2; F_Contig 3 and R_Contig 3; F_Contig 4 and R_Contig 4; F_Contig 5 and R_Contig 5; F_ubiq and R_ubiq (Table 1). For each PCR, 6 µl 10X Supertaq PCR buffer (SphareoQ, Gorinchem, The Netherlands), 5 µl 2.5 µM dNTPs (Invitrogen), 3 µl of 12.5 µM of each primer, 0.5 µl of Supertaq enzyme (5 U/µl and 42.5µl of water end volume 60 µl of the reaction). The PCR program included an initial denaturation step at 94°C for 4 minutes followed by 40 cycles of 1 minute at 94°C, 1 minute at 65°C and 1.5 minute at 72°C. An aliquot of 4 µl was taken from each reaction after 16, 19, 22, 25, 30, 35 and 40 cycles and analysed on a 1.5% agarose gel with Gelstar stain (Cambrex).

2.5 Cloning and characterization of full-length tomato genes

Total RNA was isolated from 100 mg of tomato roots infected with *G. rostochiensis* from sterile *in vitro* cultures using TRIzol Reagent according to manufacturer's

protocol (Invitrogen). Dynabeads Oligo (dT)25 (Dynal A.S, Oslo, Norway) were used to isolate mRNA. For each sequence showing up-regulation in infected root segments the 5'-end and 3'-end cDNA ends of the genes were cloned using GeneRace kit according to manufacturer's protocol (Invitrogen). Gene specific primers used for the amplification of the cDNA ends were the same as listed above for semi-quantitative RT-PCR. Fifty µl of a PCR mix included 5 µl 10X Supertaq PCR buffer, 4 µl 2.5 µM dNTPs, 3 µl of 12.5 µM of each primer, 0.5 µl of Supertaq (5 U/µl) enzyme and 34.5µl of water. A touchdown PCR program was executed on the reaction mixes, including an initial denaturation step at 94°C for 4 minutes followed by 15 cycles at 94°C for 1 minutes., 68°C for 1 minute with a reduction of 0.2°C per cycle for each next cycle, 72°C for 1.5 minute followed by 20 cycles at 94°C for 1 minute., 65°C for 1 minutes, and 72°C for 1.5 minutes, and a final elongation step for 10 minutes at 72°C. The PCR product from this reaction was used in the consecutive touch down PCR with the same conditions as specified above using nested primers (Table 1). The amplified fragments were ligated into pCR4-TOPO and transferred to *E. coli* TOP10 chemically competent cells (Invitrogen). Plasmids were purified from liquid cultures with the Wizard Plus Miniprep DNA Purification System (Promega, Madison, WI, USA) and sequenced on an Automated Laser Fluorescent DNA sequencer (Pharmacia, Uppsala, Sweden).

The computer algorithm SignalP-3.0 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the presence of a signal peptide for secretion and a corresponding cleavage site (Nielsen et al. 1997). PROSITE (ExPASy Molecular Biology Server) was used to find biologically significant sites, patterns, profiles and potential post-translational modifications of the investigated conceptually translated proteins (Appel, Bairoch, and Hochstrasser 1994).

2.6 Tissue fixation and embedding

Tomato root segments 1, 3, 5, 7, 10 and 14 days post inoculation were excised and prefixed for two hours in 4% paraformaldehyde in MSB buffer (50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$, pH 6.9). The root segments were washed four times in MSB buffer and dehydrated in a graded series of 10%, 30%, and 50% ethanol containing 10mM DTT at room temperature, and 70%, 96%, 99%, and 100% ethanol containing 10 mM DTT at 4°C for 15 minutes each. Then, the material was incubated sequentially in ethanol: BMM (80% (v/v) buthylmethacrylate, 20% (v/v) methylmethacrylate, 0.5% benzoilethylether, and 10mM DTT) at the ratios of 3:1, 1:1, and 1:3 for 2 hours each at 4°C. Root segments were subsequently washed twice with 100% BMM for 2 hours at 4°C, and then incubated overnight in 100% BMM at 4°C. The material was transferred into BEEM capsules, overfilled with 100% fresh BMM and exposed to UV light for 24 hours at -20°C. The embedded roots were sectioned to

a thickness of 2.0 µm using a Leica RM 2165 microtome (Leica, Mannheim, Germany) and adhered to MJ Research Superfrost microscope slides (MJ Research, Waltham, MA, USA) for 2 hours at 50°C on a slide warmer.

2.7 Digoxigenin-labeled cDNA probes

The primer combinations F_Cont1 and R_Cont1; F_Cont2 and R_Cont2; F_Cont3 and R_Cont3; F_Cont4 and R_Cont4; F_Cont5 and R_Cont5 (see Table 1) were designed to amplify cDNA probes from templates representing the five contigs. The PCR cycling conditions were 2 minutes at 94°C followed by 30 cycles of 94°C for 15 s, 65°C for 30 s, 72°C for 45 s and a final elongation step at 72°C for 7 minutes. Fifty microliters of a reaction volume contained 5 µl 10 x Expand High Fidelity buffer (Roche Diagnostics), 200µM of each dNTP (Amersham, The Netherlands), 0.300nM forward and reverse primers (Table 1), 20ng of a template and 2.6 units Expand High Fidelity DNA polymerase. The sense and antisense single stranded cDNA probes were synthesized separately in asymmetric PCR using PCR Dig Probe Synthesis Kit (Roche Diagnostics) according to manufacturer's instructions using 2.0µl of PCR product from the first PCR as a template either with 2.0µM of reverse or forward primer. The probes were purified using Mini Quick Spin DNA columns (Roche Diagnostics) according to the manufacturer's protocol and probe concentration was estimated on 1.0% (w/v) agarose gel.

2.8 *In situ* hybridization

In order to remove the BMM resin from roots sections the slides were incubated in a series of 5, 10 and 10 minutes in fresh 100% acetone, followed by two incubations of 5 minutes in distilled water. Rehydrated tissue sections were pre-treated with proteinase K (Sigma) (1 µg/ ml in 50 mM Tris-HCL, pH 7.5) for 15 minutes at 37°C. After rinsing twice for 10 minutes with distilled water, the sections were dehydrated in a graded series of ethanol (10, 30, 50, 70, 80, 90, and 100% ethanol for 10 seconds each) and air dried before performing *in situ* hybridization with either single stranded cDNA sense or antisense probes. Twenty microliters of the hybridization buffer, including 50% formamide (Sigma), 10% dextran sulfate (Sigma), 250 ng/ml tRNA (Sigma), 100 µg/ml poly(A) (Sigma), 300 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1x Denhards solution (Sigma), 0.01 mM DTT, and 1 µg/ml DIG labelled ss cDNA antisense or sense probe was applied. Slides were incubated overnight at 45°C in thermocycler (MJ Research). Afterwards sections were washed one time in 2x SSC buffer (Sigma) and three times in 0.2 x SSC (Sigma) buffer at 51°C, for 25 minutes each. The hybridized DIG-labeled probes were detected using Fluorescent Antibody Enhancer Set for DIG Detection according to manufacturer's procedure (Roche Molecular Diagnostics).

Table 1. List of primers

Primer name	Primer sequence (5'→ 3')	Primer name	Primer sequence (5'→ 3')
F_Contig 1	tcgtctctaatagccgctgctggcccttcttt	F_Cont4A	gtgggtccgcgttcggacgtatggctg
R_Contig 1	gccggaataacgccgttagccaccacagtct	R_Cont4A	tctccatcctcaaattccaccaaaagagaaagcc
F_Contig 2	cgcgcttctctcgggtgcttgtggttatggaaa	F_Cont5A	gccgcggcgctcgtgctcctc
R_Contig 2	tagcccaaatccacttgccatcgtaccctgctgt	R_Cont5A	ggttgccgcaggagcccatggacatgaa
F_Contig 3	gcatcgatcgtgttcaggcaagggagtaagagtgg	F_SlEXPB1	atgagtactacttttatggctctaaatccttttattgt
R_Contig 3	gccagcatccaacttccaaacagcaccccaa	R_SlEXPB1	ctagactttaaaattcaccactgatcgataagtttt
F_Contig 4	gccgcggcgctcgtgctcctc	F_SlEXPB2	atgggcttattcgacatatcgcatttagttat
R_Contig 4	ggttgccgcaggagcccatggacatgaa	R_SlEXPB2	ttactctataattgacgagggatcgatacgta
F_Contig 5	ggtagtgacggtggagcatgcgggtacg	F_StEXPB1	atgagtactactcttatggctctgaagccctt
R_Contig 5	tctccatcctcaaattccaccaaaagagaaagcc	R_StEXPB1	aagggcttcagagccataagagtagtactcat
F_Ubiq	atgcagatcttcgtgaaaaccctaacggggaa	F_StEXPB2	atgggcttattcgacatatcgcatttagttat
R_Ubiq	agcaccaccacggagacggagcacga	R_StEXPB2	ctctataattgacgagggatcgatacgtagca
F_Cont1A	caaatccaatacaaacgagtggagtgcaactaccctg	F_SlEXLA1	atgtctctcttcttccttgtcttgcttcttttcgtctc
R_Cont1A	gctgccaacctgccggaataacgcc	R_SlEXLA1	ctaattaagtttccaacttccatcatcacatggggaac
F_Cont2A	ggctatggccattgagggaaaagctcaagatgttca	F_StEXLA1	atgtctctcttcttccttgtcttgcttcttttcgtctc
R_Cont2A	caatgccccacttggcactctgcttgtatccc	R_StEXLA1	ctaattaagtttccaacttccatcatcacatggggaac
F_Cont3A	gcggttgtgatagaatttgaacaaggagatggtgatc	R_Cont3A	gccagcatccaacttccaaacagcaccccaa

2.9 The identification of homologous genes in potato

The presence of homologous genes in *Solanum tuberosum* was investigated using the PCR with genomic DNA as a template and primers designed to *S. lycopersicum* sequences showing up-regulation upon infection, in the following combinations: F_Contig 1 and R_Contig 1, F_Contig 2 and R_Contig 2, F_Contig 5 and R_Contig 5 (Table 1). The PCR consisted of 25 cycles of 40 s at 94°C, 40 s at 60°C and 1 minute at 72°C, followed by 10 minutes incubation in 72°C. The amplified fragments were ligated into pCR4-TOPO and transferred to *E. coli* TOP10 (Invitrogen). The plasmids were purified from liquid cultures and sequenced as described above. In order to investigate the expression of potato homologous root segments were collected from potato plants (*Solanum tuberosum* line RH) infected with *G. rostochiensis*. Except for the temperature (kept at 18°C), potato stem cuttings were grown under the same conditions as the tomato plants. Upon root development, plants were infected with J2s in a similar manner as previously described for tomato. Root segments (~10mm in length) were collected from control and infected plants at 3, 7 and 14-day-post-inoculation. Total RNA was isolated from these samples as described for the tomato root segments and after transcribing it to the cDNA used as a template in semi-quantitive RT-PCR with primers designed to *L. esculentum* sequences: F_Contig 1 and R_Contig 1, F_Contig 2 and R_Contig 2, F_Contig 3 and R_Contig 3 (Table 1). Full-length cDNA sequences of three up-regulated genes were cloned using the GeneRacer kit and analyzed as described above.

2.10 Phylogenetic analysis

An alignment of twenty β-expansins and expansin-like protein sequences was made in BioEdit version 5.0.9 using the ClustalW algorithm (Hall 1999; Thompson, Higgins, and Gibson 1994). The initial alignment was further optimized manually by removing gaps and ambiguously aligned sites. The putative signal peptide sequences as predicted by SignalP-3.0 were removed from all of the aligned sequences. Unrooted phylogenetic trees based on this alignment were inferred using maximum parsimony (Protopars in Phylip; (Felsenstein 1989)) and maximum likelihood (Puzzle; (Strimmer and von Haeseler 1996)) at http://bioweb.pasteur.fr/intro-ukq.html#phylo. For the maximum parsimony analysis, the character set was jumbled 7 times and the tree was evaluated with bootstrapping with 1000 replication and 7 random number seeds. For the maximum likelihood analysis, trees were inferred using the JJT substitution model with 1000 quartet puzzling steps. Three models of rate heterogeneity were used: uniform, two rate (one variable and one invariable) and gamma distributed.

3. Results

3.1 Identification of β-expansin and expansin-like sequences

Table 2. β-expansin and expansin-like tomato sequences selected from the SOL (Solanacea Genomics Network) database. BlaxtX (translated query – protein database) was used to select for most similar non-solaneceous sequences in the public domain.

SOL identifier	Given name	Most similar sequence in public domain	Accession #	Similarity	Identity	E-value
U217172	*SlEXPB1*	putative β-expansin - *Eucalyptus globulus*	DQ100335	88%	75%	3E-89
U216141	*SlEXLA1*	expansin-like protein - *Quercus robur*)	CAE12163	85%	73%	2E-102
C94561	*SlEXPB2*	putative β-expansin - *Eucalyptus globulus*	DQ100335	70%	58%	1E-47
U236451	*SlEXPB3*	putative β-expansin – *A. thaliana*	AY087228	79%	68%	3E-49
E224944	*SlEXPB4*	β-expansin 8 – *Zea mays*	AAK56131	100%	100%	5E-47

Mining of the SOL (Solanacea Genomics Network) database with β-expansins and expansin-like genes from *A. thaliana* (ATEXPB1-5 and ATEXLA1-3) resulted in the identification of 30 ESTs from tomato with an Expect (E) value < e^{-20}. Fourteen ESTs belonged to a single contig (SGN-U217172, 1,120 bp) whereas another set of 13 ESTs was assembled into unigene SGN-U216141 (1,025 bp). Here, SGN- U217172 will be indicated as *SlEXPB1,* and SGN-U216141 will be referred to as *SlEXLA1*.The three remaining singletons SGN-C94561 (481 bp), SGN-U236451 (518 bp; unigene from a

single EST), SGN-E224944 (362 bp), representing transcripts with similarities to *At-EXPB2* from roots, leaves inoculated with *Pseudomonas syringae,* and tomato flowers respectively; will be referred to as *SlEXPB2*, *SlEXPB3* and *SlEXPB4*.

3.2 Temporal expression

The temporal expression of *SlEXPB1*, *SlEXPB2*, *SlEXPB3*, *SlEXPB4* and *SlEXLA1* in nematode infected and non-infected tomato roots was analyzed by semi-quantitative RT-PCR. Primers were designed to generate family member-specific amplification products (*SlEXPB1*, 791 bp; *SlEXPB2*, 469 bp; *SlEXPB3*, 358 bp; *SlEXPB4*, 352 bp; *SlEXLA1*, 701bp), including a ubiquitin (*Ubi3*) as control (230 bp). *SlEXPB1* showed a significant level of expression at 3 and 7 dpi in the infected tomato root segments. Transcript levels of *SlEXPB2* and *SlEXLA1* were up-regulated at 7 dpi. The expression level of *SlEXLA1* was higher as compared to *SlEXPB1* and *SlEXPB2*. *SlEXPB3* and *SlEXPB4* transcripts were not detected in infected or uninfected root segments at any of the given time points. No expression of β-expansin or expansin-like genes was observed in uninfected root segments. Ubiquitin (*Ubi3*) transcript levels were similar in infected and uninfected tomato root segments at all given time points (Fig. 1).

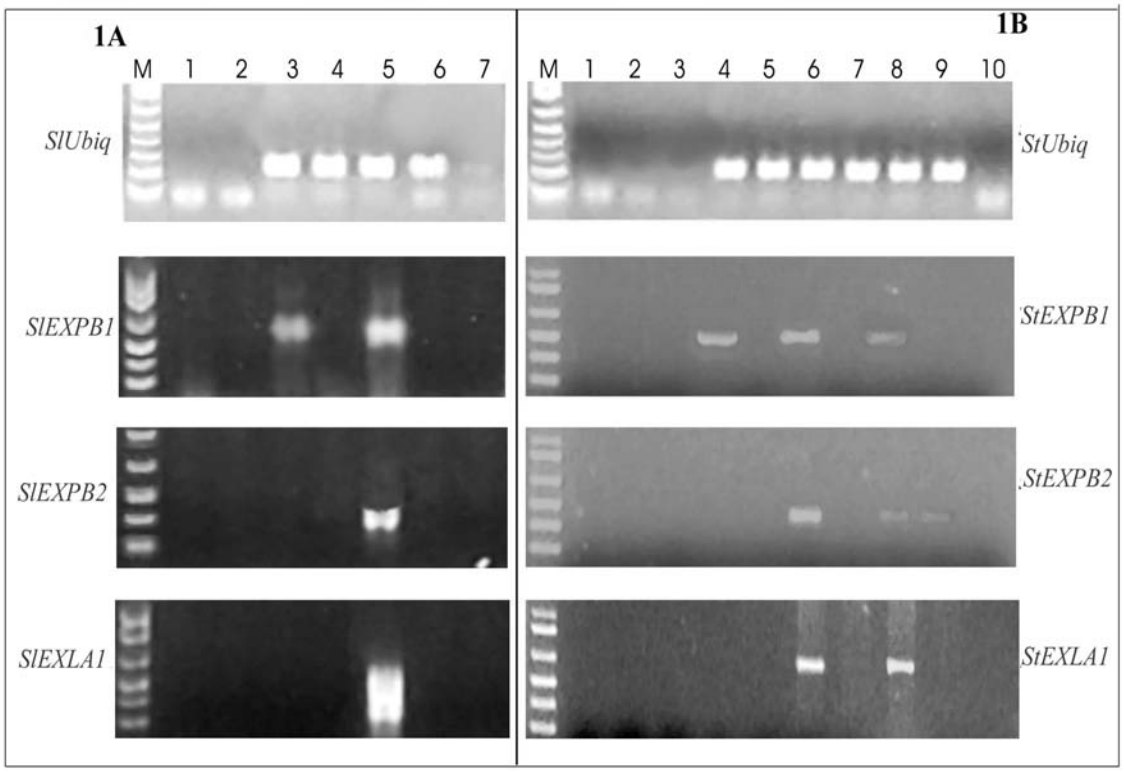


Fig 1. RT-PCR analysis of β-expansins (*EXPB1* and *EXPB2*), expansin-like transcripts (*EXLA*) and constitutively expressed control (*Ubi3*) in potato cyst nematode-infected and non-infected root segments from tomato and potato.

Left panel (1A) – Tomato. **M.** marker; **1.** Negative control - 3 day-post-infection (dpi) without reverse transcriptase (RT); **2.** Negative control - 7 dpi without RT; **3.** Infected 3 dpi; **4.** Uninfected 3dpi control; **5.** Infected 7 dpi; **6.** Uninfected 7 dpi control; **7.** Negative control - no cDNA.
Right panel (1B) – Potato. **M.** marker; **1.** Negative control - 3 day-post-infection (dpi) without reverse transcriptase (RT); **2.** Negative control - 7 dpi without RT; **3.** Negative control - 14 dpi without RT; **4.** Infected 3 dpi; **5.** Uninfected 3dpi control; **6.** Infected 7 dpi; **7.** Uninfected 7 dpi control; **8.** Infected 14 dpi; **9.** Uninfected 7 dpi control; **10.** Control - no cDNA

3.3 Cloning and characterization of full-length tomato genes

The tomato genes *SlEXPB1*, *SlEXPB2* and *SlEXLA,1* which were shown to be up-regulated as a result of a potato cyst nematode infection are derived from ESTs and represented partial gene sequences. To get full-length cDNA sequences, 3'- and 5'-end RACE was performed for each transcript using cDNA isolated from infected tomato roots (see Table 3). Full length sequences (*SlEXPB1*; GenBank # DQ234354, *Sl-EXPB2*; DQ205653, and *SlEXLA*; DQ178133) encode proteins with the domain structure of expansins, namely an N-terminal domain with similarity to glycoside hydrolase family 45 (GH-45) and a C-terminal putative polysaccharide binding domain (see table 3). All three conceptually translated proteins were predicted to be preceded by a signal peptide for secretion. The proteins encoded by *SlEXPB1* and *SlEXPB2* further include a N-linked glycosylation motif near the N- terminus (positioned between amino acids 36-39 and 35-38 respectively), whereas in the expansin-like protein from *SlEXLA1* this motif was found more to the centre of the polypeptide (between amino acids 99-102) (Table 3B). Interestingly, an ATP/GTP binding motif was predicted toward the C-terminus of the *SlEXPB1* protein.
The proteins predicted from *SlEXPB1*, *SlEXPB2*, and *SlEXLA1* include most of the conserved motifs, which have been proposed as key signatures for expansin (Kende et al. 2004; Li et al. 2002) (Figure 3). Six conserved cysteine residues regularly spaced throughout the protein, typical for the expansin superfamily, are conserved in all three genes. However, SlEXLA1 has two additional pairs of cysteine residues; one pair close to the N-terminus and another pair at the C-terminal side of the protein. In SlEXPB1 and SlEXPB2, only one additional pair of cysteines occurs near the centre of the protein sequence. Another characteristic feature of expansins is the so-called HFD–box, which is proposed to be an activity-related motif (Li et al. 2002), based on a specific similarity with GH-45 members (Cosgrove 1999), A HFD-box is found in the SlEXPB1 and SlEXPB2, but is missing in SlEXPLA1. Similarly, the putative polysaccharide binding domains in SlEXPB1, SlEXPB2, and SlEXLA1 include a series of conserved tryptophane residues, hydrophobic groups that are supposed to be involved in substrate binding.

Table 3. Domains and functional sites in tomato expansins (EXPB and EXLA) up-regulated during syncytium formation by the potato cyst nematode *Globodera rostochiensis*. Predicted cleavage site[*]: amino acid position where the signal peptide for secretion is cleaved off from the precursor protein. Domain I[#]: N-terminal domain with similarity to glycoside hydrolase family 45. Domain II[##]: putative polysaccharide binding domain. Potential phosphorylation site[+]: number of casein kinase II phosphorylation sites ([a]), protein kinase C phosphorylation sites ([b]), tyrosine kinase phosphorylation site ([c]) and cAMP- and cGMP-dependent protein kinase phosphorylation sites ([d]).

Gene	# bp	# aa	Predicted cleavage site[*]	Domain I[#]	Domain II[##]	Potential N-glycosylation sites (# and position)	Potential phosphorylation sites[+]	ATP binding motif (position)
SlEXPB1	825	279	29/30	64-174	187-269	1 (36-39)	1[a];4[b];1[c]	259-266
SlEXPB2	852	284	28/29	61-173	186-279	1 (35-38)	2[a];6[b];1[d]	
SlEXLA1	786	262	17/18	38-142	156-238	1 (99-102)	2[a];4[b];	

3.4 Tissue localization of the gene expression

To localize the expression of the *SlEXPB1*, *SlEXPB2* and *SlEXLA1* genes in nematode infected roots we preformed mRNA in situ hybridization on sections of the tomato root from 1, 3, 5, 7, 10 and 14 dpi. For *SlEXPB1* expression was detected in infected tomato from 3 dpi to 10 dpi, with a maximum level reached at 5 dpi (Fig. 2 A, B, C and D). The transcripts from *SlEXLA1* were detectable in infected tomato from 5 dpi onwards including all samples collected at the last time points in our series at 14 dpi. The highest level of expression of *SlEXLA1* was detected between 5 and 7 dpi, after which it declined to lower but consistent level at 14 dpi (Fig 2 G, H I, and J). The probe designed on *SlEXPB2* specifically labeled the cytoplasm of syncytial cells only at 5 dpi (Fig 2 E and F). The hybridization of *SlEXPB2* was relatively weak compared to *SlEXPB1* and *SlEXLA1*. For all genes transcription was localized exclusively in the cytoplasm of syncytial cells at the peripheries of the syncytium, but not in the surrounding cells. No hybridization was observed in any of the sections labeled with sense probes (Fig.2 K, L). In addition, no expression was detectable with probes designed on the two singletons, named hereafter *SlEXPB3* and *SlEXPB4*, confirming the results from the RT-PCR (data not shown).

3.5 Cloning and characterization of *SlEXPB1*, *SlEXPB2*, and *SlEXLA1* homologs from potato

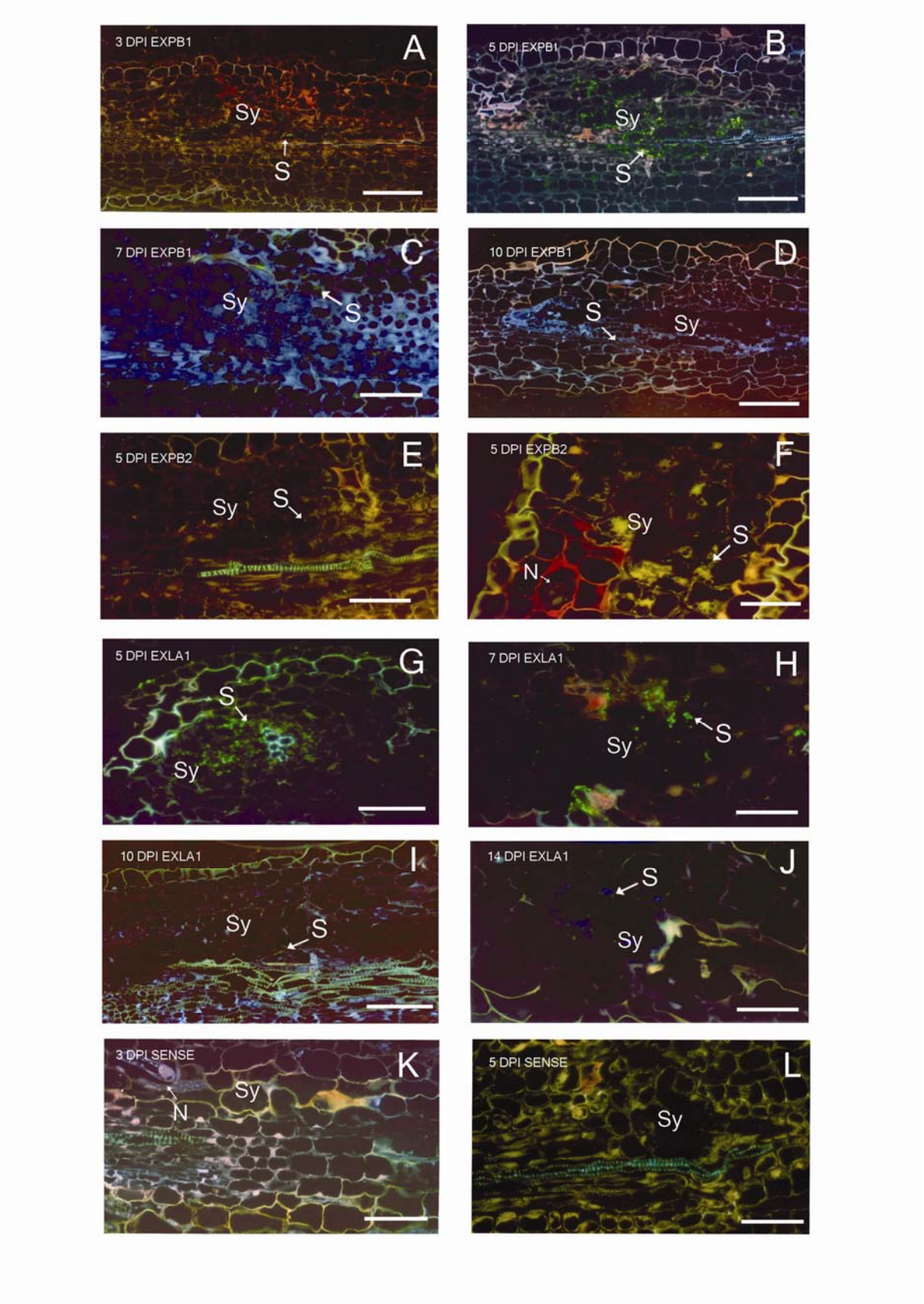
To investigate if β-expansin and expansin-like proteins are also involved in the parasitism of *G. rostochiensis* in other Solanaceaous host plants, we aimed to identify the potato homologs of SlEXPB1, SlEXPB2, and SlEXLA1. First, genomic DNA fragments were amplified from potato DNA using heterologous primers designed on *SlEXPB1*, *SlEXPB2*, and *SlEXLA1* (Table 1). Sequencing of the amplified products revealed high level of conservation between potato and tomato homologs (more than 90% identity for all homologous pairs), and consequently potato homologs were named *StEXPB1*, *StEXPB2* and *StEXLA1*.
To check the temporal expression pattern in developing syncytium a semi quantitative RT-PCR was carried out with the same set of primers as for tomato experiment (Table 1) using cDNA from infected and uninfected potato roots collected on 3,7 and 14 day post infection. The expression of *StEXPB1*, *StEXPB2* and *StEXLA1* followed the same pattern as the expression of the homologs in tomato. The *StEXPB1* transcripts were present at all the analyzed time points (3, 7 and 14 dpi). For *StEXPB2* the expression was detectable in the material collected at 7 dpi infected material, and at 14 dpi infected and uninfected material. Finally the *StEXLA1* transcripts were present at 7 and 14 dpi only in the infected material. No expression was observed for all three genes in control reactions without Reverse Transcriptase or without the template. (Figure 1B). The full length sequences (*StEXPB1* Genebank # DQ314758, *StEXPB2*, DQ314759; and *StEXLA1*, DQ314757) were cloned using 3'- and 5'-end RACE with cDNA from infected potato roots as template. The proteins encoded by the transcripts of *StEXPB1*, *StEXPB2* and *StEXLA1* shared high level of identity with their tomato homologues, viz. *StEXPB1* and *SlEXPB1* are for 94.2% identical, *StEXPB2* and *SlEXPB2* are for 93.7% identical, and *StEXLA1* and *SlEXLA1* are for 91.2% identical. The homologous protein have similar domain topology and include the same signifying motifs (see Table 4, and Fig 3).

Table 4. **A**. Most significant BlaxtP (protein query – protein database) results for potato β-expansin or expansin-like proteins up-regulated as a result of potato cyst nematode-induced syncytium formation. **B**. Domains and functional sites in potato expansins (EXPB and EXLA) up-regulated during syncytium formation by the potato cyst nematode *Globodera rostochiensis*. Predicted cleavage site*: amino acid position were the signal peptide for secretion is cleaved off from the precursor protein. Domain I: N-terminal domain with similarity to glycoside hydrolase family 45. Domain II: putative polysaccharide binding domain. Potential phosphorylation site^{+}: number of casein kinase II phosphorylation sites (^{a}), protein kinase C phosphorylation sites (^{b}), tyrosine kinase phosphorylation site (^{c}) and cAMP- and cGMP-dependent protein kinase phosphorylation sites (^{d}).

A. Gene name	Most similar seq.	Accession #	Similarity	Identity	E-value
StEXPB1	cim1 protein – soybean (*Glycine max*)	Q39802	78%	66%	8E-88
StEXPB2	Putative beta-expansin - *Eucalyptus globules*	DQ100335.1	74%	61%	2E-70
StEXLA1	expansin-like protein - *Quercus robur*	CAE12163	85%	74%	2E-105

B. Gene name	# bp.	# aa	Predicted cleavage site*	Domain I#	Domain II##	Potential N-glycosylation sites (# and position)	Potential phosphorylation sites+	ATP binding motif (position)
StEXPB1	828	276	29/30	65-175	188-270	1 (37-40)	1[a];4[b];1[c]	260-267
StEXPB2	837	279	28/29	61-173	186-273	1 (35-38)	2[a];6[b]	
StEXLA1	780	260	17/18	38-142	156-238	1 (99-102)	1[a];4[b]	

Fig 2. In situ hybridization of two β-expansins and expansin like in tomato roots infected with *G. rostochiensis.* **A)** longitudinal section through 3 days old syncytium hybridized with *SlEXPB1* antisense probe. **B)** longitudinal section of 5 days old syncytium hybridized with *SlEXPB1* antisense probe. **C)** longitudinal section of 7 days old syncytium hybridized with *SlEXPB1* antisense probe. **D)** longitudinal section of 10 days old syncytium hybridized with *SlEXPB1* antisense probe. **E)** longitudinal section of 5 days old syncytium hybridized with *SlEXPB2* antisense probe. **F)** cross section of 5 days old syncytium hybridized with *SlEXPB2* antisense probe. **G)** cross section of 5 days old syncytium hybridized with antisense probe. **H)** cross section of 7 days old syncytium hybridized with *SlEXLA1* antisense probe **I)** longitudinal section of 10 days old syncytium hybridized with *SlEXLA1* antisense probe. **J)** cross section of 14 days old syncytium hybridized with *SlEXLA1* antisense probe. **K)** longitudinal section through 3 days old syncytium hybridized with a tomato *SlEXPB1* sense probe. **L)** longitudinal section of 5 days old syncytium hybridized with *SlEXLA1* sense probe;
Sy – syncytium; S- signal; N- Nematode, The scale bar – 25 μm


3 DPI EXPB1
A
Sy
S
5 DPI EXPB1
B
Sy
S
7 DPI EXPB1
C
Sy
S
10 DPI EXPB1
D
S
Sy
5 DPI EXPB2
E
Sy
S
5 DPI EXPB2
F
Sy
S
N
5 DPI EXLA1
G
S
Sy
7 DPI EXLA1
H
Sy
S
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3 DPI SENSE
K
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N
5 DPI SENSE
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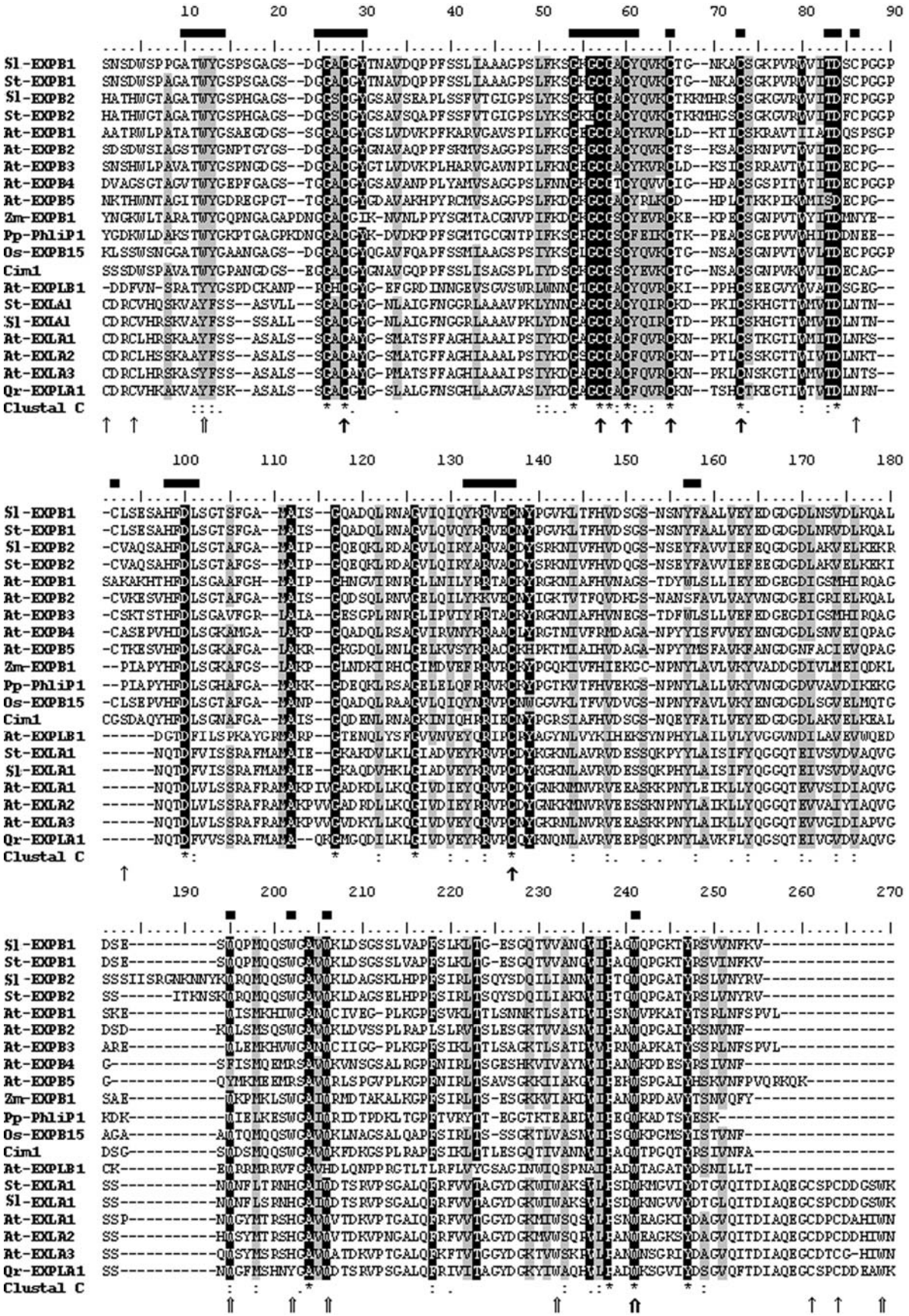
Sl-EXPB1
St-EXPB1
Sl-EXPB2
St-EXPB2
At-EXPB1
At-EXPB2
At-EXPB3
At-EXPB4
At-EXPB5
Zm-EXPB1
Pp-PhliP1
Os-EXPB15
Cim1
At-EXPLB1
St-EXLA1
Sl-EXLA1
At-EXLA1
At-EXLA2
At-EXLA3
Qr-EXPLA1
Clustal C

Fig 3 Alignment of β-expansin and expansin like sequences from : *Solanum esculentum*: SlEXPB1(Genebank #. DQ234354), SlEXPB2 (DQ205653), SlEXLA1 (DQ178133), *Solanum tuberosum*: StEXPB1 (DQ314758), StEXPB2 (DQ314759), StEXLA1 (DQ314757), *Arabidopsis thaliana* At-EXPB1 (Q9SKU2), At-EXPB2 (Q9SHY6), At-EXPB3 (Q9M0I2), At-EXPB4 (Q9SHD1), At-EXPB5 (Q9M203), At-EXLA1 (Q9LZT4), At-EXLA2 (Q9SVE5), At-EXLA3 (Q9LZT5), At- EXLB1 (O23547); *Oryza sativa* Os-EXPB15 (AAM73779), *Zea mays* Zm-EXPB1 (AAK56124), *Glycine max* cim1 (AAA50175), *Phleum pratense* Phl p1 (CAA55390), using the ClustalW-1.8 algorithm. The conserved residues are shaded in black and indicated as * on the consensus sequence, conserved substitutions are shaded in gray and indicated as : in consensus sequence, semi-conserved substitutions are indicated as . . ↑ - points at conserved cysteines, ↑-pointes at partially conserved cysteine, ⇑-tryptophan ⇑ - partially conserved tryptophan. ▄ - indicates conserved motif according to Li at el. 2002.

3.6 Phylogenetic analysis

The evolutionary relationships between tomato (SlEXPB1, SlEXPB2, SlEXLA1) and potato sequences (StEXPB1, StEXPB2, StEXLA1) and other β-expansin and expansin-like family members was studied using maximum parsimony and maximum likelihood methods (Fig. 4). Both methods resulted in the inference of trees with similar topology and relative support for the clades. The expansin homologues (SlEXPB1, SlEXPB2, StEXPB1 and StEXPB2) together with β-expansin from soybean (Genbank accession number S48032), rice (Os-EXPB15, AAM73779) and expansin B2 from *A. thaliana* (Q9SHY6) formed a separate clade. Within this clade SlEXPB1 and StEXPB1 created a separate cluster and the same was observed for SlEXPB2 and StEXPB2. The expansin-like homologues (*SlEXLA1* and *StEXLA1*) clustered separately with *A. thaliana* expansin-like sequences At-EXLA1(Q9LZT4), At-EXLA2 (Q9SVE5), and At-EXLA3(Q9LZT5) and expansin –like from *Q. robur* (CAE12163).

4. Conclusions and Discussion

The highly-directed progressive cell wall dissolution resulting in local cell wall openings and consequent protoplast fusion is essential step in the formation of a nematode-induced syncytium. In the interaction between the beet cyst nematode *Heterodera schachtii* and *Arabidopsis thaliana,* the first signs of cell wall modifications were observed as early as 18 h after the nematode had selected an initial syncytial cell (Grundler et al. 1998). There is ample experimental support for the role

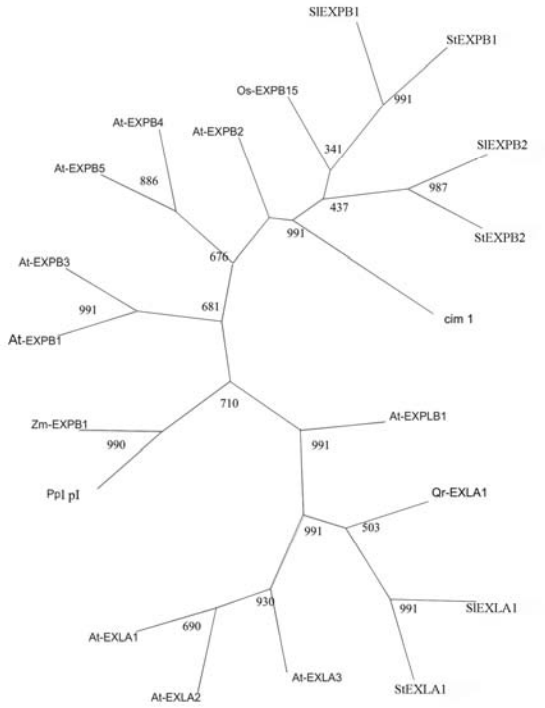

Fig 4. Phylogenetic tree of β-expansin and expansin like sequences from: *Solanum esculentum*: SlEXPB1(Genebank #. DQ234354), SlEXPB2 (DQ205653), SlEXLA1 (DQ178133), *Solanum tuberosum*: StEXPB1 (DQ314758), StEXPB2 (DQ314759), StEXLA1 (DQ314757), *Arabidopsis thaliana* At-EXPB1 (Q9SKU2), At-EXPB2 (Q9SHY6), At-EXPB3 (Q9M0I2), At-EXPB4 (Q9SHD1), At-EXPB5 (Q9M203), At-EXLA1 (Q9LZT4), At-EXLA2 (Q9SVE5), At-EXLA3 (Q9LZT5), At- EXLB1 (O23547); *Oryza sativa* Os-EXPB15 (AAM73779), *Zea mays* Zm-EXPB1 (AAK56124), *Glycine max* cim1 (AAA50175), Phleum pratense Phl p1 (CAA55390), *Quercus robur* (CAE12163).The tree was constructed using maximum parcimony method. The bootstrap values are positioned at the corresponding branches of the unrooted trees

of the cell wall-degrading enzymes in cyst nematode-induced syncytium formation (see Introduction). Apart from de-polymerizing enzymes, cyst nematodes also induce the local production of α-expansins, proteins that weaken non-covalent interactions within the plant cell wall (Golecki et al. 2002). In this paper we present data that point at a role for β-expansins in the process of feeding site formation by potato cyst nematode in their host plants tomato and potato. Contrary to the α-expansins, very little is known about the functions of β-expansins in dicotyledones, and our finding could also contribute to a better understanding of their role in dicots. We hypothesize that plant β-expansins are recruited by potato cyst nematodes – in addition to plant

cell wall-degrading enzymes - to increase the accessibility of the plant cell wall to its own cell wall-degrading enzymes.

Identification of β-expansins and expansin-like genes as plant genes contributing to nematode-induced feeding site formation adds to our current understanding of the complexity of cyst nematode-induced cell wall modifications. The timing of the expression of *SlEXPB1*, *SlEXPB2*, and *SlEXLA1* between 3 dpi to 14 dpi largely parallels the expression of the conventional cell wall-degrading enzymes and *SlEXPA5* described above. Within this timeframe the expression of the β-expansins *SlEXPB1* and *SlEXPB2* declines after 5 dpi, while the expression of *SlEXLA1* starts at 5 dpi and sustains albeit at a lower level at least until the last time-point in our sampling series at 14 dpi. When comparing the tissue specificity of the transcription of β-expansins and expansin-like proteins with that of cell wall-degrading enzymes and the α-expansin there is a remarkable difference. At later stages the expressions of cellulases, pectin acetylesterase, and α-expansin also occurs in cells immediately adjacent to the developing feeding site. In contrast, the expression of *SlEXPB1*, *SlEXPB2*, and *SlEXLA1* was specifically localized in the cytoplasm of cells newly incorporated in the syncytium, and not in surrounding cells. This implicates that the cell wall-degrading enzymes and the α-expansins may be involved in different type of cell wall modifications in syncytial cells than β-expansins and expansin-like proteins. The cell wall dissolution at the earliest stages of the inclusion of cells into a syncytium aims at the fusion of individual protoplasts, whereas other types of modifications may facilitate cell wall extension during hypertrophic swelling of syncytial cells. Immunolocalisation experiments with antisera towards the proteins encoded by *SlEXPB1*, *SlEXPB2*, and *SlEXLA1* may resolve if these proteins have sites of activity different from cell wall degrading enzymes and α-expansins.

Studies on the function(s) of β-expansins in dicotyledones and the regulation of their expression are scarce. In 1998, Downes and Crowell reported about a cytokinin-inducible β-expansin from soybean (*cim 1*). Cim 1 was shown to accumulate 20-60 fold in a cytokinin-starved soybean suspension culture upon cytokinin addition (Downes et al. 2001). More recently, three closely related β-expansins were isolated from the ephemeral flowers of four o'clock (*Mirabilis jalapa*). *MjEXPB1* was preferentially expressed during early floral development, and transcript levels dropped dramatically at the onset of expansive growth of the calyx (Gookin et al. 2003). The β-expansin expression pattern in four o'clock flowers was positively correlated with endogenous gibberellin levels. Local changes in phytohormone levels (especially auxine and ethylene – there is no reliable information about changes in cytokinin or gibberellin levels) are associated with the induction of syncytia by cyst nematodes. Clearly, it is impossible at this moment to make any statement about nematode-induced, phytohormone-mediated regulation of β-expansin expression.

Expansin-like genes share a limited set of characteristic motifs with "true" expansins. The role of expansin-like genes in plants has not been studied extensively and little is

known of their role in plant cell morphogenesis and growth. The tomato and potato expansin-like genes studied in this paper are expressed at high levels in nematode-induced syncytial cells and their expression largely coincides with the expression of other cell wall-modifying proteins in syncytium formation. Also, the presence of a signal peptide for secretion to the extracellular matrix in the plant, the polysaccharide binding domain and the overall similarity to expansins suggests that expansin-like protein are most likely involved in the cell wall modifications too. Expansin-like genes are also found in the slime mold *Dictyostelium discoideum*, in which the expression of at least one EXL parallels the expression of cellulose synthase genes suggesting that it may be involved in the synthesis and assembly of cell wall polymers (Darley et al. 2003; Eichinger et al. 2005). Cell wall modifications in nematode feeding sites also include the biosynthesis of cell wall ingrowths at the interfaces of syncytial cells and vascular tissues. It is therefore conceivable that expansin-like genes have a role in this specific event in the constitution of the syncytium. This hypothesis will be challenged in our laboratory with immunolocalisation of the expansin-like proteins at the subcellular level in developing syncytia.

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Chapter VI

General disussion and concluding remarks

General discussion and concluding remarks

1. Introduction

The main objective of this thesis was to investigate two distinct groups of proteins involved in plant cell walls modifications in the parasitism of the potato cyst nematode, *Globodera rostochiensis*, namely pectate lyases and expansins. Plant parasitism of potato cyst nematode proceeds through two main stages i.e. mobile and sessile. During the migratory phase, potato cyst nematode uses cell wall degrading enzymes and mechanical force of the stylet protruding from nematode's buccal cavity to disrupt cell walls in host plant tissue. In order to degrade plant cell wall components, nematodes produce a range of cell wall degrading and modifying proteins. Plant parasitic nematodes were the first animals for which it was shown that they are able to degrade cell wall polymers (i.e. cellulose microfibrils) without the help of symbiotic microorganisms (Smant et al. 1998). This first finding of an endoglucanase produced by *G. rostochiensis* turned out to be only a tip of an iceberg. To date, similar evidence is found in plant parasitic nematodes for the presence of enzymes capable of depolymerising the pectin matrix in plant cell walls (i.e. pectate lyase and exopolygalacturonase) (Doyle and Lambert 2002; Jaubert et al. 2002; Popeijus et al. 2000). In Chapter II, a novel putative pectate lyase gene from *G. rostochiensis* is described including a functional assay using *Agrobacterium* mediated leaf infiltration and site directed mutagenesis. In Chapters III and IV, we showed that potato cyst nematode is producing and secreting expansins, which are likely to aid the activity of cell wall degrading enzymes. Contrary to pectinolytic enzymes expansins are a relatively newly described group of proteins and there are still a lot of unresolved issues that need to be addressed with regard to their mode of action, biological functions and classification. Since their discovery in 1992, expansins were thought to be specific for land plants (McQueen-Mason et al. 1992). In this thesis, we present the first evidence of a functional expansin produced outside of the plant kingdom. We demonstrated that invasive juveniles of *G. rostochiensis* secrete β-expansin, which most likely facilitates the disruption of cell walls in the root tissues of a host by making the structural cell wall polymers more accessible to cell wall degrading enzymes.

The cell wall modifications occurring during feeding site formation by potato cyst nematode, described in the fourth chapter of this thesis, are part of the elaborate changes in the plant cell wall architecture during syncytium induction and maintenance. In contrast to the rigorous breakdown of cell walls and subsequent collapse of the protoplast during nematode migration, the modifications during feeding site formation aim at a subtle remodeling of the cell wall, including both cell wall dissolution and synthesis (Golinowski et al. 1996; Grundler et al. 1998). The

uniform structure of nematode induced syncytia suggests that these cell wall modifications are precisely controlled. A key role for the recruitment of plant enzymes (e.g. cellulases and pectin methyl esterases), by nematodes to modify plant cell wall structure to their advantage has been pointed at in several papers (Gheysen and Fenoll 2002; Goellner et al. 2001; Vercauteren et al. 2002). The data presented in Chapter V shows that these cell wall modifications in a nematode-induced syncytium are mediated by enzymes and β-expansins and expansin-like proteins of the host plant.

2. The role of pectinolytic enzymes in nematode parasitism

2.1 Pectate lyases in Potato Cyst Nematode

Chapter II describes the identification of a novel pectate lyase named Gr-PEL2. This pectate lyase is produced in the subventral oesophageal glands of the invasive juveniles of *G. rostochiensis*. Like all other pectate lyases identified in nematodes so far, Gr-PEL2 belongs to the polysaccharide lyase family 3. The polysaccharide lyase family 3 also includes pectate lyases from other plant pathogens, both bacteria and fungi. In addition, a group of hypersensitive response inducing proteins called harpins have ancillary domains that are structurally related to pectate lyases in PL family 3 (Charkowski et al. 1998). Studies on the three-dimensional protein structure of the pectate lyases (from PL families 1, 2, 3, 9, and 10) revealed an unusual right-handed helical structure build from parallel β-strands, which contrary to all other known protein structures does not contain α-helices between them. This structure is stabilised by an asparagine ladder in the core of the protein (Yoder et al. 1993).
The Gr-PEL2 protein shows highest similarity to pectate lyases from facultative plant parasitic nematodes *Bursaphelenchus xylophilus* and *B. mucronatus*, and to actinobacterial members of the PL3 family, and not to the previously identified pectate lyase Gr-PEL1 from *G. rostochiensis.* In the current analysis the pectate lyases of nematode origin seem to form three separate clades. One distinct cluster is formed by pectate lyases 2 and 3 from *Meloidgyne incognita,* Gr-PEL1 and three pectate lyases from *Heterodera glycines*. The second cluster includes pectate layse 1 from *M. incognita*, and *M. javanica*. Finally, the Gr-PEL2, together with pectate lyases from *B. xylophilus* and *B. mucronatus*, forms the third cluster. This surprising branch topology of the PL3 family, which confirms considerable divergence between Gr-PEL1 and Gr-PEL2, indicates that it is unlikely that they might have evolved from a common ancestor. Moreover, Gr-PEL2 is remarkably similar to bacterial pectate lyases produced by saprophytic actinobacteria (phylum Actinobacteria), which obtain nutrition from non-living organic material unlike the cyst nematodes - exclusive parasites of Solanacea family. Therefore, taking into account this substantial difference between the life styles of these two organisms, it seems unlikely that

similarity of the pectate lyase in *G. rostochiensis* to bacterial genes is a result of a substrate driven converging selection.

Horizontal gene transfer has been proposed as a way by which plant parasitic nematodes have acquired cellulases and possibly other parasitism genes from plant-pathogenic or saprophytic microbes (Yan et al. 1998). This hypothesis is based on a discrepancy between phylogenies of the proteins (the protein tree) and the hosting organisms (the species tree). The alternative hypothesis for this discrepancy in the occurrence of sequence similarity is vertical gene transfer conditioned by converging evolutionary forces such as similarity in substrates. Possibly the strongest argument advocating for existence of horizontal gene transfer in the development of plant parasitism in nematodes is the recent finding of the cellulase in the pine wood nematode *Bursaphelenchus xylophilus*, which is distantly related to other plant parasitic nematodes members of the Tylenchida. This cellulase belongs to glycoside hydrolase family 45 (GH45) and seems to be closely related to fungal enzymes, whereas all other cellulases identified so far from plant-parasitic nematodes belong to GH5 and show higher similarity to bacterial proteins (Kikuchi et al. 2004). *Bursaphelenchus xylophilus* is a facultative plant parasitic nematode that can both feed on plants and on fungi, and is more closely related to fungal feeding nematodes than to other nematodes strictly feeding from plants. This fact suggests that these two groups of nematodes may have independently developed the ability to break down cellulose and that genes responsible for it were acquired from plant parasitic fungi and bacteria, respectively. This suggests that if horizontal gene transfer occurred, it was not a unique event, but might be a more common phenomenon involved in evolution of nematodes.

Although it remains to be seen whether this is typical for Tylenchomorpha in general, it is striking to see that PL identified in nematodes so far belong to the same family. If they have indeed been acquired via horizontal gene transfer from actinobacteria such as members of the genus *Streptomyces*, which possess polysaccharide lyases that belong to 5 different families, shouldn't the nematode genome sequences reflect the diversity of the donor genome? Virulence factors in plant and animal pathogenic bacteria are often clustered in the same genomic region called pathogenicity islands, which differ in their G+C content, presence of repeat sequences at their ends and other mobility factors from the rest of the genome. These regions are genetically unstable and their horizontal transfer between different bacterial species is a well established part of the evolution of micro-organisms (Hacker and Kaper 2000; Morschhauser et al. 2000). One could speculate that horizontal gene transfer between bacteria and nematodes follows the same pattern and the whole pathogenicity islands or at least larger fragments of it would have been transferred rather than a specific gene. Arguments for or against this horizontal gene transfer hypothesis will be delivered with the completion of genome sequencing projects on plant parasitic nematodes and detailed analysis of the genomic location of parasitism genes.

To functionally analyze Gr-PEL2, we used a new approach which combines 3D structure homology modelling, site directed mutagenesis and transient expression *in planta* using *Agrobacterium* infiltration in leaves. From a biologist's point of view, this method allows to investigate parasitism genes in a manner more resembling the natural conditions compared to standard biochemical assays based on model substrates. It also allows studying the simultaneous effect of various cell wall degrading enzymes on the host tissue by co-infiltration of several genes encoding enzymes from different classes. Moreover, our method was sensitive enough to partially resolve ambiguities in the 3D protein model, and allowed us to exclude one of the two potential Ca^{2+} binding sites.

The significant differences between amino acid compositions (~31 % identity over 212 amino acids), especially between the number of strongly charged amino acids of Gr-PEL1 and Gr-PEL2, suggest a significant difference in their ability to recognize modified pectic polymers. Furthermore, most of the residues putatively involved in activity of Gr-PEL2, and other pectate lyases from PL3, are not conserved in Gr-PEL1. This is rather surprising considering that catalytic amino acids are usually subjected to strong evolutionary constraints. A structural comparison of other pectate lyases in polysaccharide lyase family 3 shows a high level of conservation in the immediate region around the catalytic site. The ability to recognize different substrates is attributed to the high structural diversity of the so-called substrate binding groove surrounding the catalytic site (Herron et al. 2000). This prompted us to study Gr-PEL1 in more detail to localise potential catalytic amino acids based on a model of the 3D dimensional structure of the protein. The models suggest that the residues likely to be responsible for Ca^{2+} in Gr-PEL2, are not conserved in Gr-PEL1 (thus indicating that either ligand binding in Gr-PEL1 does not require Ca^{2+}, or that bond between Ca^{2+} and Gr-PEL1 is formed by different, not identified in the model amino acids). Also, the models predict that the charge distribution between the two proteins is significantly different, with Gr-PEL2 being more charged and more acidic. These structural differences between Gr-PEL1 and Gr-PEL2 may reflect significant differences between substrate specificity, such as polymer length, charge, level of methylation, etcetera. To validate these models, *in vitro* tests of the enzymatic activity level with various substrates should be carried out. It would also be interesting to perform detailed mutagenesis experiment to identify amino acids indispensable for Ca^{2+} binding in Gr-PEL1. Activity tests of Gr-PEL2 (Chapter II) performed with complex substrate such as plant cell wall, allowed for estimation of the general biological effect that these enzymes might have in the context of plant – parasite interactions, but are not useful in substrate fingerprinting experiments. This could be performed only in highly defined *in vitro* conditions.

2.2 Pectate lyases in nematode parasitism of plants

The database searches using *Gr-Pel2* as query resulted in the finding of 23 ESTs from *H. schachtii* and 16 from *H. glycines* coding for single pectate lyase genes. A similar search performed with *Gr-Pel1* yielded 9 matching ESTs from *G. rostochiensis* coding for Gr-PEL1 and 2 ESTs from *H. glycines*. There is no *G. rostochiensis* EST corresponding to *Gr-Pel2* in the database, which suggests either a low level of expression or simply is a result of a limited amount of ESTs sequenced from this nematode (5941 ESTs). The largest number of ESTs from pectate lyase genes originate from second stage juveniles, but interestingly some of the matching ESTs from *H. glycines* are isolated from the fourth stage juveniles (Genebank access no CB299897) and virgin females (CB279891) indicating that pectate lyases might be also involved in feeding and not only in migration of the nematodes. It is plausible that pectate lyase might be also expressed in the mobile stage of adult male as was previously shown for a cellulase in root knot nematodes (Rosso et al. 1999) Unfortunately, there is no EST library from adult males of any of the plant parasitic nematodes to confirm this hypothesis.
Considering their distinctive modes of host penetration, one could assume that the intercellular migration of root knot nematode through the middle lamella is to the greater extent dependant on pectinolytic enzymes than the intracellular migration of cyst nematodes. Furthermore, in contrast to cyst nematodes, root knot nematodes have wide host ranges including hundreds of species from different families, which implies that root knot nematodes are facing a greater diversity in pectic polysaccharides. This hypothesis finds support in the significant quantitative differences in expression levels and genetic diversity of pectinolytic enzymes, as deduced from the EST database analysis. The two most comprehensive sets of ESTs are from *M. hapla* and *H. glycines* including approximately 24,000 ESTs for each species. In the *M. hapla* dataset there are 315 ESTs showing significant homology to pectate lyases, whereas from *H. glycines* only 22 ESTs show such similarity. Moreover, these 315 ESTs from *M. hapla* cluster into five groups, which are likely to represent five different genes. The twenty-two ESTs isolated from *H. glycines* breakdown into three separate groups likely corresponding to three different genes. Moreover, cyst nematodes have been reported to produce only pectate lyases whereas in root knot nematodes polygalacturonases are also found. The completion of the genome sequencing of *M. hapla* and *H. glycines* will provide better insight into the full range of pectinolytic enzymes deployed by these nematodes.

2.3 Pectinolytic enzymes in other organisms

Pectinolytic enzymes are produced by a variety of plant pathogens to reduce the cell wall rigidity (Collmer and Keen 1986). Pathogens deploy pectinolytic enzymes to get

quick access to the nutrients in the plant cell cytoplasm (De Lorenzo and Ferrari 2002; Idnurm and Howlett 2001). The severity of the symptoms caused by plant pathogens is often associated with the range and the level of expression of pectinolytic enzymes. For instance, *Xanthomonas campestris* pathovar *campestris* causing black rot secretes two pectin acetylesterases, two polygalaturonases, and four pectate lyases, whereas the closely related *Xanthomonas axonopodis* pv. *citri* agent of citrus canker lacks pectin esterases and produces only two pectate lyases. Pectin esterases cleave off lateral branches in pectins, which makes backbone chain more susceptible to the attack by pectin lyases and polygalacturonases (Collmer and Keen 1986). Therefore, pectin esterases enhance other pectinolytic enzymes and their absence from *X. axonopodis* pv. *citri* can at least partially explain the difference in severity of damage between the two closely related pathogens (Van Sluys et al. 2002). There are no reports on pectin esterases produced by sedentary plant parasitic nematodes, and considering their biotrophic mode of parasitism it is possible that they are not included in nematode's arsenal of cell wall degrading enzymes, as such extensive damage to the host tissue is of no advantage to the biotrophic plant parasitic nematodes.

The studies on pectinolysis processes are most detailed for the interactions of the necrotrophic soft rot bacterium *E. chrysanthemi* and its host plants. *E. chrysanthemi* is expressing more than 10 different pectinolytic enzymes, which allows the complete breakdown of pectic polymers (Hugouvieux-Cotte-Pattat et al. 1996). It has been shown that the expression level of pectate lyases in *E. chrysanthemi* varies depending on the host, for instance on pea plantlets only the expression of pelA seems to be required for tissue maceration, whereas the development of soft rot symptoms on chicory leaves requires expression of all pectate lyases with exception of pelE (Beaulieu and Van Gijsegem 1990). Moreover, a detailed analysis of spatial distribution of *E. chrysanthemi* pectinolytic enzymes *in planta,* revealed that two pectate lyases (PelB and PelC) are preferentially found in middle lamella and cell junctions, whereas two others (PelD and PelE) are located along the plasmalemma of cell walls (Hugouvieux-Cotte-Pattat et al. 1996). Furthermore, deletion of five major pectate lyase genes expressed by *E. chrysanthemi* did not eliminate the tissue maceration activity, revealing the existence of a whole new set of pectate lyase genes (Kelemu and Collmer 1993). Evidently, disruption of all functionally redundant genes is required for demonstrating the role of enzymes in pathogenesis. It would be interesting to perform similar experiments in plant parasitic nematodes and to investigate if pectinolysis is regulated in similarly complex manner. Obviously, this type of experiments would be most informative if performed with the root knot nematode as they may relay on various pectinolytic enzymes to the higher extent than cyst nematodes.

For a long time, pectate lyases were thought to be strictly involved in plant pathogenesis and produced exclusively by plant pathogens (Collmer and Keen 1986). This was proven wrongly with the advent of the genome sequences of *A. thaliana* (25

putative pectate lyase genes) and *Oryza. sativa* (8 putative pectate lyase genes) (CAZY 2005; (Coutinho and Henrissat 1999)). The first report of pectate lyases in a plant showed their presence in pollen where they are suggested to loosen the cell wall to enable the pollen tube emergence and penetration of the style (Taniguchi et al. 1995; Wu et al. 1996). EST sequences encoding pectate lyases were also isolated from libraries made from germinating seeds, developing flowers, ovaries, pollen, trichomes and ripening fruits, which suggests that pectate lyases gene expression is associated with diverse developmental processes in plants (Marin-Rodriguez et al. 2002). Apparently, even though the general mechanism of catalysis is shared by different pectate lyases the effect they have on plant tissue seems to be more diverse than just the overall tissue maceration. This is in agreement with the tissue changes observed on the leaves infiltrated with Gr-PEL2, which showed necrosis only within the infiltration place itself, whereas the surrounding tissue showed wrinkling and folding.

2.4 Oligogalacturonides

Plant cell walls constitute a forefront of plant defence against pathogens. They must be degraded to allow successful penetration and colonization. Analysis of the products released during degradation of cell wall polymers led to the idea that they could act as endogenous elicitors of plant defence response (Darvill et al. 1992). Products of pectin metabolism i.e. oligogalacturonides (previously referred to as oligosaccharins) were demonstrated to induce plant defences (Reymond et al. 1995). Oligomers released from potato cell walls by *E. carotovora* pectate lyases were shown to induce accumulation of phytoalexins (i.e. antimicrobial compounds) (Forrest and Lyon 1990). Oligogalacturonides also play regulatory role in plant development. The responses that the oligogalacturonides elicit in plants are quite diverse. For example, early parietal degradation during the ripening of fruit, by both the pectate lyase and the cellulase genes have been proposed to produce oligogalacturonides that promote the ethylene climacteric rise in the ripening (Trainotti et al. 2003).
The release of oligogalacturonides by pectate lyases produced by *E. carotovora* subsp *carotovora* has been demonstrated to up-regulate a WRKY-like transcription factor in potato plants (Dellagi et al. 2000). The WRKY-like transcription factors bind to the promoters of pathogenesis-related proteins, i.e. proteins coded by the host plant but induced only in pathological or related situations (Rushton and Somssich 1998). The differential up-regulation of WRKY-like transcription factor in the course of potato infection by *Phytophthora infestans* was correlated with the initial phase of penetration of the epidermis by the pathogen and later in the necrotrophic phase when a substantial amount of oligogalacturonides was generated from cell wall breakdown. During the intermediate biotrophic phase of infection when the haustorium is formed the expression of WRKY was significantly lower (Dellagi et al. 2000). The expression of one of the WRKY family members was also shown to be increased during the

infection of host plants by both root knot nematode and cyst nematodes (Barthels et al. 1997; Gheysen and Fenoll 2002; Puthoff et al. 2003). Unfortunately, the analysis concentrated only on the feeding site development phase and there is no information available as to the WRKY expression during the migration of the nematode. The expression of the WRKY transcription factor is already high in 2-day old feeding site, the level of expression is maintained until the 7th day, afterwards it decreases gradually and is almost undetectable in mature feeding site (Barthels et al. 1997). Nevertheless, the up-regulation of WRKY during the nematode infection coincides with up-regulation of plant polygalacturonases, pectin methyl esterase, and pectin acetylesterase which are recruited by the nematode in order to form functional feeding site (Puthoff et al. 2003; Vercauteren et al. 2002).
Moreover, even at very low concentration, oligogalacturonides antagonize the action of auxin such as auxin-induced growth (Branca 1988), auxin dependant somatic embryogenesis and auxin stimulated rooting in leaf explants (Fry 1993; Filippini 1992). There is ample evidence that the induction and maintenance of syncytium is strongly linked with auxin accumulation, and that nematodes are likely to manipulate the auxin household of a plant (Gheysen and Fenoll 2002; Goverse et al. 2000). Therefore, oligogalacturonides -induced responses seem to be undesirable for successful parasitism, which may have resulted in decreased reliance of nematodes on pectinolityc enzymes in order to avoid accumulation of pectin breakdown products. Alternatively, such avoidance could also have led to a limitation in time of the expression of pectate lyases, which is in accordance with an immediate shut down of subventral glands once the nematode finds a feeding site.

2.5 Pectate Enzyme redundancy

The relative importance of each of the two pectate lyases produced by *G. rostochiensis* to the successful parasitism is difficult to estimate as there are currently no protocols available for stable transformation of plant parasitic nematodes. The contribution of pectate lyases to the pathogenesis in other host–pathogen combination (e.g. pathogenic fungi *Nectria hematococca* vs. *Pisum sativum* and *Colletotrichum gloeosporioides* vs. *Citrullus lanatus* var. *lanatus*) has been analyzed to a great detail (Rogers et al. 2000; Yakoby et al. 2000). In general, the pectin-degrading enzymes were shown to contribute to symptom development especially in diseases leading to soft rot (Bauer et al. 1994; Collmer and Keen 1986). Moreover, the level of expression of pectinolytic enzymes is often positively correlated with the severity of the symptoms (Van Sluys et al. 2002). However, when it comes to a quantitative estimation of the role of specific enzymes the picture becomes more complex. In several gene disruption experiments plant pathogens carrying truncated pectate lyases did not show a substantial decrease in the virulence (Bowen et al. 1995; Gao et al. 1996; Scott-Craig et al. 1998). The most likely explanation for an unchanged

virulence is that the enzymes play only a minor role in pathogenesis (Bowen et al. 1995) However, pathogenic fungi and bacteria usually have multiple genes encoding pectinolytic enzymes with overlapping activities. This apparent redundancy complicates attempts to show the importance of individual genes. Disruption of one enzyme is not likely to cause striking decrease in virulence level, as their loss of function may be compensated by others (Kelemu and Collmer 1993). Evidently, disruption of all functionally redundant genes is required for demonstrating the role of a group of enzymes in pathogenesis (Rogers et al. 2000).
Recent developments in the gene silencing in nematodes using RNA interference may help to assess the relative importance of pectate lyases in plant parasitism of *G. rostochiensis*. Gene silencing with RNA interference mimics loss-of-function mutations and at the same time allows precise targeting of genes of interest. It is based on the ability of dsRNA to trigger the degradation of homologous cellular RNA and was proven to be a powerful tool to comprehensively study gene function in plants (Hilson et al. 2004) and animals (Maeda et al. 2001). Recently, Urwin at el. (2002) demonstrated gene silencing with RNA interference by stimulating the cyst nematodes to orally uptake the dsRNA together with a visual marker in vitro (Urwin et al. 2002). This protocol has currently been modified in several laboratories (Chen et al. 2005; Rosso et al. 2005). In natural conditions plant parasitic nematodes do not ingest until they establish a feeding site inside a plant root, therefore an artificial stimulation by chemicals is a rather invasive method that may affect the outcome of an experiment. Alternatively, dsRNA could be expressed in a host plant wherefrom it would be ingested by a feeding nematode. However, this approach can be only used to investigate the role of genes expressed in the later stages of parasitism, as there will be no dsRNA uptake until the feeding commences. Therefore, it could be only applied if pectate lyases are indeed produced in these later stages, most likely the adult males, to asses their importance to the development of the syncytium.

2.6 Functional analysis of pectate lyases

One of the objectives of this thesis was to determine the role of Gr-PEL2 in disease development. We presented a novel approach to investigate the effect that cell wall degrading enzymes may have on plant cell walls. It is likely that nematodes secrete a range of variuous enzymes at the same time. By using co-expression in plant tissue one can mimic the parasite's potential to breakdown cell wall by simultaneously testing a range of cell wall degrading enzymes with a complex substrate more closely resembling natural conditions of the parasite. Our method, combined with site directed mutagenesis, also proved to be sensitive enough to study the involvement of specific amino acids in folding and activity of Gr-PEL2 as indicated by the 3D modelling.
A drawback of our approach relates to the difficulties of making objective observations of the phenotype in a quantitative manner. To score the severity of the

phenotype, it is necessary to perform multiple observations to exclude the effect that the variation of tissue between individual leaves will have. This is illustrated by the significant differences in the severity of the phenotype that were found, depending on the age of the infiltrated leaf. To make a comparative analysis between mutant and wild-type protein it is of key importance to use leaves of the same developmental stage. Another problem in making a comparison is related to the difficulties in standardizing the amount of the protein that is being produced in the leaves. A significant improvement to the method could be made by using a reporter tag in the constructs to determine protein levels with immunodetection. Despite these disadvantages we have shown that infiltration, combined with 3D modelling, and site directional mutagenesis is relatively simple and efficient method to test the biological effect of parasitism genes on plant tissues. The general applicability of this method depends to a large extent on the nature of the putative parasitism genes. For genes encoding pioneering sequences the method could be used as a preliminary screen using a series of morphological and molecular parameters. Most likely, certain parasitism genes will simply fail to trigger detectable changes in plant tissue, and therefore lack of visible morphological changes with these pioneers does not have to point at the lack of function in parasitism. On the other hand, it is also conceivable that genes not involved in parasitism may cause phenotypic changes, which could be misleading when screening a set of genes with unknown biochemical mode of action.

3. The role of nematode expansins in plant parasitism

3.1 Expansin in Potato Cyst Nematodes

The first identification and analysis of a functional expansin gene from an organism outside the plant kingdom, in the potato cyst nematode, is presented in Chapters III and IV of this thesis. Expansins were discovered more than a decade ago as the mediators of ‘acid growth’ in plant cell enlargement. Expansins are defined by the presence of several conserved motifs in the sequence and a characteristic cell wall loosening activity. They were thought to be a group of proteins exclusively present in plants (Cosgrove 1998). Sequences with similarity to expansins had been found in other organisms before, but they lacked the characteristic expansin activity and thus were regarded as expansin-like proteins. In this thesis we provide evidence that proteins with both the structural and functional characteristics of expansins are secreted by plant parasitic nematodes. Plant parasitic nematodes were previously shown to secrete cellulose and pectin degrading enzymes to facilitate host penetration. Our current findings suggest that the action of these conventional cell wall degrading enzymes is complemented with the activity of expansins.

Expansins are cell wall proteins that have unique loosening effects on cell wall polymers and their activity is associated with plant cell growth, cell wall disassembly,

cell separation, pollen tube penetration and leaf primordium initiation (see for instance: http://www.bio.psu.edu/expansins/). Expansins increase the mobility of cell wall polymers allowing the walls to expand as the structural elements slide apart. Their action does not involve hydrolysis and they are thought to weaken the hydrogen bonds between the cellulose microfibrils and matrix polymers (Cosgrove 2000). We have shown that Gr-EXPB1 has indeed ability to increase the extension rate of cell wall polymers without detectable hydrolytic activity, and that this ability is dependent on expansin-like domain of the Gr-EXPB1 protein.

3.2 β-expansins in the nematode migration

The infective juveniles invading host roots use a combination of mechanical force of the stylet and enzymatic activity of cell wall-degrading enzymes to penetrate plant cell walls. The expansin activity most likely makes the cell wall polymers more accessible for the activity of the cellulase and the pectinases in nematode secretions. Such a concerted action of expansins and cell wall degrading enzymes has already been demonstrated during ripening of fruits (Powell et al. 2003). Gr-EXPB1 is most likely secreted by the nematode into the plant tissue, during the intracellular migration of invasive larvae, where it increases accessibility of the compact cell wall polymers to the co-secreted cell wall degrading enzymes. A comparison of homogenates from second stage juveniles and young females revealed that only proteins produced by J2s are capable of inducing polymer slippage in isolated cell walls, indicating that the presence of an active expansine is correlated with the migratory phase of the nematodes. The cocktail of cell wall-degrading enzymes and expansins, which simultaneously disrupts the covalent and the non-covalent bonds between cell wall polymers, is likely to account for the high rate of penetration by *G. rostochiensis*. Database searches suggest that expansins are common in many plant-parasitic taxa in the phylum Nematoda (Kudla et al. 2005; Roze Unpublished data).

The Gr-EXPB1 protein shows highest similarity with plant β-expansins, which have high activity on the type II cell walls present in monocots (order Poales). Extensometer assays with homogenates of the second stage juveniles showed a much higher activity on wheat coleoptiles when compared to cucumber hypocotyls. This is rather striking given that the host range of *G. rostochiensis* is limited to the members of the dicotyledonous Solanacaea family. The implications are, that either the extensometer assays as a method is not representative for the activity and the role of expansins *in vivo*, or that wheat coleoptiles and cucumber hypocotyls are poor substrates for expansins acting on root tissues, or both. Alternatively, only a very subtle action could be required as a preparation for cell wall degrading enzymes produced by invading nematodes. Future biochemical analysis on the mode of action of expansins will help us to better understand the roles of this group of proteins in biological processes.

3.3 Functional analysis of expansins

Active proteins from expansin genes are notoriously difficult to produce in heterologous protein expression systems. In order to obtain biologically active Gr-EXPB1 protein we have attempted three expression systems using bacteria, yeasts and plants as a host. Expression in bacteria and yeast resulted in the production of a significant amount, but of an inactive protein. Prokaryotes are not capable of posttranslational modifications required for most eukaryote proteins to perform their function. Expanins typically include several disulphide bridges in their structure that may not be properly established in bacteria. Our 3-D model of Gr-EXPB1 indicates that the structure of the protein is stabilized by three of these disulphide bridges. Expression in yeast does allow for posttranslational modifications, including the formation of disulphide bridges, but presents another problem with proteolysis and hyperglycosylation of recombinant proteins. The only expression system in our hands that allowed for proper processing of the protein in order to obtain active product at sufficient quantities, was expression *in planta*. The stable transformation of *N. benthamiana* plants with *Gr-EXPB1* under the control of CaMV 35S promoter resulted in high expression of biologically active protein. Interestingly, all tested lines of plants over-expressing the *Gr-EXPB1* gene displayed an impaired overall growth resulting in shorter leaves and internodes (data not shown). The specificity of this phenotype was established by plants expressing the *Gr-EXPB1* without a signal peptide for secretion to the extracellular matrix, which did not show the aforementioned phenotype. A similar phenomenon was observed by McQueenMason et al. in tomato plants over expressing α-expansin 1 from cucumber (Rochange and McQueen-Mason 2000). This finding also raises further doubts as to whether the extensometer assay is a reliable indicator for expansins activity. For instance *Gr-EXPB1* does not show significant effect on the hypocotyles of the dicot cucumber *in vitro*, whereas *in vivo* it has a profound effect on the morphology of the dicot *N. benthamiana*.

The extensometer assays measures the extension rate in isolated cell walls exposed to the putative expansins *in vitro*. As the expansins are most likely active on the interface between different polymers present in the cell walls, it is important that these experiments are carried out with highly heterogeneous substrate such as plant tissue. However, the main disadvantage of this system is in fact that tests are performed either on hypocotyls or coleoptiles, and due to the technical obstacles it is difficult to use cell walls isolated from other parts of the plant. Clearly, for analysis of the Gr-EXPB1 it would be most informative to use roots of the host plant in extensometer assay. Another drawback is the discontinuity of the tested material, as the tissue is composed of many cell walls joined by middle lamellae, which is not ideal for mechanical testing. This last problem can be solved by using pellicle of a bacterium *Gluconacetobacter xylinus* in extensometer tests, which in presence of tamarind

xyloglucan synthesizes extracellular network of crystalline cellulose and hemicelluloses resembling structure of the plant cell wall (Whitney et al. 2000). Nevertheless, one should keep in mind that this is a simplified two component structure, which therefore cannot reflect all the properties of the plant cell wall and is at best indicative for expansin activity.

Typical expansins comprise two domains, i.e. polysaccharide binding domain and a cysteine-rich region with limited sequence similarity to family-45 endoglucanases (GH45-like domain). Members of GH45 have been found in a variety of organisms, mainly fungi, but also a bacterium, a protist living in the hindgut of termites, and a mollusk (Cosgrove et al. 2002). At this time, attempts to show the production of reducing sugars associated with expansin activity have failed, and it is concluded that expansins lack hydrolytic activity on glucans (Cosgrove 2000). Moreover, certain endoglucanases of fungal origin induce extension of heat-inactivated plant cell walls in a manner only slightly different from expansins while retaining the endoglucanase activity (Yuan et al. 2001). To investigate whether Gr-EXPB1 exhibits any endoglucanase activity we tested the recombinant protein for hydrolytic activity in a cellulose plate assay. No endoglucanase activity was detected for the recombinant protein (data not shown). Therefore, the increased cell walls extension induced by Gr-EXPB1 most likely results from the reversible disruption of non-covalent bonds between cellulose microfibrils and matrix polysaccharides in the cell walls, in a manner that is typical for expansins.

3.4 Classification of expansins

The classification and the nomenclature used for members of the expansin superfamily is currently a topic of debate. Initially, to be classified as an expansin, protein had to meet the following criteria: 1) induce the acidic extension of cell walls without hydrolysis and 2) possess two domains preceded by a signal peptide for secretion, where one domain shows similarity to the GH45-like domain and another one showing similarity with polysaccharide-binding modules. Currently, it has been proposed that proteins meeting the above stated criteria, but originating from other organisms than plants should be referred to as expansin-like group X (EXLX) (Kende et al. 2004). The line of reasoning proposed by Cosgrove in support of this nomenclature is that it is not technically possible to assay the activity of every newly identified protein with sequence similarity to expansins (see: http://www.bio.psu.edu/expansins/). This is, however, not an effective criterion to achieve a unifying system in the nomenclature of expansins. For instance, according to the nomenclature proposed by Kende et al. (2004) all newly identified plant genes with similarity to expansins would be designated as expansins without necessity of testing their biochemical properties. Therefore, this may lead to the situation where expansin-like genes that have lost or changed their activity would be designated as

expansins, whereas proteins with proven expansin activity but originating outside of plant kingdom would not.
The prevailing principle, for the annotation of novel proteins that show significant homology to other functionally characterized proteins, is that the protein name is preceded by the adjective "putative" (e.g. putative cellulase) as long as its activity is not demonstrated. There is no reason why the same principle should not be applied to expansins. Moreover, no distinction based on origin is made for other groups of proteins shared by many different taxa, i.e. endoglucanases are designated as endoglucanases regardless if they are produced by plant pathogenic bacteria or by a tomato plant. Proteins should be preferably classified according to their biological function. Therefore, if the expansin in question is proven to posses the expansin activity and induces the polymer relaxation in the plant cell walls, it is most informative to designate it as an expansin. Proteins with similarity to expansin sequence alone should be referred to as putative expansins.

3.5 Expansins in other organisms

The best matches in database searches for Gr-EXPB1 are two genes with unknown function from *Amycolatopsis mediterranei* (Genebank accession number: AJ319869; E value i.e. expectation value; E value = $8e^{-11}$, identity 35%, similarity 53%) and *Streptomyces lavendulae* (AF127374; E value = $2e^{-9}$, identity 36%, similarity 50%). These proteins have not been tested for expansins activity, and nor is it clear what other function the encoded proteins may have. It would be interesting to see what the role of expansins could be for these organisms. Other proteins showing similarity to plant expansins have been found in the blue mussel (*Mytillus edulis;* CAC59694*)*, fungi (*Trichoderma reesi,* CAB92328*)*, bacteria (*Clavibacter michiganensis* AAK16222, *B. subtilis*, Z99114; *Xylella fastidiosa,* ZP_00681293) and slime molds (*Dictyostelium discoideum*; XM_633425). All of these expansin-like proteins contain N-terminal signal peptide for secretion and show different levels of conservation within motifs characteristic for plant expansins. These findings suggest that nematodes may not be unique in their ability to use expansins for degradation of cell wall polymers.
Both *C. michiganensis* and *X. fastidiosa* are biotrophic pathogens of plants. Therefore, it is possible that expansins are used by these bacteria to enhance the action of cell wall degrading enzymes in a similar manner as *G. rostochiensis*. Interestingly, the expansin-like domain in these bacteria is a part of a larger gene including a cellulase domain. The combined presence of these two domains in a single protein corroborates the hypothesis that the expansin domain enhances the hydrolytic activity of the cellulase domain (Laine et al. 2000; Li et al. 2002).
The fungal expansin-like protein isolated from saprophytic fungus *Trichoderma reeseii* was named swollenin due to its ability to swell cotton fibers without producing

reducing sugars (Saloheimo et al. 2002). It contains a polysaccharide binding domain and an expansin-like domain, however, similarly to Gr-EXPB1 the two domains are in a reversed order as compared to plant expansins. The expression of swollenin is regulated in the same way as fungal cellulolytic enzymes by plant material (Saloheimo et al. 2002). This supports the hypothesis that swollenin is involved in biomass conversion rather than in processes associated with modifications of the fungal cell wall (Saloheimo et al. 2002).

The expansin-like protein secreted into the lumen of the digestive gland of a blue mussel has been reported to possess limited expansin activity and is likely involved in digestion of plant material (Xu et al. 2001; Xu et al. 2000). However, the endogenous origin of this protein has not been confirmed.

Finally, a whole family of expansin-like proteins is expressed during the development of slime mold *D. discoideum* (Darley et al. 2003). The aforementioned proteins of non-plant origin from *A. mediterranei, S. lavendulae,* C. michiganensis, *X. fastidiosa T. reeseii* and blue mussel, are likely to be involved in degradation of plant material. Interestingly, proteins produced by slime-mold are suggested to play regulatory role in slime mold development. The slime mold forms rapidly growing stalk, which imparts the mechanical strength from the extracellular matrix containing cellulose. It appears that expansin-like proteins are involved in maintaining the fluidity and extensibility of the matrix (Darley et al. 2003).

Sequences from *A. mediterranei, S. lavendulae* and *T .reesei* show higher similarity to plant β-expansin like Gr-EXPB1(Qin et al. 2004; Saloheimo et al. 2002). In contrast, proteins from blue mussel and *Clavibacter michiganensis* are more similar to α-expansins (Laine et al. 2000; Xu et al 2001). If these proteins are indeed involved in pathogenicity and / or saprophytism it would suggest that there is no clear separation in the biological function between α- and β-expansins. It would also mean that there is no direct correlation between the type of expansin and the type of host. β-Expansins might be used by parasites of dicots and saprophytes, and α-expansins by herbivorous mussel and pathogenic bacteria thriving on both mono and dicots.

3.6 Gamma expansins - Natriuretic peptides in parasitism

G. rostochiensis also produces a secretory protein named Gr-EXPB2 (Qin et al. 2001), which shares homology with expansin-like domain in Gr-EXPB1, but lacks a polysaccharide binding domain. Recently, a novel third subfamily of γ expansins was identified comprising of proteins also with only one domain with homology with N–terminal domain of α and β expansins. The biological function of these proteins in plants is not well understood, however, initial experiments have shown that they do not exhibit cell wall loosening activity in extensometer assays (Cosgrove et al. 2002). The blight-associated protein p12 from the plant *Citrus jambhiri* is considered to

belong to the γ-expansin superfamily (Ceccardi et al. 1998).This latter plant protein is expressed in roots and stems in response to a challenge from citrus blight. Luddidi at el. (Ludidi et al. 2002) speculated that this protein may function as natriuretic peptide hormone implicated in the regulation of salt and water homeostasis and is an early host response to counteract the pathogen induced limitation of water and nutrient availability. Despite low level of homology (31% identity, 44% similarity between Gr-EXPB2 and p12), comparison between Gr-EXPB2, p12, a homolog of plant natriuretic peptides from *Xanthomonas axonopodis* (NP_642965.1), and plant natrurieti peptides (*Hedera helix*, AAO85279 and *A. thaliana* NP_849979) revealed series of conserved motifs (data not shown).
Plant natriuretic peptides cause enhanced osmoticum-dependant water uptake and therefore increase the cell turgor (Maryani et al. 2001). The homolog of plant natriuretic peptides in plant pathogenic bacteria *X. axonopodis* is believed to disturb the homeostasis in host plants to provide access to water and water soluble nutrients (Nembaware et al. 2004).
A strong increase of turgor pressure is one of the characteristics of nematode induced feeding structures. Plant assimilates and ions are continuously uploaded into the feeding structures from surrounding cells in unidirectional way and while the nematodes take up nutrients from the cytoplasm, water is being actively transferred into the feeding site. Considering the similarity between Gr-EXPB2 and the plant natriuretic peptides homolog from *X. axonopoda*, it is possible that plant parasitic nematodes make use of natriuretic peptides in a similar manner to *X. axonopodis*. To this purpose, homogenates from nematodes could be tested for the ability to increase the osmotically induced swelling of potato protoplasts (Maryani et al. 2001).

4. The recruitment of plant expansins during nematode-induced feeding site formation

In Chapters II to IV, we concentrated on nematode-produced cell wall modifying proteins that are involved in penetration of and migration in the plant root. Once a cyst nematode has selected an initial syncytial cell, it will no longer secrete cell wall modifying proteins. Syncytium induction includes a tightly controlled process of protoplasts fusion between adjacent cells. For this process, cyst nematodes recruit plant cell wall degrading enzymes. In Chapter V, we show that in addition to these enzymes, *G. rostochiensis* also recruits plant β-expansins and expansin-like proteins to establish a feeding site. Recently, Gal at el. (2006) has reported that α-expansin LeEXPA5, is specifically induced by root knot nematode in cells adjacent to the nematode feeding cells (Gal et al. 2006). Moreover, they also demonstrated that silencing of the aforementioned gene in the plant roots, has a hindering effect on the development of *M. javanica*. α–Expansins have previously been implicated in the symbiotic interaction between legume plant and *Rhizobium* spp. An α-expansin gene

(*Ma-EXPA1*) from *Melilotus alba* (white Sweetclover) was upregulated during nodule development (Giordano and Hirsch 2004). Elevated expression of α-expansins in both: nematode feeding site and nodule alike, seems to be one of many common features they share, despite one structure (i.e. nematode feeding site) being disadvantageous and the second (nodule) beneficial to the host plant (Koltai et al. 2001).
Data presented in Chapter V is the first report that links expression of β-expansin and expansin-like proteins in dicotyledonous plants to morphological changes in the architecture of the plant cell walls. The β-expansins and an expansin-like genes investigated in this chapter show different expression patterns in the developing syncytium, which suggests that they play different roles and that they are induced through different regulatory pathways. β-Expansins in rice were proposed to be specifically involved in reparation of damaged cell walls (Lee and Kende 2001). One could argue that β-expansins during nematode feeding are induced in response to damage to plant tissues. When this would also be the case in nematode-plant interactions, the expression of β-expansins would be localised along the migratory track of the nematode and not in the developing syncytium.

4.1 Role of α and β-expansins in nematode feeding site formation

β-Expansins in the developing syncytium seem to be expressed at much higher levels than the previously reported α-expansins (Fudali, Pers. Comm.). In addition, no elevated expression of any of the α-expansins has been detected in microarray experiment of *A. thaliana* infected with *H. schachtii* (Puthoff et al. 2003). In recent studies by Gal et al. (2006), tomato α-expansin LeEXP5 was shown to be upregulated in the gall tissue of root knot nematode -infected tomato roots at 4 and 10 days post infection. The expression was detected only in the gall cells surrounding the feeding cells, which might be incorporated into the feeding site in later stages. No transcripts were detected at any time point directly within the giant cells.
Infection studies performed with transgenic tomato plants with impaired expression of LeEXP5 indicated reduced ability of the nematode to succesfully establish feeding sites and complete their life cycles (Gal et al. 2006). It would be interesting to carry out similar resistance tests performed with plants with impaired expression of relevant β-expansin and expansin-like genes, which could give us insight into the individual contributions of these genes of plant origin to syncytium development.
Interesting parallels can be drawn between the role of expansins in the development of syncytium and in fruit ripening process. Cell wall changes leading to fruit ripening (e.g. in tomato) just like during syncytium formation involve the concerted and interdependent activities of a wide range of cell wall modifying proteins e.g. polygalacturonases, pectate lyases, pectin methylesterase, endoglucanases, xyloglucan

endotransglycosylase and expansins (Brummell and Harpster 2001; Catala, Rose, and Bennett 2000; Marin-Rodriguez, Orchard, and Seymour 2002). The expression of expansins during tomato fruit maturation has been studied in detail and seems to have an elaborate pattern. Transcripts of *LeEXP2* and *LeEXP4* are detectable only during the earliest stages of fruit development when cell expansion is most rapid. The decline in their expression is followed by increase in the accumulation of transcripts of *LeEXP5* and *LeEXP3*, which are present in the ripening fruit stage. The expression of the *LeEXP1* is strictly correlated with the breaker stage, when fruits do not significantly increase in size. Therefore, it was proposed that this protein is responsible for cell wall relaxation without the cell wall expansion, which is characteristic for ripening-associated cell wall disassembly (Brummell and Harpster 2001; Rose et al. 1997). It is conceivable, based on a similarly complex expression pattern, that β-expansins and expansin-like proteins might have comparably divergent roles in the formation of the syncytium as the expansins during fruit ripening.
The main difference between fruit ripening and syncytium development is a decrease in turgor in the ripening fruit caused by increasing concentrations of solutes in the cell wall space and wall loosening (Brummell and Harpster 2001). In case of syncytium, the high osmotic pressure that is maintained might be linked with the expression of natriuretic peptides. However this is merely a speculation that requires an experimental confirmation.
Numerous studies have demonstrated an important role of plant hormones in regulation of expression of various expansins. The phenomena of auxin-induced growth of the plant cells, where auxin promotes cell enlargement, is mediated by expansins (McQueen-Mason, Durachko, and Cosgrove 1992) The β-expansin from soybean (Cim1) accumulates up to 60 fold in response to auxin and/or cytokinin treatment of soybean suspension cultures (Downes et al. 2001). Expression of two *Arabidopsis* α-expansin genes (*AtEXP7* and *AtEXP18*), which are tightly linked to root hair initiation and formation was shown to be stimulated by exogenous ethylene and auxin, (Cho and Cosgrove 2002). As it has been discussed in the previous paragraph expansins are key players during the fruit maturation of many species, a process which is regulated by both auxin and ethylene (Fray and Grierson 1993). As the development of syncytium is dependant on ethylene and auxin (Goverse et al. 2000), it is tempting to speculate that up-regulation of β and α-expansin in the course of feeding site development may be caused by a change in plant hormonal balance induced by nematode.

4.2 Functional analysis of the role of expansins in nematode parasitism

According to the proposed model of plant cell wall changes occurring during the development of syncytium, the main role of plant β-expansins is to enhance the action of cell wall-degrading enzymes up-regulated by the nematode. Therefore, it is

conceivable that resistance tests performed on plants over-expressing expansin genes, and thus more prone to cell wall-degradation, could show an increased susceptibility towards nematodes. The same tests performed with plants with impaired expansins activity could show opposite effect. Various methods can be used to silence the expression of particular gene in a plant. Virus induced gene silencing, or VIGS, is a method to transiently interrupt gene function through RNA interference. The dsRNA delivered to the plant using a virus triggers systemic reaction in plant and the infected plant is supposed to display the phenotype characteristic to the loss-of-function of a host gene and not to the virus infection. Another approach, which is relatively more time consuming, is a stable transformation with constructs expressing dsRNA of plant gene of interest. Both approaches were applied to study the role of cellulases implicated in syncytium development (Karczmarek et al.). Plants with silenced expression of cellulases proved to be less susceptible to nematode infection comparing to control plants. Despite the fact that dsRNA technology still requires some adaptations to this particular biological system, it seems to be one of the most promising approaches to unravel function and significance of genes of interest to host – parasite interaction.

As it has been shown for expansins during the fruit ripening, the role of various expansins can differ significantly (Rose et al. 1997); therefore a more detailed analysis as to their effect on plant tissue would be advisable. It would be interesting to perform detailed extensometer and stress relaxation assays, which would help to determine if all of them trigger cell wall elongation and / or cell wall relaxation in a similar manner. These experiments could be complemented by the infiltration assays of expansins in combination with various cell wall degrading enzymes, which would allow us to investigate the biological effect that such mixtures have on plant tissue.

Expansins were shown to be regulated either by auxin or ethylene in ripening tomato (Rose et al 1997). Experiments with a range of auxin insensitive mutants have indicated that auxin is indispensable during the early stages of syncytium development (Goverse et al. 2000). It has also been reported that ethylene induced cell wall degradation is involved in syncytium formation (Goverse et al. 2000). However, no link has been established between particular genes expressed during this process and their hormonal regulators. Measurements of expression level of expansins during the establishment of syncytium in various relevant tomato mutants could give some insight into the hormonal regulation of expansins during parasitism.

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Podsumowanie

Nicienie pasożytnicze roślin są poważnym zagrożeniem dla wielu rodzajów roślin uprawnych. W skali światowej roczne straty w rolnictwie spowodowane przez nicienie pasożytnicze roślin ocenia się na około 100 miliardów dolarów. Praktycznie każda roślina uprawna ma swojego nicienia pasożytniczego, poczynając od drzew bananowych, a kończąc na pszenicy. Tematem niniejszej pracy doktorskiej była analiza interakcji pomiędzy roślinami wyższymi z rodziny psiankowatych (*Solanacea*), takimi jak pomidor (*Lycopersicum esculentum*) i ziemniak (*Solanum tuberosum*), a mątwikiem ziemniaczanym (*Globodera rostochiensis*) z rodziny mątwików (*Heterodera*). Objawy porażenia mątwikiem przejawiają się placowym występowaniem roślin karłowatych, przedwcześnie dojrzewających z niedorozwiniętymi pędami i liśćmi. Nawet gdy zewnętrzne objawy porażenia nie są widoczne gołym okiem, bulwy porażonych roślin są bardzo drobne. Straty spowodowane przez mątwika mogą sięgać nawet 80% plonu. Mątwiki są bardzo odporne na wszelkiego rodzaju chemiczne środki ochrony roślin. Jedynie skuteczne nematocydy, takie jak na przykład bromek metylu, są bardzo silnie działającymi i trwałymi środkami, które po wymieszaniu z glebą nieselektywnie niszczą całą faunę glebową. Użycie tych środków jest w większości krajów rozwiniętych niedozwolone przez prawo, a jedyną dostępną metodą ochrony przed mątwikiem jest stosowanie płodozmianu i uprawa roślin odpornych na porażenie. Poznanie złożonych interakcji pomiędzy tymi pasożytami a ich żywicielami na poziomie molekularnym może przyczynić się do wytworzenia w przyszłości bardziej skutecznych, a przede wszystkim bardziej selektywnych, metod ochrony.

Mątwik ziemniaczany jest typowym wewnętrznym pasożytem osiadłym systemu korzeniowego. Na korzeniach porażonych roślin można zaobserwować brunatne kuliste cysty o średnicy 0.57-0.83mm. Cysty te zawierają setki zapłodnionych jaj, które mogą przebywać w glebie bardzo długo, nawet do 20 lat, w oczekiwaniu na sprzyjające warunki. Gdy gleba osiągnie temperaturę powyżej 10^{o}C, pod wpływem wydzielin z korzeni roślin żywicielskich część osobników młodocianych (J2) opuszcza cysty i wędruje w kierunku korzeni rośliny żywicielskiej. Larwy w trakcie migracji wewnątrz korzenia, w kierunku wiązki przewodzącej, używają sztyletu osadzonego na przedniej części ciała oraz enzymów produkowanych przez dwie brzuszne komórki gruczołowe. Ta mieszanka siły mechanicznej i enzymatycznej pozwala im na przerwanie ciągłości ściany komórkowej tkanek korzenia. Po dojściu do wewnętrznej części kory pierwotnej nicień selekcjonuje jedną komórkę, która będzie początkiem wyspecjalizowanej struktury odżywczej nicienia, tak zwanego syncytium. Syncytium jest jedynym źródłem pokarmu dla rozwijających sie nicieni. Jest to struktura wielojądrowa, powstała w wyniku zlewania się protoplastów sąsiednich komórek poprzez otwory powstałe na drodze lokalnego rozpuszczania ściany komórkowej. Po zaindukowaniu syncytium nicienie przechodzą przez kolejne

stadia larwalne, aż osiągną dojrzałą formę. Płeć u mątwików jest determinowana przez czynniki zewnętrzne, głównie dostępność pokarmu, mianowicie im więcej pokarmu tym więcej rozwinie się samic. Dojrzałe samice pozostają przytwierdzone do korzenia i są widoczne jako białe i złociste kuliste formy. Robakowate samce odzyskują zdolność ruchu i opuszczają syncytia w poszukiwaniu samic. Po zapłodnieniu samice obumierają, a ich ciało przekształca się w brunatne, kuliste cysty wypełnione zapłodnionymi jajami. W ten sposób cykl się zamyka. Zazwyczaj w przypadku mątwików występuje jedno pokolenie na rok, czasem w bardzo sprzyjających warunkach mogą rozwinąć się dwa pokolenia.

Z punktu widzenia patologii roślin, w życiu mątwika ziemniaczanego najistotniejsze są dwie fazy, pierwsza to wewnątrzkomórkowa migracja przez tkanki korzenia, a druga to zakładanie syncytium. W trakcie pierwszej fazy mątwik wykorzystuje kombinację mechanicznej siły sztyletu, którym nakłuwa kolejne komórki na drodze swojej migracji, jak również mieszaninę enzymów degradujących ścianę komórkową, które są produkowane w dwóch brzusznych komórkach gruczołowych. Przez długi czas badacze byli przekonani, że zwierzęta są w stanie depolimeryzować ściany komórkowe tylko i wyłącznie dzięki symbiotycznym mikroorganizmom. Odkrycie celulazy produkowanej przez mątwika ziemniaczanego zburzyło ten długo obowiązujący dogmat. Wkrótce potem okazało się, że repertuar enzymów produkowanych przez te pasożyty jest znacznie bogatszy niż można było przypuszczać.

Charakterystyczną cechą organu odżywiającego, który nicień indukuje w korzeniu, są dramatyczne zmiany w strukturze ścian komórkowych komórek składowych. Na podstawie dotychczasowych danych, wydaje się, że procesy depolimeryzacji, które tam zachodzą są również indukowane przez nicienia ale w sposób pośredni, a nie poprzez bezpośrednią produkcję enzymów degradujących. Podczas indukcji syncytium brzuszne komórki gruczołowe, aktywne podczas migracji, zaprzestają produkcji natomiast aktywowana jest grzbietowa komórka gruczołowa. Produkty tego gruczołu są znacznie bardziej enigmatyczne w porównaniu do enzymów produkowanych w grzbietowych komórkach gruczołowych. Identyfikacja wydzielniczych protein posiadających sygnał lokalizacji jądrowej czy sekwencji kodujących peptydy sygnałowe podobne do roślinnych wskazuje na to, że nicień produkuje proteiny, które "podszywają się" pod proteiny roślinne i indukują procesy naturalnie zachodzące w komórce roślinnej w zmodyfikowany sposób wymagany do powstania funkcjonalnego syncytium.

Tematem prezentowanej pracy naukowej była analiza interakcji pomiędzy mątwikiem ziemniaczanym, a roślinami żywicielskimi, dokładniej ziemniakiem i pomidorem, na poziomie molekularnym. W pierwszym rozdziale opisany został proces identyfikacji nowego enzymu pektolitycznego (Gr-PEL2) produkowanego przez pasożytnicze formy larwalne (J2) w brzusznych komórkach gruczołowych. Pektatylaza ta została zidentyfikowana w wyniku porównania profili ekspresji genów w różnych stadiach

rozwojowych mątwika ziemniaczanego (metodą cDNA-AFLP). Ekspresja genu kudującego Gr-PEL2, została zlokalizowana w gruczołach brzusznych larw J2, aktywnych podczas pierwszej fazy pasożytniczej. Ta lokalizacja potwierdza potencjalną rolę tego enzymu w depolimeryzacji ścian komórkowych w tkance korzenia podczas migracji larwy. Trójwymiarowy model tej proteiny został skonstruowany na zasadzie homologii do pektatylazy 15 produkowanej przez bakterie *Bacillus subtilits*, której struktura jest dokładnie poznana. Według tego modelu możliwe były dwa potencjalne miejsca wiązania wapnia, który jest niezbędny dla aktywności enzymatycznej pektatylaz. Identyfikacja właściwego miejsca wiązania wapnia została przeprowadzona przez nowatorskie połączenie metody infiltracji liści z użyciem *Agrobacterium tumefaciens* oraz miejscowej mutagenezy aminokwasów potencjalnie zaangażowanych w wiązanie wapnia. Na liściach roślin infiltrowanych *Agrobacterium* zawierającym Gr-PEL2 bez modyfikacji wystąpiły zmiany w tkance liścia widoczne jako marszczenie i żółkniecie liści. Liście infiltrowane *Agrobacterium* niosącym Gr-PEL2 w którym amino kwasy wiążące wapń zostały zamienione na alaninę nie wykształciły żadnych zmian w tkance. Z kolei infiltracja konstruktami w ktorych amino kwasy nie mające znaczenia dla wiązania wapnia zostały zamienione na alaninę zakończyła się powstaniem podobnych zmian w liściach jak te obserwowane przy infiltracji z niemodyfikowanym Gr-PEL2. Dodatkowo, analiza filogenetyczna ujawniła interesujący rozdział pomiędzy enzymami pektolitycznymi produkowanymi przez nicienie pasożytnicze roślin, i tak Gr-PEL1 i Gr-PEL2 dwie pektatylazy produkowane przez mątwika ziemniaczanego są umiejscowione w dwóch odleglych galeziach drzewa filogenetycznego. Taki rozklad galęzi drzewa filogenetycznego wskazuje na możliwość zaistnienia horyzontalnego transferu genów pomiędzy bakteriami a tymiż nicieniami, gdyz malo prawdopodobnym jest aby oba geny mialy wspólnego przodka.

W rozdziale drugim przedstawiono identyfikację pierwszej ekspansyny modyfikującego ściany komórkowe roślin produkowanej nie przez rośliny wyższe (Gr-EXPB1). Ekspansyny produkowane przez rośliny, to białka ściany komórkowej posiadające zdolność rozluźniania ich struktur przez osłabianie/ rozrywanie niekowalencyjnych wiązań pomiędzy różnymi polimerami ścian (celuloza, hemiceluloza i pektyny). Znajdują one zastosowanie w różnych procesach fizjologicznych takich jak: zapylenie czy opadanie liści. Ekspansyna pochodzenia nicieniowego (Gr-EXPB1) została zidentyfikowana w wyniku porównania profili ekspresji genów pomiędzy różnymi stadiami rozwojowymi mątwika. Ulega ona ekspresji w inwazyjnym stadium larwalnym w gruczołach brzusznych, co wskazuje na jej rolę w migracji wewnątrzkomórkowej larw. Gr-EXPB1 wykazuje największe podobieństwo do β-ekspansyn, które charakteryzują się największą aktywnością w rozluznianiu ścian komórkowych roślin jednoliściennych. Badania aktywności Gr-EXPB1wyprodukowanej w transgenicznym tytoniu potwierdziły, że podobnie jak roślinne β-ekspansyny indukuje ona intensywne wydłużanie sie koleoptylu pszenicy

(roślina jednoliścienna), co jest intrygujące, jako że żywicielami mątwika ziemniaczanego są rośliny dwuliścienne. Wyniki badań zaprezentowane w tym rozdziale są pierwszym istniejącym dowodem na to, że eksapnsyny są produkowane równiez przez pasożyty roślin prawdopodobnie w celu wspomożenia działania enzymów depolimeryzujących ściany komórkowe.

W trzecim rozdziale zaprezentowano dokładniejszą analizę nicieniowej ekspansyny Gr-EXPB1. Hybrydyzacja metodą Southern'a wykazała, że najprawdopodobniej Gr-EXPB1 należy do małej rodziny genów w mątwiku ziemniaczanym, a analiza bazy danych EST ujawniła, iż podobne białka są produkowane przez różne gatunki nicieni pasożytniczych roślin. Trójwymiarowy model Gr-EXPB1 został skonstruowany w celu potwierdzenia, że to białko, pomimo różnic w sekwencji przynależy do rodziny ekspansyn. Testy aktywności przeprowadzone z użyciem CBD domeny, wyprodukowanej w ztransformowanym tytoniu, odpowiedzialnej za wiązanie ekspansyny z polimerami ściany komórkowej, pozwoliły ustalić, że domena ta nie posiada właściwości rozluźniających ścian komórkowych roślin. Analiza filogenetyczna wykazała, że Gr-EXPB1 jest najbliżej spokrewniona z proteinami pochodzenia bakteryjnego, co z kolei wskazuje iż mógl zajść horyzontalny transfer genów pomiędzy bakteriami i nicieniami.

Rozdział czwarty, opisuje identyfikację grupy β-ekspansyn i ekspansynopodobnych białek, produkowanych przez ziemniak i pomidor. Analiza bazy danych sekwencji roślin psiankowych (Solanacea Genomics Network) pozwoliła na identyfikację czterech częściowych sekwencji β-ekspansyn i jednej ekspansynopodobnej wyizolowanej z pomidora. Pełne ramki odczytu dla tych genów (*SlEXPB1, SlEXPB2, SlEXLA1*) zostały sklonowane z pomidora i posłużyły jako matryca do sklonowania homologicznych sekwencji z ziemniaka *(StEXPB1, StEXPB2, StEXLA1)*. Analiza ekspresji tych genów z użyciem RT-PCR'u i hybrydyzacji *in situ,* w korzeniach pomidora i ziemniaka porażonego przez mątwika wykazała, że dwa z tych genów nie ulegają ekspresji, natomiast trzy pozostałe są bardzo specyficznie indukowane w różnych przedziałach czasowych procesu infekcji. Dane te wskazują, że mątwiki nie tylko same produkują ekspansyny w trakcie inwazji, ale również w bardzo subtelny i specyficzny sposób wykorzystują roślinne geny w celu ukształtowania funkcjonalnego syncytium Podczas tego procesu ekspansyny prawdopodobnie przygotowują pole do działania dla enzymów depolimeryzujących ściany komórkowe w trakcie formowania syncytium.

W końcowej części niniejszej pracy przedstawiono szczegółową dyskusję na temat roli pektatylaz i β-ekspansyn w pasożytnictwie ziemniaka i pomidora przez mątwika ziemniaczanego.

Samenvatting

Nematoden – ‘aaltjes’ in gewoon Nederlands – zijn transparante draadvormige diertjes die met name in bodems zeer veel voorkomen. Aaltjes zijn zeer soortrijk, en een kleine minderheid voedt zich op planten of dieren. Dit worden de parasitaire nematoden genoemd. Sedentaire (‘zittende’) plantenparasitaire aaltjes zijn gedurende een deel van hun leven immobiel - vandaar ‘zittend’ – en gaan langdurige interacties aan met hun waardplanten. De parasitaire cyclus van deze nematoden bestaat uit twee fasen, namelijk de migratiefase en de voedingsfase. In de migratiefase dringen de nematoden de plant binnen vanuit de bodem, en migreren vervolgens kort door het plantenweefsel tot ze zich vestigen op een specifieke plek in de wortel. In dit proefschrift wordt gerapporteerd over de interactie tussen het sedentaire aardappelcystenaaltje *Globodera rostochiensis*, en zijn gastheren aardappel en tomaat. Net als andere cystenaaltjes migreert *G. rostochiensis* intracellulair, dat wil zeggen door de cellen heen. De celwanden van wortelcellen zijn hierbij voor de nematoden belangrijke barrières. Bij intercellulaire migratie, zoals bijvoorbeeld bij de wortelknobbelaaltjes, verplaatst de nematode zich tussen cellen. Het eerste doel van dit proefschrift was het identificeren en karakteriseren van nog onbekende celwand modificerende eiwitten in het speeksel van het *G. rostochiensis* in de migratie fase van de parasitaire cyclus.

Zodra de nematode zich heeft gevestigd in de wortel induceert het de vorming van een voedingscelcomplex – het syncytium. Dit syncytium ontstaat door het lokaal oplossen van celwanden met als gevolg de fusie van het protoplasma van betrokken cellen. In de interactie tussen aardappelcystenaaltjes en aardappel volgt het oplossen van celwanden een vast patroon vanuit de cortex naar de endodermis, en vervolgens binnen de vaatbundel in alle richtingen. Het syncytium groeit – cellen worden geïncorporeerd in de groeiende voedingscel - zolang de nematode actief voedingsstoffen opneemt uit het syncytium. Uiteindelijk zullen na enkele weken bij het syncytium enkele honderden cellen betrokken zijn. De voedingsfase eindigt na de bevruchting van het volwassen vrouwtje met de start van de reproductie.

Er zijn twee redenen waarom het aannemelijk is dat de lokale celwandafbraak bepaald wordt door celwand modificerende eiwitten van de plant (en niet van het aaltje). Ten eerste zijn de subventrale speekselklieren die verantwoordelijk zijn voor de productie van celwand modificerende eiwitten in de nematoden nauwelijks actief in de voedingsfase. Ten tweede vindt de lokale celwand afbraak plaats in de celwanden die het verst van de kop van de nematode (met de bron van het speeksel) verwijderd zijn, en - ten derde – is de proliferatie van de zich ontwikkelende voedingscel zeer gericht (met andere woorden, zij wijkt sterk af van het patroon waarmee een olievlek zich uitbreidt). Dit heeft geleid tot de tweede doelstelling van het proefschrift, namelijk de identificatie en de functionele analyse van celwand modificerende eiwitten van de waardplant, die betrokken zijn bij de vorming van het syncytium.

In hoofdstuk 2 is de identificatie en de functionele en structurele analyse van een nieuwe pectaat lyase gen (*Gr-Pel2*) uit *G. rostochiensis* beschreven. Analyse van expressieprofielen van het infectieuze stadium van *G. rostochiensis* heeft geleid tot de identificatie van dit gen dat verre verwantschap vertoont met leden uit de polysaccharide lyase familie 3. De hoogste similariteit heeft *Gr-Pel2* met genen van twee *Streptomyces* species, en genen uit de facultatief plantenparasitaire nematode *Bursaphelenchus*. In studies met heterologe expressie van *Gr-Pel2* met behulp van *Agrobacterium* (een bacteriesoort die gebruik wordt om lokaal een vreemd eiwit te produceren) infiltraties in bladeren van tomaat bleek dat *Gr-Pel2* een uitgesproken effect heeft op de morfologie en structuur van het blad. Dit gegeven is vervolgens gebruikt om met structuurmodellen van het eiwit en gerichte mutagenese essentiële aminozuur posities voor binding van de co-factor calcium en het substraat te bepalen. Enkele jaren geleden is een ander pectaat lyase gene (*Gr-Pel1*) gevonden in *G. rostochiensis*, en in hoofdstuk 2 is een vergelijkend onderzoek uitgevoerd tussen de twee genen. De conclusie van dit onderzoek is dat Gr-PEL2 een significant andere structuur heeft, met daarvan afgeleid een significant andere verdeling van de hydrostatisch krachten. De consequentie hiervan is dat Gr-PEL2 veel hydrofieler is dan Gr-PEL1. De positie waar het calcium ion dat fungeert als co-factor gebonden wordt ligt op een andere plek in beide eiwitten. Bovendien is de verdeling van de hydrostatische krachten in de groeve waarin de interacties met substraat plaatsvinden significant verschillend. Dit suggereert het substraat in de celwand ook verschillend zal zijn. Fylogenetische analyse van leden van de polysaccharide family 3 wijst erop dat Gr-PELl2 samen met de pectaat lyase genen uit *Bursaphelenchus* een aparte groep vormt die meest verwant lijkt met bacteriële genen. Bovendien versterkt deze uitkomst het vermoeden dat op z'n minst een aantal celwand modificerende genen in nematoden een oorsprong hebben in de Actinobacteria.
In hoofdstuk 3 staat de beschrijving en de functionele analyse van het *Gr-EXPB1* gen, dat codeert voor een expansine in het speeksel van *G. rostochiensis*. Conventionele enzymen zoals bijvoorbeeld cellulases en pectaat lyases verbreken covalente verbindingen in celwand polymeren. Expansines - tot nog toe uitsluitend gevonden in landplanten – verweken celwanden van planten door vermoedelijk aan te grijpen op de non-covalente interacties tussen celwandpolymeren (bijvoorbeeld door het verzwakken van waterstofbruggen). De expansines als groep zijn begin negentiger jaren ontdekt, en er is nog weinig bekend over het exacte werkingsmechanisme van deze eiwitten op celwandpolymeren. De expansines worden gedefinieerd door een aantal karakteristieke motieven in de aminozuur sequentie, door de domein topologie van het eiwit, en door stimulatie van een karakteristieke patroon in het uitrekken van een celwand in een extensometer. Het expansine in *G. rostochiensis* viel op in specifieke expressieprofielen van gesynchroniseerde levensstadia in cDNA-AFLP experimenten. De typische motieven in de aminozuur sequentie van het coderende gen deed vermoeden dat het ging om een β-expansine – één van de vier families binnen de

expansine superfamilie. Uit vervolgonderzoek bleek dat de eiwit topologie met een celwandbindend domein (type II) en een expansine domein ook in overeenstemming was met expansines – al zij het in een omgekeerde oriëntatie. Expansines onderscheiden zich van expansine-achtige eiwitten door hun karakteristieke effect op celwandpolymeren. Recombinant in *Nicotiana tabacum* geproduceerd Gr-EXPB1 vertoonde dit karakteristieke effect op celwanden van tarwe coleoptielen, evenals een homogenaat van nematoden in de migratiefase. Deze data maken duidelijk, dat nematode naast conventionele celwand afbrekende enzymen ook expansines uitscheiden in de migratiefase. Bovendien blijkt nu dat expansines als groep van eiwitten niet uitsluitend voorkomen in planten, maar ook in dieren en waarschijnlijk nog in andere organismen. Dientengevolge behoeft de zienswijze dat dieren genetisch slecht toegerust zijn om celwanden af te breken herziening. Het hier beschreven onderzoek toont opnieuw aan dat dit niet geldt voor plantenparasitaire aaltjes.

Hoofdstuk 4 handelt over de evolutionaire oorsprong, de verspreiding, en de structuur van Gr-EXPB1. Uit de analyse van het genomische DNA van *G. rostochiensis* bleek dat *Gr-EXPB1* lid is van een genfamilie met tenminste vijf leden. Door *Gr-EXPB1* als zoekterm te gebruiken in een database van *expressed sequence tags* van *G. rostochiensis* ontdekten we nog een gen – *Gr-EXPB2* – dat ook gelijkenis heeft met β-expansines. Gr-EXPB2 heeft ook de karakteristieke aminozuur motieven van een expansine, maar heeft geen cellulosebindend domein. Een soortgelijk zoektocht in een sequentie database van het sojaboon cystenaaltje *Heterodera glycines* leverde ook een β-expansine op, wel inclusief een cellulosebindend domein. Het is opmerkelijk, dat i) het expansine domein in Gr-expB1 (en homologen uit in nematoden) de hoogste similariteit heeft met genen uit saprofytische bodem bacteriën uit de groep van de Actinobacteria en dat ii) het cellulosebindend domein in Gr-EXPB2 de meeste gelijkenis heeft met het bacteriële type II koolhydraat bindende modules (Eng.: carbohydrate-binding modules (CBMs)). Deze bevindingen ondersteunen de hypothesis dat een aantal pathogeniteitsfactoren van nematoden verkregen zijn via horizontale gen overdracht van prokaryoten.

Uit ander onderzoek is gebleken dat cellulases en andere conventionele celwand afbreken enzymen van de plant betrokken zijn bij de lokale celwandafbraak ten behoeve van syncytium proliferatie. In hoofdstuk 5 ligt de focus op een mogelijke rol van β-expansines en expansine-achtige eiwitten van de waardplant bij dit proces. Genen die coderen voor β-expansines en expansine-achtige eiwitten waren nog niet beschreven voor tomaat, dus het eerste doel was om deze te identificeren in een database met *Expressed Sequence Tags* van tomaat. Tenminste vier genen die coderen voor β-expansines (*SlXPB1, SlEXPB2*, *SlEXPBB3, en SlEXPB4*) en één gen dat codeert voor een expansine-achtige eiwit (*SlEXLA1*) zijn gevonden, en onderzocht op expressie in het syncytium van *G. rostochiensis* met RT-PCR (RT: 'reverse transcriptase' – vertaalt mRNA in cDNA) en in situ hybridisatie. *SlEXPB1*, *SlEXPB2*, en *SlEXLA1* bleken specifiek tot expressie te komen in wortels van tomaat

geïnfecteerd met nematoden. Deze expressie is gelokaliseerd in syncytium cellen, zoals bleek met in situ hybridisatie microscopie. Bovendien was het expressiepatroon voor de drie genen in de tijd weliswaar overlappend maar niet identiek. Deze resultaten betekenen dat naast conventionele celwand afbrekende enzymen ook β-expansines en expansine-achtige eiwitten betrokken lijken te zijn bij celwandafbraak in het syncytium. Naast celwandafbraak vindt ook celwand synthese plaats in het syncytium bijvoorbeeld bij de vorming van celwandstructuren die een verhoogd transport vanuit vaatweefsel mogelijk maken. Op basis van de experimenten in hoofdstuk 5 lijkt het aannemelijk dat β-expansines en expansine-achtige eiwitten bijdragen aan dit proces van gerichte celwandafbraak. Nader onderzoek dat onder andere de immunolocalisatie van deze eiwitten op subcellulaire niveau in het syncytium zal omvatten, zal dit moeten uit wijzen.

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Acknowledgments

I'm fully aware that "acknowledgments" is probably the only part of this thesis that most people will ever read. This is also the only part that can make someone important to me smile and feel appreciated. Therefore, I think it is in a way one of the most important parts of the book, and will require at least the same amount of thought and consideration as the scientific sections. There are two main differences however: 1) that hopefully no one will ever review it 2) and /or will take its contents and style into consideration while interviewing me for a job. That means total freedom of speech!!! Let's make use of it then ☺

First of all I should start by thanking Professor Golinowski for taking a chance on me, and sending me to Holland, despite the understandable fears about what it may do to his and his group reputation. At the same time many thanks should go to my fellow students at SGGW for their complete lack of interest in this scholarship from Wageningen, which made the selection process extremely easy. On a more serious note I would really like to thank professor. Golinowski for the tremendous effort he put in removing all the administrative obstacles for me to defend my MSc thesis in time to start the PhD. Dear Professor – you are a man that truly deserves this title and all the honours that come with it, your professional and pedagogical skills are of the highest sort. You are the rare type of a teacher who makes a student want to learn just for the pure joy of it.

The person who bravely took the responsibility upon himself of making something resembling a researcher out of me was Geert Smant. It is up to you Geert to tell me if it was worth the effort, but on my side I can tell you that I really enjoyed the process. I think I told you many times that if there ever was an encyclopaedia entry entitled 'good supervisor', you would have a good chance of seeing your photo as an example. I have to thank you for your 'adjustable' supervision, which meant almost total freedom that allowed me to develop and pursue my own ideas and experiments. It gave me a good preview of what it means to be an independent researcher and made the shock of a real life out there a big deal less traumatic.

Hans Helder's input into manuscripts is highly appreciated. Jaap Bakker is gratefully acknowledged for awarding me the scholarship that resulted in completion of this thesis.

I also have to extend my gratitude to all my colleagues and co-workers that shared the time and space with me for the last 5 years. Ling, you have taught me so much about life and science. Equally. Rikus, I would be completely lost without you! Thanks a lot for being like a road sign in the thick mist of mysterious operating guides for even more mysterious machines. Hans K., your sense of humour is unforgettable, I loved our weird discussions. Erwin, I guess your life must be way much easier without my messiness. Willemien, I extremely enjoyed our horse-riding adventure.

Without Adina Milac, completion of chapters II and IV would not be possible. I am equally impressed with your professional expertise and your personal charm, and I wish you and your family, all the best for the future. I hope we will stay in touch. Sylwia Fudali from Warsaw Agricultural University deserves my very special gratitude for providing me with magnificent photos for chapter V and also for her patience with my continuous demands for more of them. Dear Anna Tomczak – I owe you so much for helping me out with the last chapter and inhaling all the toxic fumes of phenol instead of a very pregnant me. I would like also to thank Ania Kielak, with whom I had the pleasure to work on my first chapter. I remember when you were bored with our bacteria because they were producing what we wanted and when we wanted without putting up a fight. I'm pretty sure that you would appreciate it more by now– success is by no means a default pathway in science – we all learn it the hard way. Dear Patrick, I need to apologize for all the teasing, which I have to say you endured with great grace and dignity. You are and always will be my favourite German…..very much polonaised though…Viener melange without you will never taste the same. Aga, you were there in Holland since the very beginning. These were crazy times and I'm sure we both enjoyed them equally before becoming 'old and respectable women'. Pisiek, your warmth, charm and most of all humour is unbeatable. You are a great person and a great friend. Kamilko you are what I call: 'baba z jajami', and in my eyes it is really a great complement. Please try not to suffocate in your rural surroundings; it would be a great loss to me.

Anetko, you are my best friend for ages. You came with me for our Dutch adventure, and you were there with me through the bad and the good times. I'm not even sure if I would have come to Holland if I had to do it by myself. I still remember when we were standing on the balcony of Dijkgraaf and sobbing hysterically watching my Dad leave. I remember our plans and fears of coming back to Poland defeated by language and loneliness. We did not after all, and I guess it was easier than we thought it would be. You were always there when I needed you and I cannot thank you enough for that. I do cherish your friendship tremendously and I can promise you that regardless of where in the world we will end up in the future, we will stay friends forever.

I also need to extend my gratitude to my new friends I've met in the green beautiful Eire. Without them completion of my thesis would not be possible because I would most likely have a nervous breakdown by now. First of all, my lovely neighbours Asia Korzeniowska and Malgosia Chmiel. I'm so lucky to have you girls. Gosia, thanks for the crash course on the lingerie. Asia, thanks for introducing me to polish classic cuisine, it was a big surprise ☺. To both of you 'thanks a million' for showing my daughter that polish is not only a language that her mum shouts at her in, and in general thank you for being such enthusiastic aunties. Also special thanks to Aisling Doyle and William for great fun we've had together and to Catherine O'Mahony for being so extremely patient, when I continuously harass her (and probably will for a while) as a live English dictionary.

Finally and most importantly my gratitude goes to my family. Daddy, you were always my role model. Even though I will never be as ‘crazy’ as you are about science, I did my best to be at least a scientist. I thank you for your love and humour and for explaining me at the age of 8 what the binary code is. One of the greatest feelings in my geographically messed up life is the awareness that back in Poland I will always have my shelter and home with you.
Without my Mother I would never be where I’m today. Her belief in my alleged ‘greatness’ and her unconditional love made me work hard to try to live up to her dreams. I hope I’ll never disappoint you Mum. You were the greatest mum one could have wished for.
In Holland, I not only got my PhD but I also got myself a husband. A husband who is my very best friend, a husband who knows me better than I know myself, a husband without whom I cannot imagine my life. A husband who has followed me half around the world, and who has had to make so many sacrifices just to be with me. I wish you will never ever regret it Hassan. I hope that very soon we will find a place that we can both call home, and wherever that will be, I’m sure we will be very happy there as long as you love me at least in a half as much as I love you.
At last I will thank and apologise to my lovely daughter Hana. As I write this, you are exactly one and a half years old, too little to understand that your parents sentenced you to a very weird life. Half Polish, half Palestinian, born in Holland and living in Ireland. One day it will either become your curse or your blessing, I hope for the latter one, and I promise you that I’ll work extremely hard to make you feel at home wherever we are.

Publications of the author

Full papers and articles

1. Plant degradation: a nematode expansin acting on plants; Qin, L.; **Kudla, U**.; Roze, E. H.Goverse, A.Popeijus, H.Nieuwland, J.Overmars, H.Jones, J. T.Schots, A.Smant, G.Bakker, J.Helder, J; 2004 Nature, Vol 427, issue 6969, pages 30
2. Origin, distribution and 3D-modeling of Gr-EXPB1, an expansin from the potato cyst nematode *Globodera rostochiensis*; **Kudla, U.**; Qin, L.; Milac, A.Kielak, A.Maissen, C.Overmars, H.Popeijus, H.Roze, E.Petrescu, A.Smant, G.Bakker, J.Helder, J.; 2005 FEBS Letters; Volume 579, Issue 11, Pages 2451-7

Manuscripts in preparation

1 Structural and functional characterization of a novel, host penetration-related pectate lyase from the potato cyst nematode *Globodera rostochiensis;* **Kudla, U**, Milac, A. Qin, L. Roze, E. Holterman, M. Petrescu, A. Goverse, A. Bakker, J. Helder, J. Smant, G.

2 Feeding site development by the potato cyst nematode *Globodera rostochiensis* includes the recruitment of plant β-expansins and expansin-like genes; **Kudla, U.**, Tomczak, A., Fudali, S., Sobczak, M., Golinowski, W., Goverse, A., Bakker, J., Helder, J., Smant, G.

3 Epigenetics of polynucleotide repeat expansion disorders; **Kudla, U**. Colghan, A. Spillane, C.

Conference and symposia proceedings and meetings

1. Identification of an expansin from infective juveniles from potato cyst nematode G. rostochiensis; **Kudla, U.** Qin, L. Roze, E. Poppeijus, H. Helder, J. Smant, G. NONEMA Warsaw 2001:
2. *The identity and function of cyst nematode-secreted proteins in pathogenesis* Helder, J.; Qin Ling, ; **Kudla, U**.; Overmars, H.; Prins, P.; Goverse, A.; Smant, G.; Bakker,J.; In: 2001 Abstract Book Molecular Plant-Microbe Interactions, 10th International Congress : Molecular Plant-Microbe Interactions, Madison, Wisconsin, - Madison, Wisconsin : University of Wisconsin, 2001 . - p. 49.
3. Plant parasitic nematodes producers of expansin ex planta;: **Kudla, U**. Qin, L., & Smant, G. SPIT meeting; Wageninen 2001.

4. Novel pectate lyase gene from Globodera rostochiensis. In (Ed.), *Nematology Symposium abstracts Vol. 4. Nematology* (pp. 222 (283)) Leiden: Brill Academic Publishers. **Kudla, U**. Qin, L., & Smant, G. Tenerife (2002).
5. A novel pectate lyase gene from Globodera rostochiensis. Experimental Plant Sciences, Autumn School **Kudla, U**., Qin, L. Roze, Smant, G; Wageningen (2002).
6. Expansins ex planta - a novel type of parasitism factor produced by plant parasitic nematodes; **Kudla, U**., Qin, L. Roze, E. H.Goverse, A.Popeijus, H.Nieuwland, J.Overmars, H.Jones, J. T.Schots, A.Smant, G.Bakker, J.Helder; Experimental Plant Sciences Meeting; Lunteren 2003;
7. Expansin and pectate lyase 2: two essentially different parasitism factors facilitating migration **Kudla, U**., Qin, L. Roze, E. H.Goverse, A.Popeijus, H.Nieuwland, J.Overmars, H.Jones, J. T.Schots, A.Smant, G.Bakker, J.Helder; Nonema Vienna 2003:
8. Plant cell wall modifications in potato cyst nematode parasitism. In I. Tikhonovich, B. Lugtenberg & N. Provorov (Eds.), *Biology of Plant-Microbe Interactions, Molecular Plant-Microbe Interactions: New Bridges between Past and Future, Proceedings of the 11th International Congress on Molecular Plant-Microbe Interactions St.-Petersburg, Russia, July 18-26, 2003* (pp. 297-300) St.-Petersburg, Russia: Internat Society for Molecular Plant-Microbe Goverse, A., **Kudla, U**., Swiecicka, M., Karczmarek, A., Overmars, H.A., Qin, L., Filipecki, M., Bakker, J., Helder, J., & Smant, G.; St.-Petersburg, Russia (2004).
9. Modelling the structure of proteins involved in nematode-plant interaction. In (Ed.), *XXVII European Society of Nematologists (ESN) International Symposium* (pp. 56) Rome: ESN.1. Petrescu, A., Milac, A. Qin, L., **Kudla, U**., Rosso, M.N., & Helder, J.; Rome (2004).
10. : Functional analysis of pectate lyase 2 from *Globodera rostochiensis* (Gr-EPL2) using transient expression in planta; **Kudla, U**., Milac, A., Qin, L. Roze, Smant, G; NONEMA Gent, Belgium 2004
11. : Plant β-expansins in the early stages of syncytium development in cyst nematode infected potato and tomato, **Kudla, U**. Tomczak, A. Fudali, S. Smant, G.; SPIT meeting; Antibes, France, 2004
12. Functional analysis of pectate lyase 2 from Globodera rostochiensis (Gr-PEL2) using transient expression in planta; **Kudla, U.**, Milac, A. Qin, L. Helder, J. Smant, G; SPIT meeting Antibes, France 2004

Curriculum Vitae

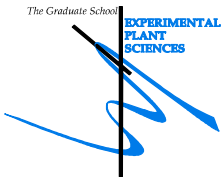
Urszula Kudla was born on the 19th of October 1976 in Bialystok, Poland. She attended primary, secondary and high schools in Warsaw. After passing the scientific stream high school state exam in 1995, she enrolled in the Interdisciplinary Faculty of Biotechnology at Warsaw Agricultural University. In 1999 she carried out eight-month long research project at the Laboratory of Nematology of Wageningen University and Research Centre in The Netherlands. In the year 2000 she returned to Warsaw Agricultural University and in May 2000, she obtained an MSc degree in Biotechnology. Shortly after that, she was awarded a PhD fellowship from the Laboratory of Nematology of Wageningen University in The Netherlands. After four years of experimentation, she fulfilled the requirements of a PhD degree in Plant Sciences (compatible interactions between plant parasitic nematodes and host plants) and defended her thesis on the 17th of May 2006. Currently she is employed as a Post Doctoral researcher in the Genetics & Biotechnology Laboratory of the Biochemistry Department at University College Cork in Ireland.

Pushy, Pushy, Pushy

By HENRY FOUNTAIN
OBSERVATORY
(New York Times Jan 6, 2004, page D3)

Parasites are pushy creatures, invading their hosts in one way or another. The golden nematode, a parasite that invades tomato and potato plants, takes pushiness to an extreme, penetrating a host plant at a rapid rate (about two minutes per cell layer).
Now, scientists in the Netherlands and Scotland may have discovered the secret to its speed. The nematode is pushy in a novel way: it produces proteins that make plant cell walls loosen up as it moves in.The proteins, a type called expansins, had previously been identified only in plants. They induce the cell walls to extend and relax and are thought *to* play a role in both plant growth and the disassembly of cell walls. Although the precise details of their function is not known, they weaken bonds between a wall's cellulose fibrils and compounds called glucans that hold the fibrils together. The researchers identified a nematode gene responsible for the proteins and showed that they were produced in a juvenile stage of the parasite, by esophageal glands.
Their research was reported in the journal Nature. Many organisms that get their food from plants use enzymes called glycanases to break down cell walls completely. The researchers found that golden nematodes produce glycanases, too, at the same time that they produce the expansins. The researchers suggest that by loosening up the walls, the expansins make the wall's component parts more accessible to the glycanases. So the plant tissue is broken down faster and the parasite can enter sooner.

Experimental Plant Sciences



Issued to: **Urszula Kudla**
Date: **17 May 2006**
Group: **Laboratory of Nematology, Wageningen University**

1) Start-up phase	*date*
► **First presentation of your project**	
First Functional Expansin of Non-plant Origin	May 2001
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Feeding site development by the potato cyst nematode Globodera rostochiensis includes the recruitment of plant beta-expansins and expansin-like genes	Oct 2003
► **MSc courses**	
► **Laboratory use of isotopes**	
Safe Handling with Radioactive Materials and Sources	Apr 2001
Subtotal Start-up Phase	*9.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
Wageningen	25 Jan 2001
Wageningen	24 Jan 2002
Utrecht "A new dimension to networking"	27 Mar 2003
► **EPS theme symposia**	
Interactions between Plants and Biotic Agents Pathogenicity Factors	07 Feb 2002
Interactions between Plants and Biotic Agents Pathogenicity Factors	12 Dec 2003
Interactions between Plants and Biotic Agents Pathogenicity Factors	17 Feb 2004
► **NWO Lunteren days and other National Platforms**	
ALW Discussieplatform Experimentele Planten Wetenschappen (EPW) Lunteren	Apr 2002
ALW Discussieplatform Experimentele Planten Wetenschappen (EPW) Lunteren	8 Apr 2003
► **Seminars (series), workshops and symposia**	
Flying Seminars Signal transduction from genome to cell to plant	11 Mar 2003
Frontiers in Plant ScienceCell cycle control and plant development'	27 Feb 2003
Frontiers in Res. on Interact. between Plants and Biot. Agents Mech.of Mi-mediated Resist. to Nemat. in Tomatoes	30 Oct 2003
The Arrangem. of Interph. Chrom. Territ. in Arabid. thal. and their Alter. by the Transg. Tandem Repet. Chrom. Tags	17 Dec 2004
► **Seminar plus**	
► **International symposia and congresses**	
Fourth International Congress of Nematology, Tenerife, Spain	08-13 Jun 2002
Nonema International Project Meeting, Warsaw, Poland	May 2001
Nonema International Project Meeting, Vienna, Austria	May 2002
Nonema International Project Meeting, Gent, Belgium	Feb 2004
International SPIT Meeting, Wageningen, The Netherlands	May 2001
International SPIT Meeting, Antibes, France	Oct 2004
► **Presentations**	
Fourth International Congress of Nematology, Poster presentation	Jun 2002
Nonema International Project Meeting, Oral Presentation	May 2001
Nonema International Project Meeting, Oral Presentation	May 2002
Nonema International Project Meeting, Oral Presentation	Feb 2004
International SPIT Meeting, Oral Presentation	May 2001
International SPIT Meeting, Oral Presentation	Oct 2004
Discussieplatform Experimentele Planten Wetenschappen, Oral Presentation	01 Apr 2003
Autumn School Disease Resistance in Plants, Oral Presentation	Oct 2002
► **IAB interview**	
► **Excursions**	
Subtotal Scientific Exposure	*13.8 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Interactions betweenplants and attacking organisms	16-18 Oct 2000
Winterschool Bioinformatics	11-15 Dec 2000
Disease Resistance in Plants	14 -16 Oct 2002
Molecular Basis of Microbe-Plant interactions	05-07 Jun 2003
► **Journal club**	
journal club "Nematology"	Sep 2000-Feb 2005
► **Individual research training**	
Subtotal In-Depth Studies	*7.2 credits**

4) Personal development	*date*
► **Skill training courses**	
Presenting in English (15 lessons)	24 Nov - 8 Dec 2003
English Scientific Writing (12 lessons)	4 Oct 2001 - 7 Feb 2002
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.8 credits**

TOTAL NUMBER OF CREDIT POINTS*	**33.8**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

The Chapters II and IV in this thesis were conducted as a joint efforth between Laboratory of Nematology of Wageningen University in The Netherlands and Institute of Biochemistry of the Romanian Academy, in Bucharest, Romania.

The Chapter V described in this thesis was conducted as a joint effort between the Laboratory of Nematology of Wageningen University in The Netherlands and Department of Botany of Warsaw Agricultural University in Warsaw, Poland.

This research was sponsored by funding from the European Union project QLK5-1999-01501, which is gratefully acknowledged

Cover design: Urszula Kudla

Printing: Grafisch Service Centrum Van Gils B.V