Dissecting host plant manipulation by cyst and root-knot nematodes

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Dissecting host plant manipulation by cyst and root-knot nematodes

by

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Propositions

1. Contrary to interactions between endoparasitic nematodes and legumes, *ENOD40* plays no role in the infection of non-legumes.
    (This Thesis)

2. Host plant resistances based on the specific and subtle interference in the molecular interaction between a pathogen and its host will contribute to a more positive perception of GM (genetically modified) crops.
    (This Thesis)

3. The fact that the potato cyst nematode *Globodera rostochiensis* secretes during plant penetration an endo-β-1,4-D- glucanase with a family 2 carbohydrate binding module (CBM) and recruits during feeding cell induction a plant endo-β-1,4-D- glucanase with a similar CBM is unlikely to be coincidental.

4. According to the Red Queen theory organisms with sexual reproduction have an advantage in the cyclic arms race with their competitors; sex will allow them to accommodate faster. Hence, it is remarkable to see that root knot nematodes - predominantly asexual organisms - are currently the most successful group of plant parasitic nematodes. The Red Queen theory predicts a successful future for the - predominantly sexual - cyst nematodes.

5. Nowadays, great results make up half of the ingredients needed for a publication in a high-impact scientific journal. The other half consists of guts, a convincing writing style and the identification of appealing selling points.

6. There is more to life than life sciences.

7. Integration would not be such a problem if more people realized that freedom of speech does not give them the right to offend.

8. Patria est ubicumque est bene.
    (Pacuvius)

Propositions belonging to the thesis:
"Dissecting host plant manipulation by cyst and root-knot nematodes"
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Darkowi, Danielowi i Dziadkowi

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Abstract

Cyst (*Globodera* spp. and *Heterodera* spp.) and root-knot nematodes (*Meloidogyne* spp.), one of the most damaging crop pests, are a perfect example of highly adapted, sophisticated root parasites. These nematodes induces specialized feeding structures (cyst nematodes-syncytia, root-knot nematode-giant cells) within the host root and benefits from guaranteed continues supply of food and water from their host plant and protection within the cyst or gall for their progeny. This dissertation aims at elucidation of the molecular mechanisms controlling the induction and maintenance of those feeding structures. This knowledge is indispensable to make novel advances in constructing durable host plant resistance.

A number of different observations suggested a primary role of the phytohormone auxin in the feeding cell development. However, it has never been directly proven. Therefore, we have used the DR5 promoter element fused to *gusA* reporter gene to visualize spatial and temporal changes in auxin distribution, during nematode infection in *Arabidopsis thaliana* roots infected with cyst or root-knot nematodes. DR5 promoter element is considered to be a specific indicator for auxin and is activation points at an increase of the perceived auxin concentration. For both, cyst and root-knot nematode species, strong GUS expression was observed at the very onset of parasitism, already at 18 hours post inoculation. This high expression level was maintained until 3-5 days post inoculation and then gradually was reduced. Semi-thin sections of infected roots were investigated microscopically, revealing the presence of GUS inside the initial cells, and in case of cyst nematodes, in cells to-be incorporated into syncytium. This implies that expansion of syncytium is marked by the regions with high *gusA* expression. Activation of DR5 promoter element can be explained by: an accumulation or an increased sensitivity to auxin. However, based on formerly gathered data, a local accumulation seems to be the more probable explanation. Moreover, the feeding structures of cyst and root-knot nematodes arise by distance mechanisms, hence the highly similar DR5 activation patterns in both feeding structures, is remarkable. This prompt the conclusion that notwithstanding the highly dissimilar structure and ontogeny, both nematodes interfere with similar signal transduction pathways in root morphogenesis.

Apart from phytohormones, small peptide signalling plays a very important role in diverse aspects of plant growth and development, and such a molecule(s) could be potentially involved in the establishment of nematode feeding cell. Early nodulin (*ENOD*) genes have been defined as genes expressed in legumes during nodule formation. Recent studies, however, revealed a number of homologues of *ENOD40*s in non-legume plants. In both plants types, *ENOD40* expression associates with the development of the vascular tissue. Feeding site development by root-knot and cyst nematodes is accompanied by the distortion of the vascular tissue, therefore a local increase in *ENOD40* expression could be anticipated. This holds truth in interaction between legume *Medicago truncatula* and root-knot nematodes. However, in non-legume tomato infected with cyst and root-knot nematodes, no change in *ENOD40* transcript levels could be detected using RT-PCR. In addition, activation of the *Sl-ENOD40* promoter was only observed in infected regions showing endogenous *ENOD40* expression. Similar results were obtained for a legume *ENOD40* promoter (soybean) in a non-legumes background (*Arabidopsis*), and - contrary to *M. truncatula* - over-expression of *Gm-ENOD40* in *Arabidopsis* had no effect on nematode development. Hence, *ENOD40* expression in

tomato is basically unaffected by the induction of feeding sites by root-knot and cyst nematodes. This unexpected difference in nematode-induced *ENOD40* is apparently plant background-dependent, and suggests the existence of a legume-specific factor that boosts the *ENOD40* expression in legume hosts-nematode interaction.

One of the most striking characteristic of the cyst nematode induced feeding structure (syncytium) is the extensive cell wall dissolution, as the syncytium expands towards the vascular bundle and along the xylem vessels. Endo-1,4-ß-glucanases (cellulases) reside among plant enzymes that are involved in this progressive cell wall dissolution. RT-PCR experiments in tomato revealed that the potato cyst nematode induces the expression of two out of the eight described cellulases, precisely, *Sl-cel7* and *Sl-cel8*. *In situ* hybridization and immunodetection studies showed that both cellulases are expressed inside and adjacent to nematode-induced syncytia. Transgenic potato plants carrying hpRNA-silencing constructs for *cel7* and *cel8* were infected with cyst nematode which resulted in a significant arrest of cyst nematodes development. Reduced development of juveniles into females (up to 60%) and increase in the fraction of females without eggs (up to 89%) was observed. This indicates that the recruitment of specific plant cellulases by potato cyst nematodes is essential for their development, and demonstrates how detailed knowledge of compatible plant-parasite relationships can lead to host plant resistance.

The genomic sequence of potato, *cel7* and *cel8a* was cloned and characterised, in order to enable the RNA interference experiments in the natural host of potato cyst nematodes. The analysis of sequences suggests the presence of multiple isoforms of *cel8* in potato. That corresponding proteins consist solely of a catalytic core domain (St-CEL7) or of a catalytic domain linked to a carbohydrate binding module (CBM) (St-CEL8a). Only recently, the CBM domain of Sl-CEL8a was shown to belong to CBM family 2 and on the basis of shared features between CBM domains in Sl-CEL8 and St-CEL8a it can be concluded that St-CEL8a harbours a cellulose binding domain as well. Therefore, those findings imply that the potato cellulases recruited by the potato cyst nematode are able to hydrolyse both xyloglucan (CEL7) and crystalline cellulose (CEL8), and apart from cloning novel potato cellulases genes, provides an explanation why nematodes recruit exactly these two members of the cellulase family. Moreover, potato cellulase sequences were used to mine EST databases and this information was used for the generation of a phylogenetic tree of the *Solanaceous* endo-β-1,4-D-glucanase gene family.

In this dissertation we take an effort to understand better the intimae relationship between the obligatory plant parasites, cyst and root-knot nematodes and its host plants. We show that such a detailed knowledge can be beneficial in better understanding of plant physiology and can be used as a highly specific and bio-safe approach for pathogen management.

Chapter 1

General Introduction and Thesis Outline

Nematodes

Nematodes are extremely numerous; presumably 80% to 90% of all multicellular animals on earth belong to the phylum Nematoda (Bongers, 1988). These simple, fusiform animals are found from the bottom of the deepest ocean to near the tops of the highest mountains, and from the polar regions to the tropics (Cobb, 1914; Ditlevsen, 1918; Mann, 1948). Cobb (1914) said that if everything on the earth were to disappear except nematodes, the contour of everything would still be visible: the lakes, oceans, the plants and the animals would be outlined by the nematodes living on every habitant. The estimated number of species in the phylum Nematoda varies from 40,000 to 100 million species (Dorris, *et al.* 1999), however only 20,000 has been described so far. The inaccuracy of estimation of the species number is in part due to the lack of informative morphological characters. Nematodes are relatively simple, colorless and unsegmented roundworms mostly lacking appendages. Most of the nematodes are of microscopic size, however, a few animal parasitic species can be as long as several meters. The majority of this phylum consists of free living and predaceous nematodes, feeding on materials as varied as algae, fungi, bacteria and fecal matter. Nowadays the most famous worm is certainly *Caenorhabditis elegans*, a bacterivorous nematode that has been used as model organism during the past 3 decades. It became the first animal whose complete genome was sequenced completely (1998). A relatively small group of nematodes adapted themselves to a parasitic lifestyle. Members of the nematode genera *Steinernema* and *Heterorhabditis* are entomopathogenic, and are used as biocontrolling agents. Some parasitic species, however, cause important diseases in livestock, animals, humans and plants. Over 10% of world population suffers form the hookworm infection, and 5,8% children living in poor conditions are expected to die of helminthiasis before their fifth birthday (Hirst and Stapley, 2000). Nematodes also occur as parasites in a great number of plant species and create extensive crop losses worldwide. Plant parasitic nematodes exploit the root as a food source. Ectoparasites stay outside the plant root and use their stylet to feed from rhizodermis and cortical cells. On the other hand, migratory endoparasitic nematodes invade the root, and feed form the cortical or stelar cells. The most sophisticated relationships between plant and the parasite is found within the infraorder *Tylenchomorpha*. Sessile endoparasitic nematodes spend most of their life inside the root and transform host plant cell(s) into specialized feeding structures. This feeding structure is the only source of nutrition throughout the nematode's life, and therefore successful induction of feeding cell is crucial for nematode survival and reproduction.

Cyst and root knot nematodes

Sedentary endoparasites such as root-knot (*Meloidogyne* spp.) and cyst nematodes (*Heterodera* spp. and *Globodera* spp.) are the most damaging nematodes on a worldwide basis and are responsible for billions of dollars in crop losses each year. Just as, e.g., the animal parasitic nematode *Trichinella spiralis* (Despommier, 1998), they do not kill the host cell from which they feed. Instead, they are remodeling the host root cell into highly specialized feeding structures by reprogramming the host gene expression. Both nematode genera start their lifes outside the root. Pre-parasitic second-stage juveniles (J2) hatch from the eggs retained in the protective cyst (cyst nematodes) or gelatinous egg sac produced by *Meloidogyne* females. For some oligophagous cyst nematodes such as *Globodera rostochiensis* and *G. pallida* hatching is induced by the root diffusate of host plants (Perry, 1987). Plant parasitic nematodes have oesopheal glands: one dorsal and two subventral glands. The dorsal gland ends near the base of the nematode stylet whereas the subventral glands empty into the lumen posterior to the metacorporal pump chamber. The shape and metabolic activity of the glands change during the life cycle: subventral glands are most active just prior to the host invasion whereas the dorsal gland increases its activity at the very onset of parasitism, just before the induction of the feeding structure. The root-knot nematode enters the root in/near the meristematic zone, whereas cyst nematodes prefer the elongation or the differentiation zone. The plant cell wall is penetrated by the combination of two methods: mechanical piercing by stylet (a hollow needle-like device) thrusts and the enzymatic softening. Subsequently, the pre-parasitic J2 migrates intracellulary (cyst nematodes) or intercellulary (root-knot nematodes) within the host root, towards the vascular tissue in order to find a cell competent for feeding site induction. Remarkably and in contrast to cyst nematodes, *Melodogyne* juveniles appear to be unable to penetrate the vascular cylinder directly. Once inside the root, J2 of root-knot nematodes, orient themselves towards the root tip. When they reach the meristem, they turn around, and migrate back up into the differentiating vascular cylinder until they arrive at the zone where protoxylem starts to develop (Sijmons, *et al.*, 1991; Wyss, *et al.*, 1992).

One of the most intriguing features of nematode-plant interaction is the induction and maintenance of the feeding structure. In this process, plant cells are forced to re-differentiate into a completely new structure. Therefore, feeding site formation can be regarded as the modification of the original plant developmental plan. The fact that, those different types of feeding cells, induced in different hosts, are remarkably similar (syncytium to syncytium/giant cell to giant cell) suggests that the nematode interferes with conserved signal-transduction pathways in root morphogenesis. The criteria underlying initial feeding cell selection by the nematode are unknown. Initiation of syncytium and giant-cell formation is believed to occur in response to molecules produced in the dorsal glands and secreted by

the infective juvenile, through the stylet, that is partially inserted into the initial feeding cell. Once infective juveniles from cyst nematodes have injected gland secretions into a selected plant cell, the plant cell will start to re-differentiate. These changes include increased density of the cytoplasm, intensification of the cytoplasmic streaming, proliferation of the cellular structures, and polyploidisation of the nucleus (Wyss and Zunke, 1986). Highly directed cell wall breakdown results in protoplast fusion with neighbouring cells. The initial syncytium spreads towards the vascular tissue and from there it expands along the xylem vessels by progressive cell wall breakdown and coalescence of protoplasts. Finally, up to 200 cells can be incorporated into a syncytium. This extensive modification of dozens of differentiated plant root cells is one of the most remarkable features of the syncytium. The syncytium becomes increasingly hypertrophied and acts as metabolic sink from which the nematode continuously takes up food. This syncytium will be the only source of food throughout the nematode's life. Shortly after the feeding process started, the nematode becomes immobile and undergoes three additional moults before reaching maturity. Males of cyst nematodes become mobile again after the third moult, and leave the roots to fertilize the swollen females. After fertilisation the female dies and her cuticle turns into a protective structure for embryonated eggs. Giant cells are formed by root knot nematodes and the initial event in giant cell formation is the induction of the repeated mitosis without cytokinesis in a selected number of plant cells surrounding the head region of the nematode (Bird, 1975). Each infective juvenile triggers the development of five to seven giant cells, each containing up to 100 nuclei (Sijmons and Atkinson, 1994). Nuclear and cellular hypothrophy is accompanied by the changes in the cell wall structure. Transfer cell-like cell wall ingrowths are formed by the deposition of secondary cell wall material (Bleve-Zacheo, *et al.*, 1997). Notwithstanding differences in their ontogeny giant cell and syncytium share the same physiological function: supplying nematode with water and food. As a result of giant cell formation, the surrounding tissue starts to divide. This gives rise to the characteristic gall. Reproduction in most root-knot nematodes species is by parthenogenesis, although males are also commonly found. The adult female produces several hundreds of eggs deposited as an egg masses within a secreted gelatinous matrix. After completion of its life cycle, the nematode dies and the giant cell and gall tissue degenerate.
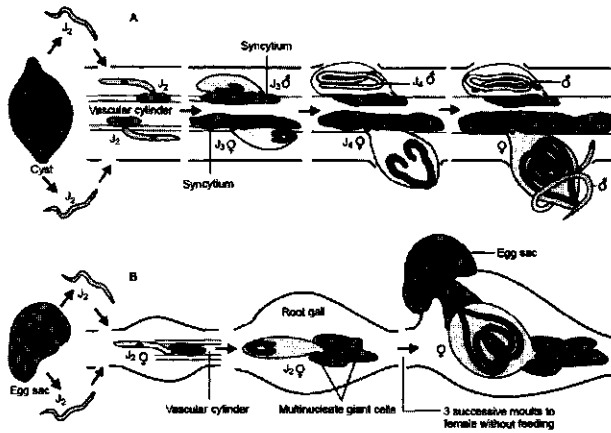
Figure 1:
The life cycle of cyst (**A**) and root-knot nematodes (**B**). J2, J3, J4-juveniles in the second, third and fourth developmental stages. (Jung, *et al.*, 1999)

Nematode induced plant gene expression

Feeding site formation by sedentary plant parasitic nematodes is a very fascinating and complex process that received a lot of scientific attention. Elucidation of this poorly understood interaction involves characterisation of *proteins secreted by the nematode* via its oesophageal glands cells that could alter the normal plant cell program and *plant genes* whose expression levels are affected by the intimate plant-parasitic interaction. Nematode secretions could be active on different levels: either by interfering with existing metabolical and transductional pathways, or by migrating into the nucleus and triggering (transient) changes in the transcriptome. Molecular analyses of the genes expressed in nematode dorsal glands resulted in identification of the MjCM-1 (CM - chorismate mutase) gene, expressed in dorsal glands on *Meloidogyne,* and secreted into the plant tissue during the parasitism. MjCM-1 gene could be involved in the reprogramming of the plant cell developmental fate, upon nematode feeding (Doyle and Lambert, 2003).

Molecular approaches to study the plant gene expression in nematode feeding site
The key to the understanding of the molecular interaction between the nematode and its host is time and labour-intensive molecular research. Recent development of various molecular techniques allowed scientists to study in detail the effect of nematodes infection on the plant genes expression patterns. Approaches vary from detailed studies on single genes to transciptome comparison using *e.g.* cDNA-AFLP. Discovery of 'novel' plant genes involved

in plant-nematode interactions can be done by techniques like promoter tagging or differential display:

Promoter tagging

A promoter tagging method is based on the random integration of the *gus* reporter gene in the plant genome. In this way the plant promoters that are activated during migration or feeding site formation could be spotted. This laborious method performed on *Arabidopsis* lines infected with the root-knot nematode *M. incognita,* resulted in identification of plant genes like RPE (Favery, *et al.,* 1998) *AtFH6* (Favery, *et al.,* 2004) and *pyk20* (Puzio, *et al.,* 1999). In a similar experimental set up six differentially activated tags in cyst nematode feeding structure were isolated (Barthels, *et al., 1997*).

Differential display

To obtain more comprehensive information of the changes in plant gene expression associated with the nematode infection different display techniques were employed. Those techniques are based on the comparison of the transcriptomes of infected and non infected plant parts. Subtractive cDNA cloning resulted in identification of fifty eight cDNA clones up-regulated in the root tissue infected with root-knot nematodes (Bird and Wilson, 1994). The identified clones encoded the Myb transcription factors, plasmalemma proton ATPase and subunits of RNA polymerase II. cDNA-AFLP performed on tomato roots infected with cyst nematode *Globodera rostochiensis* revealed up and down regulation of hundreds of the plant genes (Pierzgalska, unpublished).

In the compatible interaction between the cyst nematode *Heterodera schachtii* and *Arabidopsis thaliana* differential display yielded 11 down – and 13 up-regulated plant genes (Hermsmeier, *et al.,* 2000) and in the *A. thaliana -Meloidogyne incognita* interaction six genes with an altered expression pattern during feeding site formation were found (Vercauteren, *et al.,* 2001). Based on the extensive knowledge of the *A. thaliana* genome, DNA microarrays were produced that allow the comparison of expression patterns between roots infected with cyst nematodes *H. schachtii* (which is able to develop feeding sites in *A. thaliana*) or *H. glycines (*which is not able to develop feeding sites in *A. thaliana),* and uninfected roots. Eighty two genes were induced and forty six down regulated in the compatible interaction between *A. thaliana* and *H. schachtii,* whereas only 12 transcripts with changed expression were detected following infection with *H. glycines* (Puthoff, *et al.,* 2003).

Promoter-reporter gene fusion

Promoter–reporter gene fusion was successfully used to study the spatial and temporal the activation of plant promoters in the course of the feeding site formation. These experiments revealed the differential regulation of the plant *ENOD40* gene in legume and no

legume background, during infection with parasitic nematodes. In *Medicago truncatula* roots infected with the root-knot nematode *M.incognita* ENOD40 gene was expressed in/around giant cells (Koltai, *et al.*, 2001; Favery, *et al.*, 2002), however its expression in tomato (no legume) roots infected both with root and cyst nematodes was coincidental (this thesis, Chapter 3). Moreover promoter-reporter gene fusion experiments revealed up-regulation of wound inducible (Hansen, *et al.*, 1996) genes, genes involved in cell cycle activation (De Almeida Engler, *et al.*, 1999; Goverse, *et al.*, 2000a) vascular tissue differentiation (Koltai, *et al.*, 2001; Favery, *et al.*, 2002) activation of auxin-responsive promoters (Hutangura, *et al.*, 1999; Goverse, *et al.*, 2000b; Karczmarek, *et al.*, 2004) and sugar transporters genes (Juergensen, *et al.*, 2003).

RT-PCR, in situ hybridisation, RNA blotting

Information about nematode-induced feeding structures can also be used to formulate working hypotheses about candidate plant genes that could possibly be involved in feeding site morphogenesis. To confirm or discard such hypotheses, RNA-based techniques can be employed. Messenger RNA is isolated from the infected tissues and RT-PCR experiments revealed for instance the up-regulation of *Sl-cel7* and *Sl-cel8* in syncytia induced in tomato roots (this thesis, Chapter 4). Detailed *in situ* hybridisation and immunolocalisation experiments confirmed those findings (this thesis). More candidate genes such as α and β expansins (Kudła, unpublished, Gal, *et al.*, 2006) and ENOD40 (this thesis, Chapter 3) (Favery, *et al.*, 2002) were found to be differentially regulated in the course of the feeding cell development.

Plant genes differentially regulated in nematode feeding site formation

Plant genes that are differentially expressed in the feeding site morphogenesis can be divided into several classes related to their metabolical and developmental functions (Gheysen and Fenol, 2002).

Wound and defence response genes

Nematode infections result in the wounding of the plant tissue. As a result we observe the induction of wound and defence response genes in the infected root tissue. In the presence of the appropriate resistance gene, the defence response will be fast and strong and will result in a so-called hypersensitive response. On the other hand, slow and weak defence responses - in the absence of an effective resistance gene - will be observed as a compatible interactions (Williamson, 1999). Interestingly, most of the genes are expressed both in compatible and incompatible interaction, although differences in timing and the level of expression are observed depending on type of the interaction (Williamson and Hussey, 1996). The

nematode-induced defence response of plant is not only limited to the up-regulation of the various defence genes (extensins, proteinase inhibitor, chitinase, peroxidase, lipoxygenase) (Williamson and Hussey, 1996) but includes also activation of pathways leading to phytoalexin biosynthesis (Kaplan, *et al.* 1980) and the deposition of callose or lignin as a physical barrier (Balhadere and Evans, 1995) for the nematode infection.

Transcription factors

Feeding site formation is accompanied by the extensive modification of the original developmental plan. Transcription factors direct the gene expression resulting in cell differentiation. Identification of specific transcription factors present in the nematode feeding structure may contribute to the identification of signal transduction pathways leading to feeding site formation. Transcription *WRKY* factor assigned as (Att0001), forms a big family of important transcription factors with multiple roles, is auxin upregulated (Gheysen and Fenoll 2002) and was identified in a screen for plant regulatory elements activated in the nematode feeding structure (Barthels, *et al.*, 1997). Another transcription factor, up-regulated in syncytium only, is ABI3, the global regulator of the cell fate involved in cellular differentiation and meristem quiescence (Giraudat, *et al.*, 1992). Another group of possibly relevant transcription factors *KNOX* and *PHAN* are induced in giant cell formation (Koltai, *et al.*, 2001). Both genes are required for the normal functioning of the meristem, and *KNOX* mutations phenocopy certain *PHAN* mutations (Schneeberger, *et al.*, 1998; Timmermans, *et al.*, 1999). Moreover elevated *KNOX* expression level was linked to elevated auxin and cytokinin levels (Tsiantis, *et al.*, 1999). Therefore it was postulated that *Mt-knox-1* expression in giant cells of *M.incognita* might be involved in the regulation of phytohormone levels (Koltai, *et al.*, 2001).

Hormonal response

Regulation and coordination of the growth, metabolism and morphogenesis of plant is associated with the activities of five (abscisic acid, auxin, ethylene, cytokinin, gibberellin) groups of phytohormones. They have a central role in communication inside the plant, and in the adjustment of growth and development to environmental stimuli. Each of the phytohormones has the ability to influence a remarkable variety of developmental and physiological processes. Above all, auxin has received most scientific attention, since this simple molecule can bring about very diverse responses. Indole-3-acetic acid (IAA) is the most abundant naturally occurring auxin. IAA is a simple small molecule that is chemically similar to the amino acid tryptophan and can be produced by releasing IAA from conjugates or via *de novo* synthesis by conversion of tryptophan or indirectly in tryptophan-independent manner (Bartel, 1997). Interestingly, auxin has been hypothesized as being one of the key regulators in feeding site morphogenesis (Hutangura, *et al.*, 1999; Karczmarek, *et al.*, 2004). Auxin responsive genes have been reported to be down or up regulated in the nematode

feeding structure (Gheysen and Fenoll, 2002). Genes coding for cell wall-modifying enzymes responsible for the extensive cell wall modification in the NFS are often (also) regulated by auxin (some cellulases and expansins accumulate in the presence of auxin) (Catala, *et al.*, 1997; Catala, *et al.*, 2000). Also genes responsible for the cell cycle reactivation in the NFS, like *cdc2a, cdcb1* are induced by auxin (De Almeida Engler, *et al.*, 1999). Another plant hormone that is thought to play a role during NFS formation is ethylene. Ethylene-overproducing *A. thaliana* mutants are hyper susceptible to beet cyst nematodes (Wubben, *et al.*, 2001; Goverse, *et al.*, 2000b). Ethylene can also modify the shape of the cell by inducing changes in the cytoskeleton (Shibaoka, 1994). Cytokinins are though to have essential in the controlling of the plant cell cycle. Interestingly, high cytokinin levels correlate with the plant susceptibility to root-knot nematodes (Sawhney and Webster, 1975). Moreover, *Melodoigyne* spp. are able to produce biologically active cytokinin (De Meutter, *et al.*, 2003). In summary, nematode-induced changes in plant gene expression are likely to be mediated by local changes in phytohormone levels and current insights point at a role for at least auxin and ethylene in this process.

General metabolism

One of the main characteristics of nematode feeding structures is a very dense cytoplasm, and the proliferation of cellular organelles point at high levels of metabolic activity. Nematode is completely dependent on this structure for its growth and reproduction. The quality of the feeding site, its size, protein content and amino acid composition will determine the sex of the nematode (Grundler, *et al.*, 1991). It is not surprising to see the up-regulation of an extensive set of house keeping and stress response genes. For example the enzyme hydroxylmethyl-glutaryl-CoA reductase is accumulated in the giant cells from the onset of parasitism till the end of egg maturation (Bleve-Zacheo, *et al.*, 1997). This protein is a key enzyme in the phytosterol biosynthesis (Chappell, 1995) and may be used in the intense vesicle traffic needed for the extensive cell membrane biogenesis. Moreover the fact that this enzyme and the endoplasmatic reticulum co-localise with tubulin and actin gave rise to the assumption that vesicles charged with this enzyme travel to the cell surface to support the extra tubulin and actin production in giant cells (Bleve-Zacheo, *et al.*, 1997).

Cell cycle genes

Feeding cell formation and development is accompanied by the re-activation of the plant cell cycle. Giant cells undergo rounds of repeated mitosis without cytokinesis, whereas syncytial cells undergo endoreduplication. Considering the nuclear changes that occurs during the feeding site development it is not surprising that 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). Genes like *ccs52*, an ubiquitous regulator of the cell cycle transition, cell cycle kinases like cdc2a, cdc2b and cyclins are commonly found to be activated, both in syncytia and in giant cells (Niebel, *et al.*, 1996; De Almeida Engler, *et al.*, 1999; Goverse, *et al.*, 2000a)

Cell wall

One of the most striking characteristics of feeding site proliferation are the directed and extensive cell wall modifications, cell wall dissolution, and local cell wall thickening. No surprise, a range of cell wall-related enzymes is activated both in syncytia and giant cells. Tomato cellulase 7 and 8 are expressed already in the initial syncytial cell, and if knock-out, syncytium development is hampered, underscoring the importance of the cell wall modification in its development (this thesis, Chapter 4). The *Arabidopsis cell* promoter is exclusively activated in giant cells, and not in syncytia (Goellner-Mitchum, *et al.*, 2004), which illustrates the difference in the sets of cell wall-modifying proteins that are recruited during syncytium or giant-cell proliferation. The activation of other cell wall modifying proteins of plant origin such as pectinacetylesterases, polygalacturonase (Mahalingam, *et al.*, 1999; Vercauteren, *et al.*, 2002) and expansins (Kudła, unpublished, Gal, *et al.*, 2006) emphasises the importance of cell wall modification in the feeding site development.

Outline of the thesis

Obligatory plant parasitic nematodes (cyst and root-knot) establish specialized feeding cells within the host's roots to supply nutrients for their growth and reproduction. This thesis aims to increase our understanding of the molecular mechanisms underlying the compatible interactions between the plant parasitic nematode and its host plant. This knowledge can be applied to engineer new durable host plant resistance against parasitic nematodes.

Initiation of the feeding cells is believed to occur in response to molecules secreted from the nematode. Modification of the normal developmental cell development by nematodes is accompanied by dramatic changes in the gene expression. A number of different observations suggested a role of the phytohormone auxin in the onset of the feeding cell development. However, direct evidence of auxin involvement in feeding cell establishment is lacking. DR5, a synthetic auxin response element derived from the GH3 promoter, is currently the best-known and most used auxin marker. Therefore, in **Chapter 2** we describe the use of the DR5 promoter-*gusA* fusion to visualize changes in auxin distribution during nematode infection in *Arabidopsis thaliana* roots.

Feeding cell development by cyst and root-knot nematodes strongly correlates with lateral root formation. A similar high correlation was observed between lateral root formation and nodule formation in the *Rhizobium*-legume plant interaction. Rhizobial bacteria, as well

as nematodes, direct the host cell to form a complex novel structure in the root tissue. This process is accompanied by local accumulation of the *ENOD40* gene product. Interestingly, in nematode legume-host interaction *ENOD40* was also found to be present in the feeding structure. In **Chapter 3**, I investigated the expression pattern of the early nodulin gene *ENOD40* in two non-legumes host plants, tomato and *Arabidopsis*, upon infection with the cyst nematodes *Globodera rostochiensis* and *Heterodera schachtii*, and the root-knot nematode *Meloidogyne incognita*.

The most striking characteristic of the cyst nematode induced syncytium is the extensive cell wall dissolution, as the syncytium expands towards the vascular bundle and along the xylem vessels. Enzymes of plant origin were implicated to have a main role in the fascinating process. In **Chapter 4** expression patterns of the tomato endo-β-1,4-D-glucanases are examined, and two family members up-regulated in the developing syncytium (*Cel7* and *Cel8*) were subjected to a more detailed study: *in situ* hybridization and immunolocalization *in planta*. Moreover, the gene-silencing experiments were conducted to assess the importance of *Cel7* and *Cel8* up-regulation in the process of syncytium development. **Chapter 5** describes cloning of the two novel cellulases from potato (*Solanum tuberosum*): homologues of the tomato cellulases *Cel 7* and *Cel 8*.

Finally, in **Chapter 6** the role of cell wall-modifying enzymes in feeding site development, and the possible mechanisms underlying this process are discussed.

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Chapter 2

Feeding cell development by cyst and root-knot
nematodes involves a similar early, local and transient
activation of a specific auxin-inducible promoter
element

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Abstract

To study the role of the phytohormone auxin in nematode feeding cell induction and early development, the transcriptional regulation of the auxin-responsive promoter element DR5 was monitored in *Arabidopsis thaliana* roots infected with cyst or root-knot nematodes. For both nematode species, a specific and strong activation of DR5::*gusA* was observed inside the initial cells at 18 hours post inoculation, pointing at an increase of the perceived auxin concentration. This high expression was maintained until 3-5 days post inoculation and subsequently the GUS staining was reduced. Cyst and root-knot nematodes are distantly related and the feeding sites they induce are highly dissimilar. In this respect, the similarities between the two nematode-induced DR5 activation patterns in *A. thaliana* roots are remarkable. A transient and local increase in auxin perception could be due to an accumulation or to an increased sensitivity. Based on previously published data, a local auxin accumulation seems to be the more probable explanation. The observed specific and transient increase of the perceived IAA concentration in the initial feeding structure could be an important clue in the elucidation of the molecular mechanisms underlying feeding cell induction by plant parasitic nematodes, a fascinating but still poorly understood phenomenon.

Introduction

Both cyst nematodes (*Globodera spp. and Heterodera spp.*) and root-knot nematodes (*Meloidogyne spp.*) are obligatory plant parasites that establish an intimate relationship with their host plants. Upon infection, the pre-parasitic second stage juveniles (pre-parasitic J2) penetrate the roots of a suitable host and migrate intracellularly (cyst nematodes) or intercellularly (root-knot nematodes) towards the vascular cylinder in order to find a competent plant cell for the induction of a multinuclear feeding cell complex. Depending on the nematode-host plant combination, a differentiated or undifferentiated cell is selected as a starting point for feeding cell induction (Golinowski, *et al.*, 1997). In *Arabidopsis*, a procambial or cambial cell is selected by the beet cyst nematode *Heterodera schachtii* (Golinowski, *et al.*, 1997), whereas the root-knot nematode *Meloidogyne incognita* selects 2-12 parenchymatic xylem cells in the differentiation zone of the root (Wyss, *et al.*, 1992).

Although cyst and root-knot nematodes use a comparable strategy to parasitise the plant, the feeding structures they induce are highly dissimilar. Cyst nematodes induce a so-called syncytium, whereas root-knot nematodes induce a giant cell complex. In case of a syncytium, the initial selected feeding cell (ISC) expands towards and within the vascular tissue by progressive cell wall dissolution resulting in the fusion of the protoplasts of neighboring cells (Golinowski, *et al.*, 1997). In case of a giant cell complex, several progenitor cells are selected around the nematode's head. Cell enlargement and repeated rounds of mitosis without cytokinesis result in the formation of a cluster of multinuclear giant cells (Huang, *et al.*, 1985). Hypertrophy of the surrounding tissue results in the formation of typical galls on the infected roots (Jones and Northcote, 1972; Jones and Payne, 1978).

The formation of a feeding cell is associated with dramatic changes in gene expression (Gheysen and Fenoll, 2002) and several studies point at the role of plant hormones in this process (Hutangura, *et al.*, 1999; Goverse, *et al.*, 2000a; Goverse, *et al.*, 2000b; Wubben, *et al.*, 2001). The phytohormone auxin, indole-3-acetic acid (IAA), is involved in many aspects of plant differentiation and growth, including root development (Coenen and Lomax, 1997; Sabatini, *et al.*, 1999; Casimiro, *et al.*, 2003). The expression of auxin-inducible genes such as the *Arabidopsis* ribosomal protein S5 (Goverse, *et al.*, 2000b; Weijers, *et a.l*, 2001) in early syncytium development, and the observation that the auxin insensitive tomato mutant *diageotropica (dgt)* is *de facto* resistant to both the potato cyst nematode *G. rostochiensis* (Goverse *et al*, 2000b) and the root-knot nematode *M. incognita* (Richardson and Price, 1984) suggest a role for auxin in feeding cell development.

To study the role of IAA in nematode-induced feeding site induction in more detail, a specific, well-characterized set up should be used with a relatively high spatial resolution. The auxin-responsive reporter DR5-β-glucuronidase (GUS) was shown to be a valuable tool to study a number of developmental processes in plants (Aloni, *et al.*, 2003; Scarpella, *et al.*, 2003; Tang, *et al.*, 2003). DR5 is a synthetic auxin-responsive promoter element consisting of

a tandem repeat of a modified natural auxin-responsive element (AuxRE). This element originates from the soybean promoter *GH3* (Hagen, *et al.*, 1991). As it does not contain any other *cis* element that can regulate gene transcription, DR5 is considered to be a specific indicator for auxin, and this element is more sensitive to auxin than *GH3* (Ulmasov, *et al.*, 1997). In this paper, we show that both giant cell and syncytium induction is accompanied by an early, transient and very local activation of DR5, and the consequences of this finding in relation to feeding site induction by nematodes are discussed.

Results

To monitor role of the phytohormone auxin during nematode-induced feeding cell development, the transcriptional regulation of the artificial auxin-responsive element DR5 was studied in *A. thaliana* roots infected with cyst and root-knot nematodes. The *gus* reporter gene was used to visualize the spatial and temporal regulation of the DR5 element.

In uninfected roots, *gus* expression was observed specifically in the meristematic zone of the root tips and in lateral root primordia (Fig. 1A and B). After inoculation with the beet cyst nematode *H. schachtii*, a strong and local activation of the DR5 element was observed at the infection sites at 18 hpi and at 42 hpi (Fig. 1C and D). No changes in *gus* expression pattern were observed in the nematode migratory track as compared to control plants (Fig. 1D and E). Apparently, the observed transcriptional activity is a specific response to nematode feeding cell induction and not due to wounding. At 3-5 dpi, a slow gradual decrease of the intense blue GUS staining was observed (Fig. 1E). No activity was observed at 10 and 14 dpi (Fig. 1F). Apart from the infection site itself, additional GUS staining was observed in the lateral root primordia induced near the nematode feeding site (Fig. 1F). It is concluded that cyst nematode-induced syncytium formation in *Arabidopsis* roots is accompanied with an early, localized and transient activation of the auxin-responsive element DR5.

To determine the cellular localization of the *gus* expression in developing syncytia, semi-thin sections were investigated microscopically. Intense GUS staining was detected inside the young syncytia initiated by *H. schachtii* (Fig. 1G and H) and a more diffuse staining pattern was observed in the surrounding cells, including the cortex and vascular bundle. Observations at later time points suggest that the majority of those cells will be incorporated into the developing syncytium during its expansion towards the vascular bundle. Interestingly, a change in the spatial distribution of this *gus* expression pattern was noticed around 3 dpi. The level of *gus* expression decreased in the developing syncytium and the zone of intense GUS staining was shifted basipetally towards the distal end of the expanding syncytium (Fig. 1I and J). Therefore, the staining patterns suggest that the radial and longitudinal expansion of the syncytium is preceded by a local accumulation and/or increased sensitivity towards IAA.

In a similar experimental set up, the effects of root knot nematode (*Meloidogyne incognita*) on DR5-induced *gus* expression was investigated. At 18 hpi, intense GUS staining was detected specifically at the nematode infection site (Fig. 1K). This high expression level was maintained until 3-5 dpi and at 7 dpi, the expression gradually decreased (Fig. 1L-M). From 10 dpi onwards (Fig. 1N), no *gus* expression was detected at the infection site. The level of GUS staining in the feeding sites was comparable to *gus* expression levels in the root tips of uninfected plants. Also in this interaction, feeding site induction was accompanied by the induction of lateral root primordia in its direct vicinity, and strong transcriptional activation was observed in these developing roots (Fig. 1M and N). Interestingly, a reduction in *gus* expression was observed in the primary root meristem upon giant cell formation suggesting that the basipetal auxin transport towards the root tip is hampered upon nematode infection (Fig. 1K).

Before any typical morphological features of giant cell formation were visible, microscopic observations of feeding sites induced by *M. incognita* revealed the presence of GUS particles inside cells surrounding the nematode head region (Fig. 1O and P). This indicates that auxin plays role at a very early stage in the initial giant cells. At 3 dpi, the homogeneous *gus* expression was still present inside the young giant cells and in the surrounding procambial cells. At later stages, the level of GUS decreased drastically (Fig. 1Q-R) and around 10 dpi, no *gus* expression was visible in or around the examined giant cells. These data demonstrate a local and temporal increase in the perceived IAA level inside the giant cell progenitor cells and in a layer of surrounding cells.
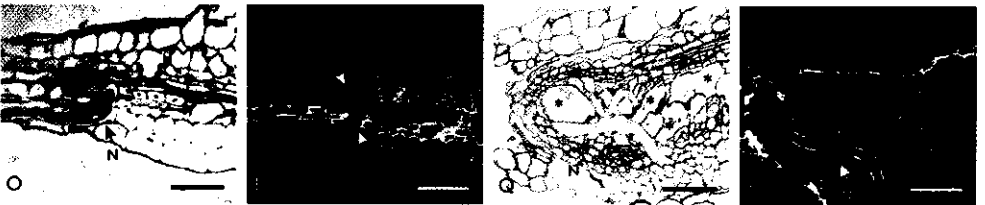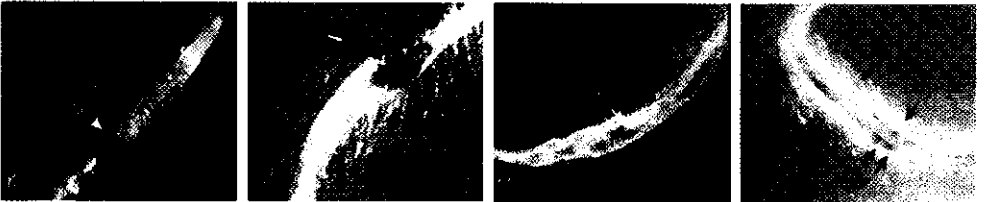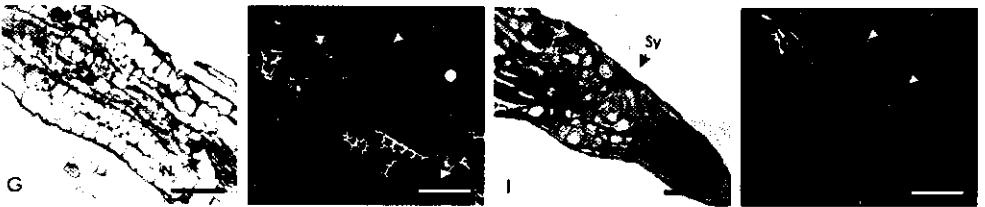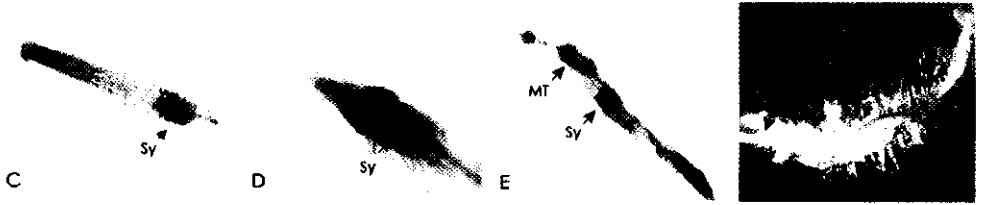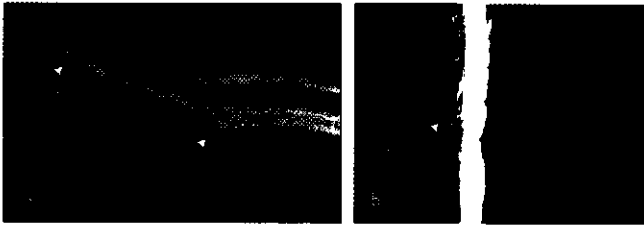
Figure 1:

Transcriptional regulation of the auxin-inducible DR5 element in transgenic *A. thaliana* roots infected with the beet cyst nematode *H. schachtii* or the root-knot nematode *M. incognita* using the *gusA* reporter gene. Semi-thin sections were investigated using bright **(G, I, O, Q)** or dark field **(H, J, P, R)** microscopy, resulting in respectively a blue or a red staining of the GUS particles. For bright field microscopy, sections were stained with toluidine blue.

A and B, Uninfected control; GUS staining is observed only in the root tip and the lateral root primordia. **C-E,** Infection with *H. schachtii* (18 hpi, 42 hpi, 3 dpi respectively); strong *gus* expression was observed in young developing syncytia. Root penetration and intracellular migration of the nematode resulted in necrotic cells. **F,** From 10 dpi onwards, no GUS staining was visible anymore in the syncytium. **G and H,** At 42 hpi, intense GUS staining was observed in the young expanding syncytium (marked with an asterisks) and in cells surrounding the syncytium including vascular tissue and the cortex. **I and J,** Weak *gus* expression was visible in the syncytium (marked with an asterisk) and surrounding cells at 3 dpi. Intense GUS staining was observed in the cambial and cortical cells basipetally to the syncytium. **K and L,** Infection with *M. incognita*; strong GUS staining was observed in the nematode infection site at 18 hpi and 3 dpi. In **K,** a reduced *gus* expression was observed in the root tip. **M,** Weak GUS staining was observed in the gall tissue at 7 dpi. **N,** From 10 dpi onwards, no GUS staining was detected in gall tissue except for the LRP. **O and P,** GUS staining was observed in the initial giant cells around the nematode head at 18 hpi. **Q and R,** At 7 dpi, weak GUS staining was observed in the giant cell complex (marked with asterisks) and the surrounding cells.

Abbreviations: Sy = syncytium, G = gall, CN = cyst nematode, RKN = root-knot nematode, LRP = lateral root primordium, MT = migratory track. In G, I, O, Q bar = 25 µm; in H, J, P, R, bars = 33 µm.

Discussion

The phytohormone auxin was postulated to be involved in the initiation and development of nematode feeding structures in the early 1960s (Balasubramanian and Rangaswami 1962; Viglierchio and Yu 1965), and a range of mainly correlative studies have been published since (Bird, 1975; Hanschke, *et al.*, 1975; Matsui and Nakagawa, 1977; Sinhababu and Sukul, 1983). Here, the auxin-responsive reporter DR5::*gusA* was used to monitor the role of auxin during feeding site induction by cyst and root-knot nematode-induced. This experimental set up showed a transient transcriptional activation of DR5 in early stages (18 hpi till 7 dpi) of feeding cell induction both by cyst and root-knot nematodes. Histological analysis revealed that the formation of syncytia and giant cells in roots of *Arabidopsis* is accompanied by an activation of the DR5 element inside the initial feeding cells.

Although the soybean promoter *GH3* and its DR5 element are both auxin responsive, they are apparently activated in different stages of the infection process. Only after 48 hours, infection of white clover with the root-knot nematode *Meloidogyne javanica* resulted in

expression of *GH3::gusA* (Hutangura, *et al.*, 1999). In *Arabidopsis*, DR5 was activated as early as 18 hpi in feeding sites of the root-knot nematode *M. incognita* and the beet cyst nematode *H. schachtii*. Although we cannot exclude that the timing of changes in the perceived auxin levels as a result of nematode infection depends on the host-nematode combination, it seems more likely that this discrepancy reflects differences in sensitivities of these promoters towards auxin; the synthetic promoter element DR5 shows a substantially higher responsiveness to auxin than the *GH3* promoter (Ulmasov, *et al.*, 1997). Hence, the observation that cyst and root-knot nematodes induce a local increase of the perceived IAA concentration at a very early stage of the infection process (< 48 hpi) is probably valid for other endoparasitic nematode-plant interactions as well.

Cyst nematode infection resulted in a similar activation pattern of DR5 as observed for the auxin-inducible (Weijers, *et al.*, 2001) promoter region of the ribosomal protein S5 (Goverse, *et al.*, 2000b), which is specifically expressed in proliferating cells that are about to be incorporated into the syncytium. This suggests a role for auxin in preconditioning of cells prior to integration into a multinuclear feeding cell complex. For root-knot nematodes, the DR5 activation pattern in cells surrounding the giant cells was comparable to the *GH3* activation in white clover (Hutangura, *et al.*, 1999). In case of giant cells, surrounding cells are not incorporated in the nematode feeding structure. It is concluded that gall formation (= cell enlargement and division) in *Arabidopsis* is accompanied by increased IAA perception as well.

Do cyst and root-knot nematodes locally increase the sensitivity of plant cells to IAA, or do they bring about a locally elevated IAA concentration? In case of an increased IAA sensitivity, the nematode would secrete components that could induce such a change. In an earlier study we provided evidence that naturally-induced cyst nematodes secretions did not change the sensitivity of tobacco protoplasts towards this hormone (Goverse, *et al.* 1999). Alternatively, infective juveniles of plant parasitic nematodes could induce a local IAA accumulation. As a first option, infective juveniles could release auxin or auxin-like compounds into the initial feeding cells. However, no indications were found for the presence these components in concentrated secretions from *Globodera rostochiensis* (Goverse, *et al.* 1999). Alternatively, cyst and root-knot nematodes could produce proteins that affect the auxin level in initial feeding cells. Such a protein could change the local IAA level directly (effect on conjugation, degradation or synthesis). Recently, (Doyle and Lambert, 2003) suggested that the release of chorismate mutase by the root knot nematode *M. javanica* could cause a local IAA depletion due to a reduced IAA biosynthesis. This hypothesis, however, is apparently contradictory with various observations that point at a locally increased auxin perception (Goverse *et al.*, 2000b; Gheysen and Fenoll, 2002; this chapter). A local increase of the IAA level could be the result of the manipulation of polar auxin transport. Hutangura *et al.*, (1999) showed an accumulation of intracellular flavonoids in developing root galls (not in the giant cells themselves) in white clover roots infected with *M. javanica*. Some flavonoids

are known to be potent inhibitors of IAA efflux carriers, and the authors suggested a role for these components in IAA accumulation. In this study, a reduction of DR5 activity was observed in the primary root tip of root-knot nematode infected plants suggesting also that the infective juvenile hampers polar auxin transport. As argued-above, both syncytium and giant cell induction are presumably accompanied by a local accumulation of IAA. We are currently investigating whether this could be the result of an increased influx or a decreased efflux of IAA.

Materials and methods

Plant material

Seeds of transgenic *A. thaliana* plants carrying the DR5::*gus*A construct were incubated for 72h at 4°C and subsequently surface sterilized in a 1:4 mix of a hypochloride solution (40g l^{-1}) and 96% EtOH for 15 min. Thereafter, seeds were washed 3 times with EtOH and dried in a flow cabinet. Sterile seeds were transferred with a toothpick to plates (∅ 9 cm, 10 seeds per plate) containing KNOP medium (Sijmons, Grundler *et al.* 1991) supplemented with 2% sucrose and 0,7% Daichin agar (Brunschwig, The Netherlands). Slightly tilted plates were incubated at 22°C with 16h of light.

Nematode inoculation

In vitro cultures of 14 day old *A. thaliana* plants were inoculated either with surface-sterilized pre-parasitic J2 of the beet cyst nematode *H. schachtii* (Goverse, *et al.*, 2000b) or with pre-parasitic J2 of the root-knot nematode *M. incognita*. For the latter, egg masses were collected from sterile cultures of *M. incognita* and transferred to 70 μm cell strainers (Falcon 2350), which were placed in a Petri dish (∅ 3,5 cm) partially filled with sterile tap water. Eggs were incubated in the dark for 1 week at 24°C. Thereafter, hatched nematodes were collected, counted and concentrated by centrifugation at 2000 rpm for 5 min. Sterile water was added to the nematode suspension to adjust the volume. Plants were inoculated with approximately 500 J2 (in 150 μl) per plate using siliconized tips.

Histochemical GUS assay

Infected and uninfected root segments were collected at 18 and 42 hours post inoculation (hpi) and at 3, 5, 7, 10 and 14 days post inoculation (dpi). The histochemical GUS assay was performed essentially according to (Barthels, *et al.*, 1997). The samples where incubated for 2h at 37°C. GUS staining was observed in the roots using a dissecting microscope (Wild, Heerbrugg, Switzerland).

Histology of nematode feeding sites

Semi-thin sections of infected and uninfected roots were prepared according to Goverse *et al.,* (2000b) and analyzed using bright and dark field microscopy (Zeiss, Oberkochen, Germany).

Acknowledgments

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Chapter 3

ENOD40 is not involved in the interaction between endoparasitic nematodes and non-legumes host plants

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Abstract

ENOD40, best known as a small protein required for nodule formation in legumes, is present in numerous legumes and non-legumes. In both types of plants, *ENOD40* expression is associated with vascular tissue development and functioning. Feeding site development by root-knot and cyst nematodes implies a physical distortion of the vascular tissue, and consequently a local increase in *ENOD40* expression could be anticipated. This has been shown for the legume *Medicago truncatula* upon infection with root-knot nematodes. Here, we investigated the role of *ENOD40* in non-legumes upon infection with cyst and root-knot nematodes. Contrary to nematode-infected *M. truncatula*, the expression of *ENOD40* was essentially unaffected by nematode parasitism. In tomato, no change in *ENOD40* transcript levels could be detected using RT-PCR and activation of the *Sl-ENOD40* promoter was only observed in infected regions showing endogenous *ENOD40* expression. Similar results were obtained for a legume *ENOD40* promoter (soybean) in a non-legumes background (*Arabidopsis*), and - unlike *M. truncatula* - over-expression of *Gm-ENOD40* in *Arabidopsis* did not affect nematode development. This unexpected, apparently plant background-dependent, difference in nematode-induced *ENOD40* expression patterns prompted us to postulate the existence of a legume-specific factor that boosts *ENOD40* transcription.

Introduction

Co-evolution between nematodes and plants resulted in obligatory endoparasites that have established a sophisticated and intimate relationship with their host plant. Cyst *(Globodera* and *Heterodera* spp.*)* and root-knot nematodes *(Meloidogyne* spp.*)* became developmentally dependent on the plant cells they modify to form a private food source. After penetration and migration, the infective juveniles (J2) select a competent cell in vicinity of the vascular tissue to establish a permanent feeding site (Golinowski, *et al.*, 1997). Mitotic stimulation of the infected root cells by endoparasitic nematodes results in the development of two typical new root structures *viz.* a syncytium (cyst nematodes) and a giant cell complex (root-knot nematodes) (De Almeida Engler, *et al.*, 1999). Syncytium development involves progressive cell wall breakdown of the neighboring cells resulting in a multinuclear cell complex (Grundler, *et al.*, 1998), whereas giant cells form a cluster of discrete enlarged multinucleate root cells that are embedded in a gall (Bleve-Zacheo, *et al.*, 1997). Notwithstanding the differences in their ontogeny, feeding cell development is initiated by re-activation of the cell cycle and nuclear changes. Syncytia are characterized by several rounds of endoreduplication, whereas giant cells are formed by karyokinesis without cytokinesis (De Almeida Engler, *et al.*, 1999). In addition, cells surrounding the syncytium and giant cells become mitotically activated prior to incorporation into the expanding syncytium or the formation of a typical gall structure, respectively (Bleve-Zacheo, *et al.*, 1997; Golinowski, *et al.*, 1997).

Interestingly, feeding cell development by cyst and root-knot nematodes strongly correlates with lateral root formation (Grymaszewska and Golinowski, 1991; Magnusson and Golinowski, 1991). For example, the auxin resistant tomato mutant *dgt*, which is lateral root deficient, is *de facto* resistant to the potato cyst nematode *G. rostochiensis* and the root-knot nematode *M. incognita* (Richardson and Price, 1984; Goverse, *et al.*, 2000). A similar strong correlation was observed between lateral root formation and nodule formation in the *Rhizobium*-legume plant interaction (Nutman, 1948; Nutman, 1956). The modification of root cells into nodules is accomplished by the re-initiation of cell division and differentiation of (mitotically activated) pericycle and cortical cells. These observations and several molecular studies point at overlapping signal transduction pathways (Mathesius, 2003). For example, the cell cycle regulators *cdc2, ccs52*, the early noduline gene *ENOD40*, the transcription factor *KNOX* and its possible regulator *PHANTASTICA*, and the auxin responsive promoter *GH3* are induced during nodule, feeding cell and lateral root development in various legumes plants (De Almeida Engler, *et al.*, 1999; Hutangura, *et al.*, 1999; Koltai, *et al.*, 2001; Gheysen and Fenoll, 2002).

ENOD40 encodes a transcript necessary for cortical cell division during nodulation (Crespi, 2000). Initially, these so called noduline genes have been defined as genes that are

exclusively induced during nodule formation in legumes plants (Kammen, 1984)Recent studies, however, revealed homologues of noduline genes in non-legumes plants, suggesting a more general role for *ENOD40* in plant development (Sande, *et al.*, 1996; Kouchi, *et al.*, 1999; Vleghels, *et al.*, 2003). In legumes, *ENOD40* was suggested to be involved in feeding cell development by the root-knot nematode *M. incognita* in roots of *Medicago truncatula* (Favery, *et al.*, 2002; Koltai, *et al.*, 2001). In this paper, however, we show that *ENOD40* is not involved in feeding cell formation in non-legumes plants upon infection by both cyst and root-knot nematodes. Possible differences in the roles of *ENOD40* in nematode feeding cells of legumes and non-legumes are discussed.

Results

Effect of nematode infection on a non-legume *ENOD40* promoter

To visualise the expression of *ENOD40* in and around nematode-induced feeding structures, tomato plants harbouring a *gusA* reporter gene driven by an endogenous *Sl-ENOD40* promoter were infected with the root-knot nematode *M. incognita* or the potato cyst nematode *G. rostochiensis*. In the uninfected control plants, *gusA* expression was observed in the meristematic regions of the primary and lateral roots. In differentiated regions of the root, strong *gus* expression was restricted to the site of lateral root emergence resulting in an irregular GUS-staining pattern (Fig. 1A). Histological analysis of those regions revealed that *gus* expression was associated with vascular tissue development (Fig. 1B-D) both in the main and lateral root.

Potato cyst nematode-infected roots were stained for GUS activity at 1, 3, 7 and 14 day post inoculation (Fig. 1E-F). GUS staining was only observed when the syncytium was located in the region of lateral root emergence. Syncytia induced in between these active regions did not show any GUS staining. This expression pattern shows that *ENOD40* promoter activity in syncytia correlates with endogenous *ENOD40* expression in the roots. No *gus* expression was detected at the penetration site or during nematode migration.

To study the spatial distribution of *Sl-ENOD40* expression in cyst nematode-induced feeding cells, semi-thin sections of syncytia were investigated microscopically. In case syncytia were induced in root segments without visible *ENOD40* expression, no GUS staining was observed in syncytia either (Fig. 1G-H). However, when syncytia were induced in GUS-stained root sections, GUS particles were also present inside and around the nematode-induced feeding site (Fig.1 I-J).

The effect of root-knot nematode (*M. incognita*) infection on *Sl-ENOD40* expression in tomato roots was investigated in a comparable experimental set up, and – again – no *gusA* expression was detected at the penetration site (in the elongation zone) or during root-knot nematode migration. The observed expression pattern resembled the expression pattern observed for the potato cyst nematode *G. rostochiensis* (Fig. 1K-L). The time course

experiment showed a similar variable transcriptional activation of the *ENOD40* promoter in the galls, which seems to reflect the endogenous expression pattern in the tomato root system. To determine the localisation of the GUS particles in the stained galls, semi-thin sections of infected tissue were investigated microscopically. GUS activity was confined to the giant cells and the vascular tissue directly surrounding the giant cells (Fig. 1M-N; P-Q).

The absence of a correlation between *ENOD40* promoter activity and nematode infection in a non-legume was supported by semi-quantitative RT-PCR on tomato roots infected with *G. rostochiensis* or *M. incognita*. Total RNA was extracted from root segments containing young syncytia or giant cells at 3 dpi, and – as a control - corresponding non-infected root segments were included. Transcript levels of *Sl-ENOD40* did not significantly differ between nematode infected root segments and the uninfected controls. Thus, similar *ENOD40* expression was observed irrespective whether non-infected tomato roots were investigated, or roots infected with either cyst or root-knot nematodes (Fig. 2).

Effect of nematode infection on a legume *ENOD40* promoter in a non-legume background

As compared to a homologous legume promoter, a non-legume *ENOD40* promoter could have distinct regulatory elements. Favery and co-workers (2002) tested the activation of a 1.2-kb fragment of the *MtENOD40* promoter in stably transformed *M. truncatula* plants, and they clearly showed promoter activation in the close vicinity of root-knot nematode-induced giant cells, but not in the giant cells themselves. Therefore, we tested the effect of nematode infection on the soybean (*Glycine max*) *ENOD40* promoter in *Arabidopsis*, an alternative non-legume that lacks *ENOD40* homologues. *A. thaliana* plants carrying the *Gm-ENOD40::gusA* construct were infected with cyst (*H. schachtii*) or root-knot (*M. incognita*) nematodes. In non-infected *Arabidopsis* roots, *gusA* expression was restricted to the vascular tissue and particularly strong in the area of lateral roots emergence. Microscopic observations revealed occasional activation of the *Gm-ENOD40* promoter at the infection sites of both cyst and root-knot nematodes (data not shown), resembling the activation patterns of the *Sl-ENOD40* in tomato. Thus, no differences were noticed between the effect of nematode infection on the activity of the legume promoter *Gm-ENOD40* in *Arabidopsis* and the endogenous, non-legume *Sl-ENOD40* promoter in tomato. These data indicate that *ENOD40* transcription is not specifically induced during early syncytium and giant cell formation in non-legumes irrespective whether this transcription is driven by a legume or a non-legume promoter.
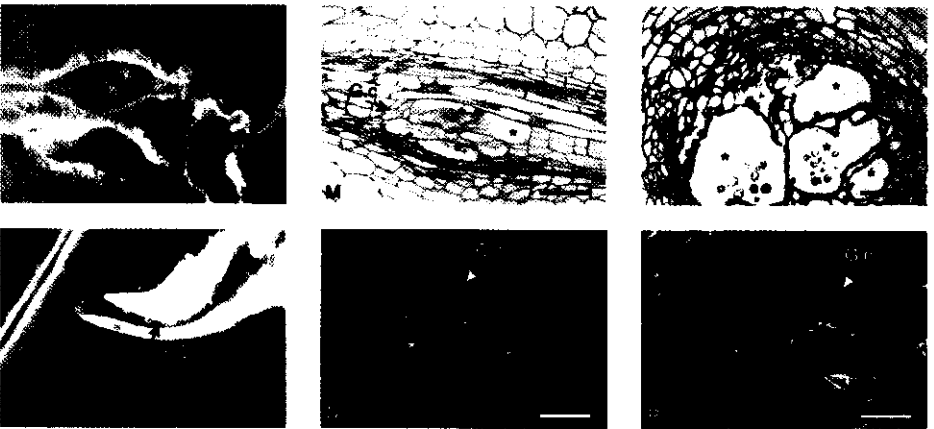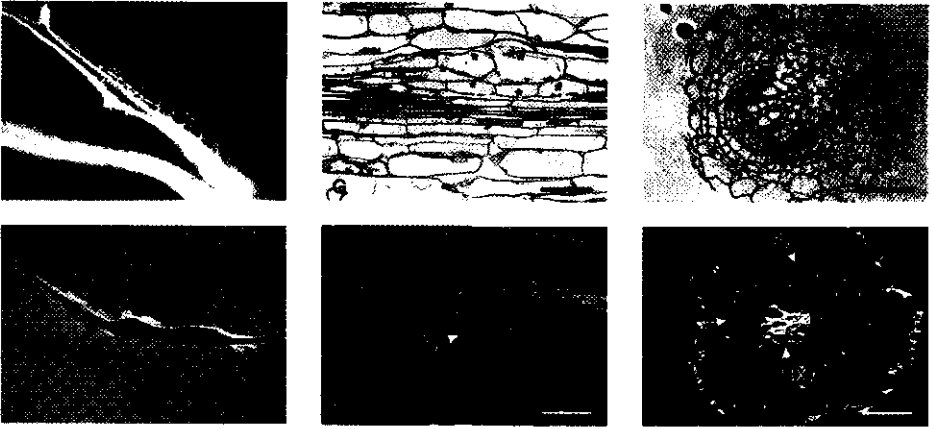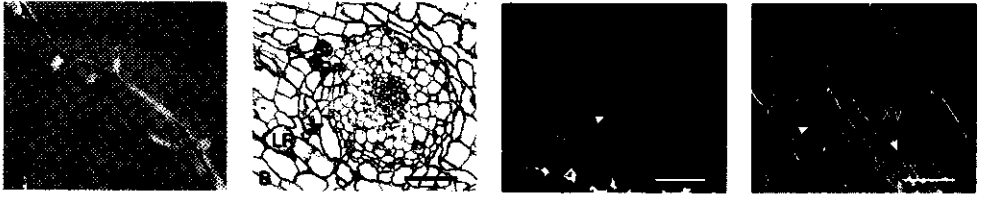
Figure 1:
Transcriptional regulation of the *Sl-ENOD40* promoter in tomato roots infected with the potato cyst nematode *Globodera rostochiensis* or the root-knot nematode *Meloidogyne incognita* using the *gusA* reporter gene. Spatial and temporal expression was investigated in whole root systems using binoculars (**A, E, F, K, L**) and in semi-thin sections using light (**B, G, I, M, O**) and dark-field microscopy (**C, D, H, J, N, P**). This results either in the detection of a blue or a red staining of the GUS particles, respectively. **A**, In uninfected roots, *Sl-ENOD40* promoter activity was observed in root meristems and the vascular bundle, specifically at the sites of lateral root emergence. **B-D**, Cross sections showed that GUS staining was present in the lateral root primordia and the pericycle. **E-J**, Upon infection with *G. rostochiensis*, no change in *Sl-ENOD40* promoter activity was observed in the roots. The detection of *Sl-ENOD40* promoter activity in nematode feeding sites was corresponding with the sites of lateral root emergence, reflecting the endogenous *ENOD40* expression pattern. No GUS expression was observed when feeding cells were established in between sites of lateral root emergence (**E**, dpi = 7), whereas GUS was observed in feeding sites induced within regions where lateral roots emerged (**F**, dpi = 14). Longitudinal and cross sections of GUS-positive and GUS-negative syncytia were analysed microscopically. **G-H**, Example of a syncytium without *gus* expression (dpi = 3). **I-J**, In GUS-positive syncytia, *Sl-ENOD40* promoter activity was detected in and around the nematode feeding cell. **K-P**, A similar expression pattern was observed for root-knot nematode-induced feeding sites. Some galls showed GUS staining (**K**, dpi = 14), whereas others did lack the typical blue colour (**L**, dpi = 7). Longitudinal (**M-N**) and cross sections (**O-P**) of GUS-positive galls show that the *Sl-ENOD40* promoter is active in and around young developing giant cells at 3 dpi. GUS expression within the giant cells was relatively weak (marked with an asterisk) compared to intense GUS staining in the cambial and cortical cells surrounding the giant cells.
Abbreviations: Sy = syncytium, G = gall, G.c = giant cell, LR = lateral root, Xy = xylem, MXy = metaxylem. Scale bars 25μm in B, C, D, G, H, I, J and Q; 33 μm in M, N, O, P.

Effect of *ENOD40* overexpression on nematode infection

It has been shown that overexpression of *ENOD40* in the legume *M. truncatula* stimulates gall formation by *M. incognita* (Favery, *et al.*, 2002), and this observation prompted us to investigate whether overexpression of *ENOD40* would affect feeding site formation in a non-legume as well. To this end, three independent transgenic *Arabidopsis* lines expressing *ENOD40* under the control of the CaMV 35S promoter (Sande, *et al.*, 1996) were infected with *H. schachtii* or *M. incognita*, and no significant difference in nematode development was observed compared to the wild type plants (Fig. 3). It is noted that - like rice and unlike *M. truncatula* (Dey *et al.*, 2004) - the phenotype of the transgenic plants was similar to the wild type plants. This observation is in line with our data on the expression of *ENOD40* in tomato and *Arabidopsis* roots upon infection with endoparasitic nematodes. Apparently, *ENOD40* is not involved in syncytium and giant cell development in non-legumes plants.
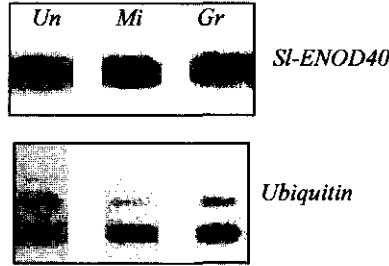
.

Un	Mi	Gr	
			Sl-ENOD40

| | | | Ubiquitin |

Figure 2:
Semi-quantitative RT-PCR was performed to determine *Sl-ENOD40* transcript levels in tomato root segments upon infection with the root-knot nematode *M. incognita* (Mi) or the potato cyst nematode *G. rostochiensis* (Gr). As a control, corresponding root segments were collected from uninfected tomato plants (Un) at the same developmental stage (dpi = 5). No significant differences were observed in *ENOD40* expression upon nematode infection compared to the control. The amount of mRNA was normalized using ubiquitin primers
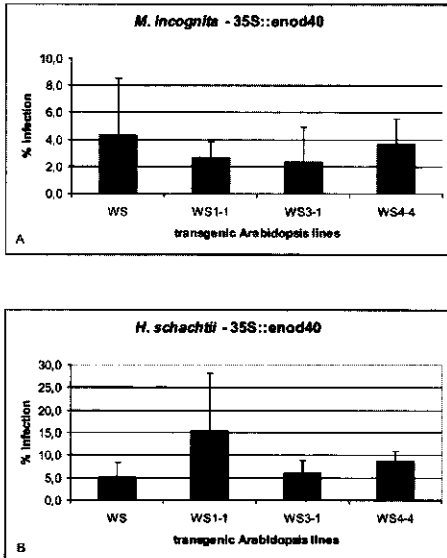
Figure 3:
Cyst and root-knot nematode development on three independent transgenic *Arabidopsis* lines (WS1-1, WS3-1 and WS4-4) overexpressing *Gm-ENOD40* under control of the constitutive CaMV 35S promoter and on wild type control plants (WS). No significant differences were observed in the development of the root-knot nematode *Meloidogyne incognita* (A) and the beet cyst nematode *Heterodera schachtii* (B). Nematode development was expressed as the percentage of infective pre-parasitic second stage juveniles that developed into adult females at 5 weeks post inoculation in three independent experiments.

Discussion

As indicated by their name, early nodulin genes such as *ENOD40* have been associated mainly with nodule formation in nitrogen-fixing plants by rhizobial bacteria. However, its presence in non-leguminous monocotyledons and dicotyledons points at more ancient roles including the differentiation and/or function of vascular tissue (Larsen, *et al.*, 2003). Feeding site formation by endoparasitic nematodes results in a distortion of the vascular system (Amus, *et al.*, 2003; Golinowski, *et al.*, 1996; Yeboah, *et al.*, 2004), and in an attempt to restore vascular continuity a local increase in *ENOD40* expression could be anticipated. Recently, the role of *ENOD40* was investigated in the interaction between the root-knot nematode *M. incognita* and the legume *M. truncatula*. *ENOD40* expression was found in (Koltai, *et al.*, 2001) or exclusively around (Favery, *et al.*, 2002) giant cells. In this paper, *ENOD40* expression was investigated in the interaction between endoparasitic nematodes and two non-legumes. Unexpectedly, no correlation was found between *ENOD40* expression and feeding cell development by both cyst and root-knot nematodes.

In legumes *ENOD40* has gained an additional and essential function during nodulation. Activation patterns of legume (*Gm-ENOD40*) and non-legume (*Sl-ENOD40*) promoter regions in a non-legume background appear to be identical (Vleghels, *et al*, 2003; Kouchi, *et al.* 1999; this paper) indicating that the regulatory sequences are conserved both in legume and non-legume promoters. Therefore, (Vleghels, *et al.*, 2003) suggest that legumes harbours a specific factor that is responsible for the unusual high *ENOD40* expression levels in the symbiosis between legumes plants and *Rhizobium*. The incongruity between our results and the nematode-induced *ENOD40* expression profiles presented by (Koltai, *et al.*, 2001; Favery, *et al.*, 2002) could relate to the leguminous *versus* non-leguminous plant backgrounds. Here, we postulate that the presence of a specific factor(s), which interacts directly or indirectly with *ENOD40* promoter regions, is required for nematode-induced expression in legumes. The absence or - at least - the non-functionality of such an unknown factor in non-legumes could explain the deviant expression profile observed in nematode-infected non-legumes.

In this context it is interesting to take the role of *ENOD40* in the interaction between arbuscular mycorrhiza and (non-)legumes into consideration, and compare it with effects on nematode infectivity. In the legume *M. truncatula*, arbuscular mycorrhiza (AM) fungal growth (and nodulation) is accelerated in plants that overexpress *MtENOD40*, whereas co-suppression of *MtENOD40* slowed down fungal colonization (Charon, *et al.*, 1999; Rhijn, *et al.*, 1997; Sinvany *et al.*, 2002). The same transgenic alfalfa overexpressor lines were used by (Favery, *et al.*, 2002), and infection with *M. incognita* resulted in a significant increase of the number of galls. On the other hand, severe reduction of *ENOD40* transcript levels in a non-legume, *Zea mays*, had no effect on mycorrhizal colonization of roots (Compaan, *et al.*,

2003). In this paper overexpression of *ENOD40* in the non-legume *Arabidopsis* was shown to have no detectable effect on nematode infection. This finding supports our hypothesis that legumes have gained a specific factor in the regulation of *ENOD40* expression, which is not present in the more ancient non-legumes plants. Although experimentally hard to proof, our results suggest that *Rhizobium*-mediated nitrogen fixation by legumes is accompanied by an increased susceptibility to parasitism by nematodes.

Materials and methods

Plant material

Seeds of non-transgenic and transgenic tomato plants (*Solanum lycopersicum cv* Money Maker) carrying the *Sl-ENOD40::gus*A construct were surface sterilised in a 1:4 mix of a sodium hypochloride solution (40g l^{-1}) containing 4% of the detergent and sterile tap water for 20 min. Thereafter, seeds were washed 3 times with sterile water and transferred with sterile forceps to the plates (Ø 9 cm, 10 seeds per plate) containing Gamborg's B5 medium (Duchefa, The Netherlands) supplemented with 2% sucrose and 1,5% Bacto-agar (Difco). Slightly tilted plates were incubated at 22°C in the dark.

Seeds of transgenic *A. thaliana* plants carrying the *Gm-ENOD40::gus*A and the *35S::Gm-ENOD40* (ecotype Wasilewskija) construct were incubated for 72h at 4°C and subsequently surface sterilized in a 1:4 mix of a hypochloride solution (40g l^{-1}) and 96% EtOH for 15 min. Thereafter, seeds were washed 3 times with EtOH and dried in a flow cabinet. Sterile seeds were transferred with a toothpick to plates (Ø 9 cm, 10 seeds per plate) containing KNOP medium (Sijmons, *et al.*, 1991) supplemented with 2% sucrose and 0,7% Daichin agar (Brunschwig, The Netherlands). Slightly tilted plates were incubated at 22°C with 16h of light.

Nematode infection

In vitro cultures of 7 day old tomato and 14 day old *Arabidopsis* plants, carrying a *Sl-ENOD40::gusA* or *Gm-ENOD40::gusA* construct, respectively, were inoculated either with sterile pre-parasitic J2 of the cyst nematode *Globodera rostochiensis* (Goverse, *et al.*, 2000) and *Heterodera schachtii* (Goverse, *et al.*, 2000) or with pre-parasitic J2 of the root-knot nematode *Meloidogyne incognita*. For the latter, egg masses were collected from sterile cultures of *M. incognita* and transferred to 70 µm cell strainers (Falcon 2350), which were placed in a petri dish (Ø 3,5 cm) partially filled with sterile tap water. Eggs were incubated in the dark for 1 week at 24°C. Thereafter, hatched nematodes were collected, counted and concentrated by centrifugation at 2000 rpm for 5 min. Sterile water was added to the nematode suspension to adjust the volume. Plants were inoculated as follow: tomato plants with approximately 500 J2 (in 150 µl) of *G. rostochiensis* or *M. incognita*, *A. thaliana* plants

with 200 J2 (in 100 μl) of *H. schachtii* or *M. incognita* per plate using siliconized tips. For RT-PCR, plates of wild type tomato seedlings (Moneymaker) were inoculated with 75 J2 for both *G. rostochiensis* and *M. incognita*. Furthermore, three independent transgenic *Arabidopsis* lines containing the CaMV *35S::Gm-ENOD40* construct and the corresponding wild type plants were infected *in duplo* with both *M. incognita* (180 J2 per plate) and *H. schachtii* (300 J2 per plate) as described above. The number of adult females was counted 5 weeks post infection in three independent experiments.

Histochemical GUS assay

Infected and uninfected root segments were collected at 3, 5, 7, 10 and 14 days post inoculation (dpi). The histochemical GUS assay was preformed essentially according to (Barthels, *et al.*, 1997). The samples where incubated for 2h at 37°C. GUS staining was observed in the roots using a dissecting microscope (Wild, Heerbrugg, Switzerland).

Histology of nematode feeding sites

Semi-thin sections of infected and uninfected roots were prepared according to Goverse *et al.*, (2000) and analyzed using bright and dark field microscopy (Zeiss, Oberkochen, Germany).

RNA isolation and RT-PCR

In vitro sterile tomato root segments containing infection sites of *M. incognita* and *G. rostochiensis* were collected at 3 days post inoculation for RNA isolation according to Pawlowski *et al.*, (1995). Uninfected root tissue in a comparable developmental stage was used as a control. After Dnase treatment of the isolated RNA, the amount of *ENOD40* transcript in the three samples was determined in a semi quantitative reverse transcriptase PCR (RT-PCR) according to Vlegels *et al.*, (2003). The RNA samples have been standardized for tomato ubiquitin gene.

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Chapter 4

Silencing of plant endo-β-1,4-D-glucanases in potato severely hampers growth and development of cyst nematodes

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Submitted

Abstract

A novel approach is presented to engineer resistance by disrupting essential elements in the molecular interaction between an obligate parasite and its host. RT-PCR experiments in tomato indicated that the potato cyst nematode *Globodera rostochiensis* induces the expression of two out of the eight described endo-β-1,4-glucanases (EGase), namely *Sl-cel7* and *Sl-cel8. In situ* hybridization and immunodetection studies demonstrated that both EGases are specifically expressed inside and adjacent to nematode-induced feeding sites. Silencing of these members of the EGase family in potato (a close relative of tomato) through RNA interference resulted in a reduced development of juveniles into females (up to 60%) and in a sharp increase in the fraction of females without eggs (up to 89%). Our results show that the recruitment of specific plant EGases by potato cyst nematodes is crucial in their life cycle, and illustrate how detailed knowledge of plant-parasite relationships can lead to host plant resistance.

Introduction

Many parasites and pathogens are entirely dependent on plants to complete their life cycle and have evolved sophisticated mechanisms to manipulate their hosts. These intimate relationships have often culminated in highly specialized feeding structures to extract nutrients from plants, such as haustoria formed by mildews and rusts or feeding cells induced by nematodes. Effective management of these plant pathogens with a minimal negative impact on the environment is an important issue in modern agriculture. Especially the control of soil-borne pathogens raises problems, because the use of chemical soil disinfectants has or will be abandoned in most countries.

Root parasitic nematodes – mainly cyst and root knot nematodes - cause estimated worldwide losses of about US $ 125 billion annually (Chitwood, 2003). Cyst nematodes are obligate oligophagous parasites of a number of major crops: the soybean cyst nematode *Heterodera glycines* is one of the main yield limiting factors in soybean, potato production is worldwide limited by the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* and sugar beets are extensively parasitized by *H. schachtii*. As a common denominator, the main production areas of these crops are at a large distance from the areas where co-evolution between the host plants and their parasites took place. The lack of natural enemies might be one of the factors that underlies the very substantial damage caused by these organisms. Crop rotation and the growth of resistant varieties are in most areas inadequate for an appropriate control.

Successful infection of a host plant by a nematode depends on its ability to penetrate the plant cell wall. Plant parasitic nematodes are equipped with a protrusible stylet (~ a hollow needle) that is used to puncture the cell wall in a highly coordinated manner. For host invasion nematodes use a combination of mechanical piercing and enzymatic softening. Unlike most other herbivores, plant parasitic nematodes do not depend on symbiotic microorganisms for the production of cell wall-degrading or modifying proteins such as endo-β-1,4-endoglucanases(Smant, *et al.*, 1998), pectate lyases (Popeijus, *et al.*, 2000) and expansins(Qin, *et al.*, 2004). Once an infective second stage juvenile (J2) has entered the plant root, it selects an initial feeding cell (IFC). Highly directed cell wall dissolution between the IFC and adjacent cells results in the formation of a syncytium that is - for the better part - situated within the stele. Finally the multinucleate syncytium may include up to 200 plant cells (Jones, *et al.*, 1981). Syncytial cells are re-differentiated metabolically active plant cells that constitute the sole food source for the developing nematode. Although infective juveniles produce a range of cell wall-degrading enzymes, the highly coordinated and selective incorporation of plant root cells make it unlikely that nematode cell wall-modifying proteins are used for syncytium proliferation. In the interaction between the beet cyst nematode (*H. schachtii*) and *Arabidopsis*, a gradual widening of plasmodesmata between the initial syncytial cell and neighboring cells was the starting point for protoplast fusion, and

it was suggested that plant EGase activity was involved in the observed cell wall dissolution (Grundler, *et al.*, 1998).

The hypothesis that plant cell wall modifying proteins are recruited by parasitic cyst nematodes is supported by gene expression surveys showing a local up-regulation of plant EGases (Goellner, *et al.*, 2000), pectinmethylesterases (Vercauteren, 2002), and extension (Niebel, *et al.* 1993). Goellner *et al.*, (2001) showed a local up-regulation of five endo-β-1,4-endoglucanase genes of plant origin in tobacco cyst nematode-infected tobacco roots. In this report, we studied EGase recruitment by potato cyst nematode (*G. rostochiensis*) in tomato. This host plant harbors at least eight endo-β-1,4-endoglucanases. Here we demonstrate that only two isoforms, *Sl-cel7* and *Sl-cel8*, are up-regulated in young expanding syncytia, and we show that both the relevant transcripts and proteins are only present in or very near to the developing syncytium. The importance of EGase recruitment by potato cyst nematodes for their pathogenicity was assessed by post-transcriptional gene silencing. Remarkably, silencing of *cel7* or *cel8* caused a reduction of the number of juveniles that developed into females, and resulted in a sharp increase in the fraction of abnormally developed females. Our findings suggest that individual members of plant endo-β-1,4-endoglucanases could be efficient and environmentally sound targets for cyst nematode control.

Results

Endoglucanase expression in nematode-infected tomato roots

To see whether the transcription of tomato EGases is affected by infection with potato cyst nematodes, mRNA levels of individual EGases were determined in nematode-infected and uninfected root segments using semi-quantitative RT-PCR. Family member-specific primers were designed (Tab. 1) and ubiquitin expression levels were determined in parallel to normalize the EGase expression data. The cDNA sequence of *Sl-cel6* is only partially known and no specific primers were designed to monitor the expression of this family member. Whereas expression levels of EGases were relatively low in young tomato root segments, transcripts of five out of the seven EGase genes under investigation were detected in infected root tissue at 7 dpi (*Sl-cel1* and *Sl-cel5* transcripts were not detectable). Two members of the EGase gene family were specifically up-regulated in nematode-infected roots, namely *Sl-cel7* and *Sl-cel8* (Fig. 1).
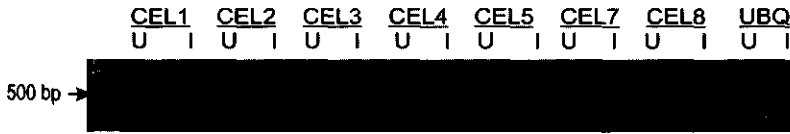
CEL1		CEL2		CEL3		CEL4		CEL5		CEL7		CEL8		UBQ	
U	I	U	I	U	I	U	I	U	I	U	I	U	I	U	I

500 bp →

Figure 1:

Semi-quantitative RT-PCR analysis of seven tomato endo-β-1,4-glucanase (EGase) transcripts in tomato root segments. RT-PCR products were amplified from corresponding uninfected (U) and potato cyst nematode-infected (7 dpi, I) root segments. Ubiquitin transcript levels were used to normalize the data. Transcripts of *Sl-cel7* and 8 are specifically up-regulated in infected root segments, whereas transcripts levels of other EGases did not show major changes upon nematode infection.

Spatial and temporal distribution of *Sl-cel7* and *Sl-cel8* transcripts

Based on the RT-PCR results, the spatial and temporal distribution of transcripts from the tomato EGases *Sl-cel7* and *Sl-cel8* were investigated by *in situ* hybridization and *in situ* RT-PCR followed by hybridization. Neither *Sl-cel7* nor *Sl-cel8* transcripts could be detected in non-infected tomato roots (Fig. 2A, E). The expression of *Sl-cel7* was up-regulated from 1 dpi onwards with the highest transcript level at 5 dpi in young syncytial elements (Fig. 2B-D). There was no visible fluorescent signal in the cells surrounding the young proliferating feeding site. In older feeding sites (10-14 dpi), a high *Sl-cel7* transcripts level was observed only in the distal parts of the syncytium (= cells recently incorporated into the syncytium) and in cells directly next to the developing syncytium (data not shown). In the part of the syncytium close to the nematode head, none or only a very weak fluorescent signal was detected. Syncytium grows apically by highly directed, cell wall dissolution-driven protoplasts fusion, and these expression data suggest that *Sl-cel7* is involved in syncytium proliferation. *Sl-cel8* expression was consistently following *Sl-cel7* expression, and was observed from 3 dpi onwards (Fig. 2F-H). The strongest *Sl-cel8* transcript signal was present in distal syncytial elements while the expression level in the older parts of the syncytium was relatively low. *Sl-cel8* was preferentially expressed in parenchymatous vascular cylinder cells surrounding the syncytium and in the terminal syncytial elements. Only a weak signal was present in the IFC and in the cortex derived part of the syncytium next to the juvenile's head. *In situ* localization of *Sl-cel8* transcripts in older stages of the infection process (7-14 dpi) revealed their presence in the cytoplasm of distal syncytial elements and in the cells neighboring the syncytium. The *Sl-cel8* transcripts level was higher in the adjacent cells than in syncytial elements (data not shown). There was no visible fluorescent signal in other cells of the infected root. The hybridization with sense probes for *Sl-cel7* and *Sl-cel8* did not give any signal on the sections of syncytia (data not shown).
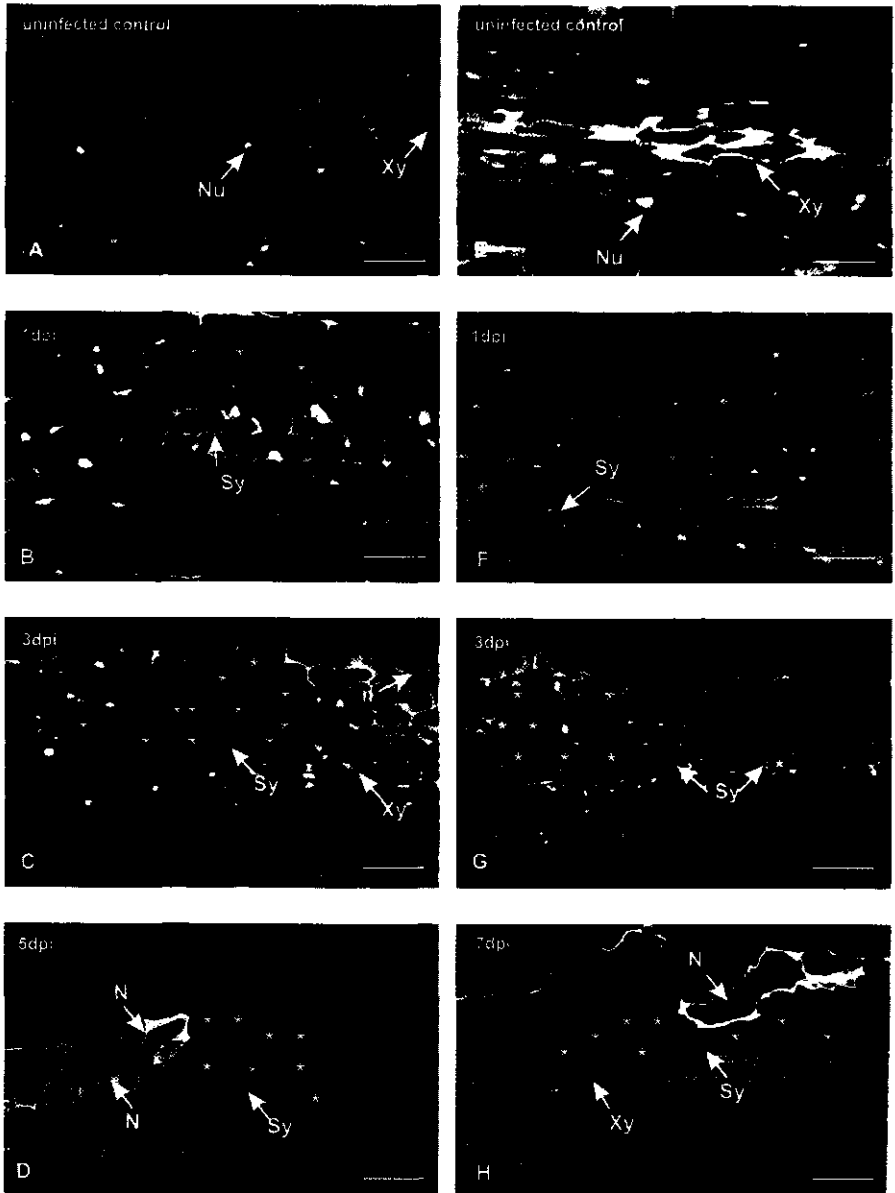
Figure 2:
Detection of *Sl-cel7* (**A-D**) and *Sl-cel8* (**E-H**) transcripts in longitudinal sections of potato cyst nematode-induced syncytia in roots of the susceptible tomato cultivar cv. Moneymaker. (A, C, E, G, H) *in situ* RT-PCR followed by *in situ* hybridisation (**B, D, F**) *in situ* hybridisation alone. (**A**) No endogenous *Sl-cel7* expression was observed in uninfected tomato root segments. (**B, C, D**) Upon nematode infection, accumulation of *Sl-cel7* mRNA was observed as a green fluorescent signal within

young developing syncytia at 1 (**B**), 3 (**C**) and 5 (**D**) days post infection (*dpi*). No expression was observed in cells surrounding the syncytium. (**E**) No endogenous *Sl-cel8* expression was observed in uninfected tomato root segments. (**F**) No *Sl-cel8* transcripts could be detected in the initial syncytial cell at 1 dpi. (**G**) *Sl-cel8* transcripts appeared in developing syncytia from 3 dpi onwards. (**H**) *Sl-cel8* mRNA level decreased in older parts of syncytium from 7 dpi onwards, but it remained at high levels in the distal parts of the syncytium. Abbreviations: N = nematode, Nu = nucleus, Xy = xylem, Sy = syncytium (marked with asterisks), dpi- days post infection. Scale bars: 30 μm in A, B, E and 60 μm in C, D, E, G, H.

Localization of *Sl-cel7* and *Sl-cel8* in developing syncytia

Antibodies against distinctive parts of *Sl-cel7* and *Sl -cel8* were raised in chicken, and after purification polyclonal IgY's were checked for specificity. Preimmune serum (used later as a negative control) was checked for absence of reactivity with cellulases (Fig. 3A). Labeling with anti-*Sl-cel7* and anti-*Sl-cel8* antibodies revealed a highly syncytium-specific signal. In the case of *Sl-cel7* a weak signal was observed already in 2-day-old syncytia (Fig. 3B). The labeling intensity was substantially stronger in 7 to 10-day-old syncytia, and it was restricted to paramural cytoplasm and to syncytial areas with dense cytoplasm (Fig. 3C, D). The labeling was most intense in distal syncytial elements located inside the vascular cylinder. In syncytial elements derived from the cortex - being the oldest part of the syncytium - labeling was weak (Fig. 3C). Older syncytia (21 dpi) still retained relatively high levels of *Sl-cel7* (data not shown).

Although its distribution pattern was similar, *Sl-cel8* appeared later as compared to *Sl-CEL7*. In 2-day-old syncytia no signal for *Sl-cel8* was observed (Fig. 3E). Specific labeling in the syncytium was observed from 7 dpi onwards (Fig. 3F, G). In older syncytia (21 dpi), immunolabeling showed a similar pattern and intensity (data not shown).
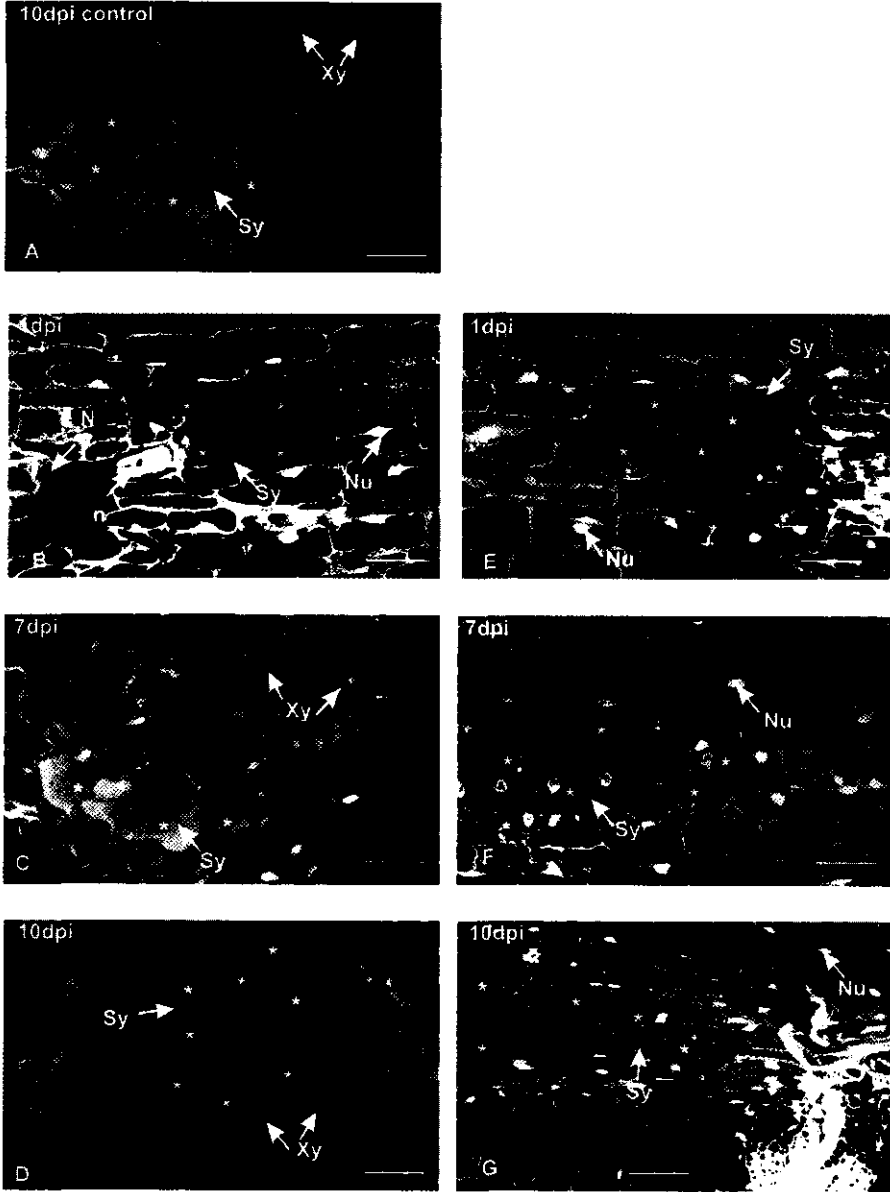
Figure 3:

Immunodetection of *Sl-cel7* (**B-D**) and *Sl-cel8* (**E-G**) in syncytia induced in susceptible tomato cv. Moneymaker roots by potato cyst nematodes in longitudinal (**B, E, F, G**) and cross sections (**A, C, D**). (**A**) No signal was observed in syncytia (7 dpi) incubated with pre-immune serum. (**B, C, D**) High levels of *Sl-cel7* were detected in syncytia at 2 (**B**), 7 (**C**) and 10 (**D**) dpi. (**E, F, G**) Contrary to *Sl-cel7*, no *Sl-cel8* could be detected at 2 dpi (**E**), whereas relatively high *Sl-cel8* levels were observed in syncytia at 7 (**F**) and 10 (**G**) dpi. Abbreviations: N = nematode, Nu = nucleus, Xy = xylem, Sy = syncytium (marked with asterisks), dpi- days post infection. Scale bars: 30 μm in A-F and 60 μm in G.

Post transcriptional silencing of *cel7* and *cel8*, and effects on nematode growth and development

To assess the importance of *Sl-cel7* and *Sl-cel8* recruited by potato cyst nematodes in the process of syncytium formation, the individual genes were silenced. Contrary to tomato, potato cyst nematodes are a major problem in potato cultivation. As potato EGases showed 87-95% identity to their tomato equivalents at the transcript level (Karczmarek *et al.*, unpublished results), we transformed potato with *Sl-cel7* (360 bp) and *Sl-cel8* (379 bp) hairpin constructs. After *Agrobacterium*-mediated potato transformation, six and eight independent lines harboring respectively *Sl-cel7* and *Sl-cel8* hairpin constructs were regenerated. The silencing efficiency was verified by RT-PCR experiments and strong suppression was found in three hp-*Sl-cel7* and hp-*Sl-cel8* lines (Fig. 4A). Apart from occasional transformation effects, no obvious silencing-related aberrant phenotype could be observed in *in vitro* grown plants.

Silenced potato plants and the corresponding empty vector controls were *in vitro* inoculated with potato cyst nematodes (10 J2-s per root tip). Root penetration and migration in the roots of the transgenic plants was apparently unaffected in the silenced plants. Five weeks after inoculation, nematode development in EGase-silenced plants was compared to its development on control plants. Two parameters were scored: (1) the total number and (2) the phenotype of adult females. On control plants, the total number of J2-s that developed into females was 136±14 (average ± SE) (Fig. 4B, grey and black bars taken together). On silenced plants with a normal phenotype, the largest reductions in total number of females ranged from 60% in *cel7*-silenced potato plants (line *s7.1*; 55±11 ♀♀) to 31% in *cel8*-silenced plants (line *s8.2*; 94±4 ♀♀) (Fig. 4B). Microscopic observations showed that nematode development stopped in most cases at the J2/J3 life stage (Fig. $4c_4$, c_5). Under normal conditions the posterior part of the female body is spherical and opaque (Fig. $4c_1$) as it is filled with eggs. On EGase-silenced plants, the percentage of fully developed females had dropped by 89% and 78% in line *s7.1* and *s8.1* (respectively 15±4 and 29 ±13 fully developed females). Poorly developed females are smaller, and the posterior part of their body is transparent and saccate (Fig. $4c_2$, c_3) as it does not contain eggs. Growth on EGase-silenced potato plants resulted in a sharp increase of the number of poorly developed females (40±19 for s7.1 and 82±28 for s8.1 as compared to 5.5±2 in control; black bars in Fig. 4B). Hence, the post transcriptional silencing of *St-cel7* or *St-cel8* in potato resulted in a reduced number of female nematodes, but - more importantly - the majority of the females that could develop showed a severely aberrant phenotype.
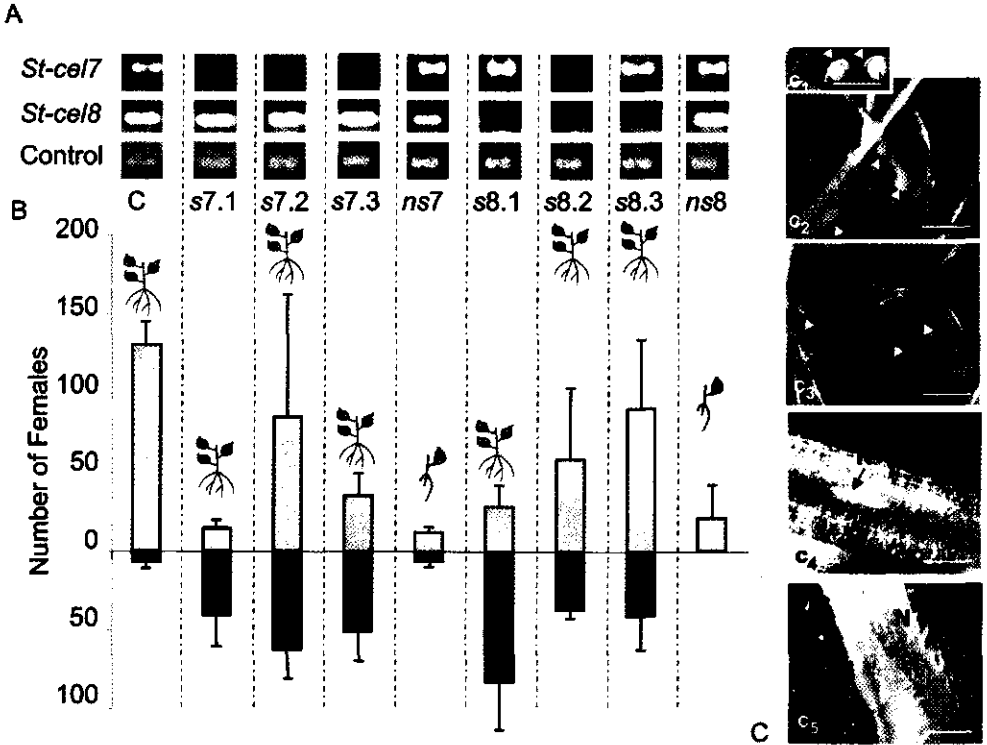
Figure 4:
Effect of dsRNA-based endo-β-1,4-glucanase silencing on the development of potato cyst nematodes. Hairpin constructs of *Sl-cel7* and *Sl-cel8* specific fragments were used to silence their homologues (*St-cel7* and *St-cel8*) in potato. For each construct, four independent potato lines (*s7.1-s7.3*, *ns7* and *s8.1-s8.3*, *ns8*) were infected with potato cyst nematodes. (**A**) Silencing efficiency was determined in transformed potato lines by semi-quantitative RT-PCR experiment using *cel7* and *cel8*-specific primers. Transcript levels were normalised against a constitutively expressed *LRR*-containing potato gene. Specific silencing was observed in potato lines *s7.1*, *s7.2*, *s7.3*, *s8.1* and *s8.3*. Line *s8.2* showed cross silencing for *St-cel7*. Potato lines *ns7.4* and *ns8.4* showed no reduction in *St-cel7* and *St-cel8* transcript levels. (**B**) Effect of silencing on potato cyst nematode development was tested *in vitro*. Except for *ns7.4* and *ns8.4* (two non-silenced lines; dwarf plants with poorly developed root system), potato plants showed no aberrant phenotype (indicated as pictograms above the bars). For each potato line, the average number of adult females was determined (see Methods). Two categories were distinguished: normally developed ♀♀ (spherical and opaque; grey bars) and poorly developed ♀♀ (saccate and transparent; black bars). (**C**) Normally developed adult females at 5 weeks post inoculation (c_1). Poorly developed females on *St-cel7*-silenced (c_2) or *St-cel8*-silenced (c_3) potato plants at 5 weeks post inoculation. A substantial part of the infective juveniles was arrested in the parasitic J2/J3 stage (c_4 - *St-cel7*-silenced and c_5 - *St-cel8*-silenced plant). Juveniles (n) are marked with arrows. Scale bars: 5mm (c_1-c_3), 1 mm (c_4, c_5).
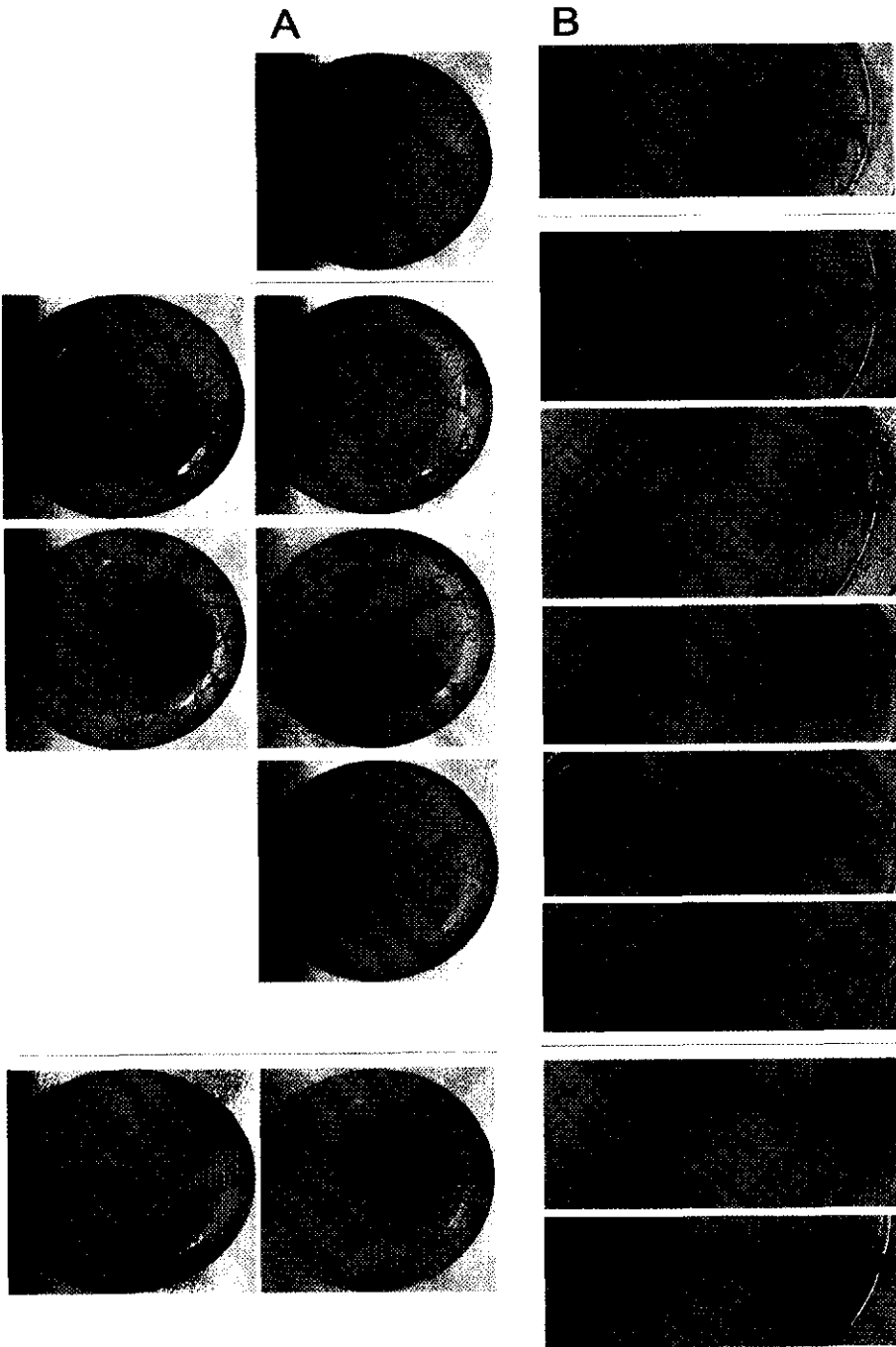
Figure 5 :
Phenotyping *in vitro* potato plants harbouring dsRNA constructs directed towards cel7 (*s*7.1-*s*7.3, *ns*7) or cel8 (*s*8.1-*s*8.3, *ns*8). No obvious differences between silenced and not silenced plants (control) plants was observed, exept for *ns*7.4 and *ns*8.4 (two non-silenced lines; dwarf plants with poorly developed root system). **(A)**: one-month-old roots system **(B)**: one-month-old shoots

Discussion

In this paper we show that detailed knowledge about the intimate interaction between the nematode and its host provide us with subtle and effective handles that can be used to create host plant resistance. In potato (*Solanum tuberosum*), we silenced two genes encoding endoglucanase *St-cel7* and *St-cel8* that were shown to be recruited by potato cyst nematodes for feeding site induction in tomato (*Solanum lycopersicum*), a close relative of potato. Post transcriptional gene silencing of these individual plant endo-β-1,4-glucanase (EGase) - members of a gene family with at least eight representatives in tomato (Catala and Bennett 1998) - significantly hampered cyst nematode development. This loss in susceptibility shows that specific EGases of plant origin are essential for syncytium formation by cyst nematodes.

In an attempt to inhibit the softening of tomato fruit, Lashbrook *et al.,* (1998) and Brummell *et al.,* (1999) inhibited the accumulation of tomato EGases *Sl-cel1* and *Sl-cel2* transcripts by using an antisense approach. Although suppressed lines showed a 99% decrease in mRNA levels, no change in the ripening behavior of the tomato fruit was observed. Fruit ripening in tomato involves at least three EGase family members, *Sl-cel1*, *Sl-cel2* and *Sl-cel5*, and the absence of an effect on fruit softening suggests functional redundancy among these ripening-induced EGases (Brummell and Harpster, 2001). Our results show that a set of EGases is expressed in tomato roots, namely *Sl-cel2, Sl-cel3, Sl-cel4* and low levels of *Sl-cel7* and *Sl-cel8*. Remarkably, only *Sl-cel7* and *Sl-cel8* were shown to be strongly up-regulated upon potato cyst nematode infection. It is imaginable that the effect of silencing of one of these nematode-induced plant EGases would have been compensated by the other, or by other family members. However, *Sl-cel8* is so far the only tomato EGase with a putative carbohydrate binding module. Consequently, its functional replacement by another EGase seems to be unlikely. Here we demonstrate that silencing of either *St-cel7* or *St-cel8* resulted in a significant arrest of cyst nematode development, and consequently there is no indication for a functional redundancy among potato cyst nematode-induced EGases.

Essentially, there are two targets for genetic engineering-mediated endoparasitic nematode control: the nematode or its feeding site. The anti-nematode strategy aims to interfere with its physiology, development or behavior in order to block invasion of the plant root, migration, feeding site formation or feeding. Up till now, a range of anti-nematode components have been tested including exotoxins of *Bacillus thuringiensis* (Devidas and Rehberger, 1992), lectins (Ripoll, *et al.,* 2003) and peptides with acetylcholinesterase-inhibiting properties (Winter, *et al.,* 2002). In the field of plant engineering for nematode

resistance, the use of protease inhibitors has received most attention (Atkinson, *et al.*, 2003). Serine and cysteine protease inhibitors (and derivates thereof) produced in various plants were shown to interfere with the digestive proteinases of plant parasitic nematodes. In case of cysteine protease inhibitors (cystatins), rice genes were used (*Oc-I* and *Oc-II*) and - more specifically - a high affinity mutant of OC-I, namely Oc-IΔD86. Constitutive expression of Oc-IΔD86 in *Arabidopsis thaliana* resulted in a retardation of nematode development and a reduction of nematode offspring both in case of *H. schachtii* (cyst nematode) and *Meloidogyne incognita* (root knot nematode) (Urwin, *et al.*, 1997). In potato, partial resistance was obtained by the expression of Oc-IΔD86 (from rice), a chicken egg-white or a sunflower cystatin (Urwin *et al.*, 2003). This rather generic anti-feeding approach raised concerns about its environmental safety. Cowgill *et al.*, (2002) showed that the risk of cystatins to non-target invertebrates depends on the level of expression. Although recent field experiments in the central Andes with transgenic potato did not detect harm to non-target organisms, gene flow occurred to wild relatives of potato (Celis, *et al.*, 2004).

Alternatively, feeding cell development can be obstructed by the expression of cytotoxins (Jung and Wyss, 1999; Gheysen and Fenoll, 2002) or by preventing the recruitment of plant genes involved in feeding site induction (De Almeida Engler *et al.*, 2005). For growth and development sedentary endoparasitic nematodes fully depend on a feeding site and in case of cyst nematodes, poor syncytium development will result in a strongly male-biased population and in *de facto* plant resistance. Feeding cell induction and proliferation is accomplished by a dramatic local change in plant gene expression (Gheysen and Fenoll, 2002). A substantial part of these so-called susceptibility genes is essential for the host plant (*e.g.* genes involved in the reactivation of the cell cycle), and manipulation of such genes will predictably give rise to undesirable off-target effects. However, manipulation of individual members of gene families will in some cases have minor or no effects to plant fitness. Here, we demonstrate the loss-of-susceptibility of potato plants towards potato cyst nematodes due to the manipulation of two EGases that are apparently essential for syncytium induction in the absence of an obvious aberrant plant phenotype (under the conditions tested).

Feeding site proliferation by cyst nematodes is equivalent to protoplast fusion due to cell wall dissolution between the IFC and neighbouring cells. Our finding that two particular EGases, namely *Sl-cel7* and *Sl-cel8*, are recruited by the potato cyst nematode is consistent with the observation that infection of tobacco with tobacco cyst nematodes (*G. tabacum*) induces the local expression of *Nt-cel7* and *Nt-cel8* (Goellner, *et al.*, 2001). This is a first indication that distinct cyst nematode species recruit similar sets of cell wall-degrading enzymes in different *Solanaceous* host plants. Morphological and histological studies have shown that cyst nematode-induced syncytia are almost identical irrespective of the host plant species (Goverse, *et al.*, 2000a). Hence, it is expected that this approach could be applicable

as well for the management of other cyst nematodes such as the soybean cyst nematode *H. glycines* and the beet cyst nematode *H. schachtii.*

In this paper, we demonstrated and characterized the local recruitment of EGase *cel7* and *cel8* by potato cyst nematode in two agronomically important *Solanaceous* plant species, tomato and potato, and we showed that these plant genes are essential in the process of feeding site proliferation. More in general, these results demonstrate that detailed knowledge about the molecular interaction between a plant and a pathogen may result in the identification of the Achilles' heel of such a pathogen. A next step would be to avoid transgene expression outside the plant roots. In this case, it would imply the replacement of the 35S CaMV promoter by, *e.g.*, a root-specific promoter. The rolD promoter from *A. rhizogenes* (Elmayan and Tepfer, 1995) that was tested in tomato by Grichko *et al.,* (2005) could be a reasonable alternative, although its responsiveness to cyst nematodes in plant species like potato and tomato is unknown. Recently, more specific promoter elements were identified in *Medicago truncatula* and in *M. sativa*, namely *MtPT1* and *MsPRP2* (Winicov, *et al.*, 2004; Xiao, *et al.*, 2005). Properly targeted prevention of the recruitment of susceptibility factors by a pathogen could be a highly specific and relatively bio-safe approach for pathogen and pest management.

Materials and methods

Plant material and nematode inoculation

Seeds of the tomato (Solanum lycopersicum L. cv. Moneymaker) were surface sterilized with 0.8% commercial bleach (v/v) for 25 min and rinsed 3 times in sterile tap water. Seeds (five per plate) were germinated in a Petri dish (Ø = 9 cm) containing 1.5% (w/v) water agar in the dark at 18 °C. Germinating seeds were transferred into Petri dishes containing Gamborg's B5 medium supplemented with 2% (w/v) sucrose and 3% (w/v) Gelrite (Duchefa, The Netherlands). Tomato plantlets were grown on slightly tilted plates at 18 °C under 16-h light and 8-h dark cycles. Potato plants (S. tuberosum 6487-9; (Horsman, *et al.*, 2001) were grown on Murashige and Skoog basal medium containing 2% (w/v) sucrose and 0.8% (w/v) Daishin agar (Brunschwig Chemie BV, The Netherlands) at 24 °C under 16-h light / 8-h dark cyclus. Surface-sterilized preparasitic J2-s from the cyst nematode Globodera rostochiensis (pathotype Ro1, Mierenbos) were prepared as described by Goverse *et al.*, (2000b). Approximately 10 individual J2/per root tip were transferred to 10-12 days old tomato or potato plants using sterile siliconized pipette tips. For the nematode resistance test of the transgenic potato plants, 9 plantlets (3 plantlets per Petri dish) of each line were inoculated as described above. After inoculation plants were kept in the dark at 18 °C. Five weeks after infection, the number of adult females was determined per Petri dish, and the phenotype of the females was scored.

Semi-quantitative RT-PCR

At 7 days post inoculation (dpi) tomato root segments (length ≈ 1 cm) from both infected and non-infected roots were collected and frozen in liquid nitrogen. For total RNA isolation, 50-100 mg of frozen root material was incubated in 0.75 ml TRIzol Reagent (Gibco/Life Technologies, The Netherlands). Glass beads (425-600 μm) were used to homogenize the segments in a Silamat S5 shaker (Vivadent, Austria). Oligo $(dT)_{25}$ Dynabeads (Dynal, Norway) were used to isolate mRNA. For first strand cDNA synthesis the GeneAmp RNA PCR Kit (Applied Biosystems) was used. On the basis of the alignment of cDNA sequences of 8 known tomato EGases (*cel1* U13054, *cel2* U13055, *cel3* U78526, *cel4* U20590, *cel5* AF077339, *cel6* Q9S946 (partial sequence), *cel7* Y11268, *cel8* AF098292) specific primers were designed (Tab. 1). Individual EGase transcripts were amplified in AmpliTaq Gold DNA polymerase-driven PCR reactions (Applied Biosystems). The following profile was used: 95°C for 10 min, subsequently 39 cycles of 30 s at 94°C, 45 s at 53°C, and 45 s at 72°C. Products were analysed on a 1.2% (w/v) agarose gel.

Plant material for *in situ* examination

Root segments containing 1, 3, 5, 7, 10 and 14-day-old syncytia were excised and fixed in 4% (w/v) paraformaldehyde in 50 mM PIPES buffer (pH 6.9) for 2 h at room temperature. After washing the segments 4 times for 30 min in the same buffer, they were dehydrated and embedded in butyl-methyl-methacrylate (BMM) resin (De Almeida Engler, *et al.*, 2001). Embedded material was serially cut into semi-thin (2 μm) sections with a Leica RM2165 microtome. The sections were adhered to Superfrost 3-Chamber Slides (MJ Research, USA) on a slide heating plate (2 h at 50°C). For further experimentation the BMM resin was removed and sections were rehydrated, digested with Proteinase K (Sigma, Germany) and dehydrated again.

In situ hybridization in tomato root sections

DIG-labeled ss-cDNA probes for *Sl-cel7* and *Sl-cel8* were prepared in 2 successive amplification reactions (Expand High Fidelity PCR System, Roche) using primer pairs *Sl-cel7-A* or *Sl-cel8-A* (Tab. 1). In a 1st reaction, ds-cDNA fragments were generated by one of the primer pairs using a TOPO vector harbouring cDNA of *Sl-cel7* or *Sl-cel8* as template. In a 2nd reaction, a PCR DIG Probe Synthesis Kit (Roche) was used to synthesize antisense or sense ss-cDNA probes with 2 μl of the 1st PCR product as a template and 2 μM of a single primer. Both reactions were performed on a MJ Research PTC-100 thermal cycler using the following PCR profile: 1 cycle at 95 °C for 2 min followed by 35 cycles of 15 s at 95 °C, 30 s at 54 °C, and 45 s at 72 °C, followed by 7 min at 72 °C. Unincorporated nucleotides were

removed with a Mini Quick Spin DNA Columns (Roche). Both amplification products and purification steps were verified on agarose gel.

For *in situ* hybridizations, frame-seal incubation chambers (MJ Research, USA) were attached to slides with root sections pre-treated as described above. Into each slide-well, 20 µl of hybridization mix was pipetted. This mix consisted of 50% (v/v) formamide, 10% (w/v) dextran sulfate, 250 ng/ml tRNA (Sigma, Germany), 100 µg/ml Poly(A) (Sigma, Germany), 0.3 M NaCl, 0.01 M Tris-HCl (pH 7.5), 1 mM EDTA, 1x Denhard's solution (Amersham/Pharmacia), 0.01 mM DTT and 2 µg/ml DIG labeled ss-cDNA antisense or sense probe. Chambers were sealed and kept overnight at 45 °C. Afterwards sections were washed in 2x SSC buffer (saline-sodium citrate buffer, pH 7.2) for 25 min at room temperature, followed by 3 washes with 0.2x SSC at 53 °C for 25 min each. Finally, slides were rinsed 3 times with sterile distilled water for 1 min and subjected to immunodetection of DIG. Immunodetection of DIG was carried out with Fluorescent Antibody Enhancer Set for DIG Detection (Roche). After air-drying in the darkness slides were mounted with Immuno-Fluore Mounting Medium (ICN, USA). Slides were viewed using an AX70 "Provis" microscope (Olympus, Japan) equipped with a U-M61002 fluorescence filter set.

In situ RT-PCR followed by *in situ* hybridization.

Before *in situ* RT-PCR reaction pretreated slides were treated with DNase I solution (1 kU/ml DNase I (RNase-free); Roche), 100 mM sodium acetate buffer (pH 5.5), 5 mM MgSO$_4$, 1 kU/ml RNase Inhibitor (Roche) for 8 h at 37 °C. *In situ* RT-PCR was carried out with a Titan One Tube RT-PCR Kit (Roche) supplemented with the tomato EGase primers pairs *Sl-cel7-A* or *Sl-cel8-A* (2 µM each) and 1x Self-Seal Reagent (MJ Research, USA). Following RT-PCR profile was used: reverse transcription for 30 min at 50 °C was followed by 10 cycles of 30 s at 94 °C, 30 s at 54 °C, and 2 min at 68 °C. This was followed by 40 cycles of 30 s at 94 °C, 30 s at 54 °C, and 2 min at 68 °C with an addition of 5 s per cycle. After the final cycle, slides were kept for 10 min at 68 °C, for 1 min at 92 °C and stored at 4 °C. After amplification, slides were washed for 5 min in 2x SSC, followed by the addition of hybridization mix (composition as described above whereas ss-cDNA probes were replaced by 1 µg/ml of DIG-labelled ds-cDNA probes). The probes were produced as described above for ss-cDNA using PCR DIG Probe Synthesis Kit (Roche) but both primers were added to the 2nd reaction mix. Amplified cDNA and DIG-labelled ds-cDNA probes were denatured at 95 °C for 10 min and hybridized overnight at 45 °C. Thereafter, DIG was detected as described above.

Antibody production

Based on the alignment of eight tomato EGases, specific fragments for *Sl-cel7* and *Sl-cel8* were amplified using primer pairs *Sl-cel7-B* and *Sl-cel8-B* (Tab. 1). Isozyme-specific protein fragments were produced in a pBAD/Thio-TOPO expression system (Invitrogen), and recombinant fragments were purified on a Ni-NTA spin column (Qiagen, Germany). Purified antigen ($\approx$ 50 µg) was injected into chicken, and eggs were collected after 4 weeks. IgY-s were precipitated from the yolk by PEG 6000 and affinity-purified on a HiTrap IgY column (Amersham/Pharmacia). Dialyzed fractions of anti-*Sl-cel7* and anti-*Sl-cel8* were used for immunolocalization studies.

Primer pair	Primer (forward) 5'→3'	Primer (reverse) 5'→3'	Product size (bp)
Sl-cel1	tgctgattcatttatttgtgg	atagtgttctccttctttggt	574
Sl-cel2	tttctcatccccaagtccaat	acaacaacaacacaataatag	590
Sl-cel3	ttaatcgcacaaaaggag	gtaattagaacggacatcac	428
Sl-cel4	tatgttacctccacttcttcctg	cattcttgattctactacacaccttgac	487
Sl-cel5	ctatgggtgcttaattgattgatgact	tgcactggatggtagagatgaac	463
Sl-cel7	cttatgtcaagggagaagcgtg	tgtaagtagctggctccgaat	567
Sl-cel8	ggcaactagttcatgggctctg	ctttcttcctccgacttctccaca	497
Sl-ubq	atgcagatccttcggtgaaaacc	accaccaccggagaacggag	250
Sl-cel7-A (ss cDNA probes)	gattcattcaaacaaagagcggaag	gcattgccaaagaaggtaaagatg	360
Sl-cel8-A (ss cDNA probes)	tggtcacagcggttacaatc	cgattgcaggtgaagcagtgtg .	382
Sl-cel7-B (Ab production)	ttgagagcaacaaatgacatttc	tgtataaggtgagttgggtagtac	213
Sl-cel8-B (Ab production)	aatgggaagacttactacagatac	ggagacgattggcaggtgaag	219
Sl-cel7-C (plant transformation)	ggggacaagtttgtacaaaaaagcag gcttcgattcattcaaacaaagagc	gggaccactttgtacaagaaagctgg gttcattgccaaagaaggtaaag	360
Sl-cel8-C (plant transformation)	ggggacaagtttgtacaaaaaagcag gcttctgagctcctctcattcgc	ggggaccactttgtacaagaaagctggg ttcaactggaaggagctgattg	379

Table: 1
List of primers

Tissue preparation and immunocytochemical localization

Root segments with syncytia of 2, 5, 7, 10 and 21 dpi were fixed in 2% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M PBS (pH 7.0) for 2 h, washed 4 times with PBS and embedded in BMM resin. Three µm thick sections were collected on silane-coated slides and the resin was removed (De Almeida Engler, *et al.*, 2001). After rehydration in PBS, unspecific epitopes were blocked by incubation for 1 h in PBS supplemented with 5% (w/v) non-fat dried milk and 0.05% (v/v) Tween 20. Thereafter, slides

were treated with primary antibody (anti-*Sl-cel7* or anti-*Sl-cel8*) diluted 1:10 or 1:20 in PBS supplemented with 1% (w/v) non-fat dried milk and 0.05% (v/v) Tween 20 (1 h at 37 °C). Subsequently, slides were washed twice in PBS with 0.1% (v/v) Tween 20, and once in PBS. In a next step, sections were incubated in 5 µg/ml goat anti-chicken IgG conjugated with Alexa Fluor 488 (Molecular Probes, USA) in PBS (1h at 37 °C) and washed 3 times in PBS as described above. Samples were counterstained with 1 µg/ml DAPI for 10 min, with 0.01% (w/v) Toluidine Blue for 1 min, and mounted with Immuno-Fluore Mounting Medium (ICN, USA). In negative controls primary antibodies were replaced by pre-immune chicken serum. The sections were examined as described above for *in situ* analysis.

dsRNA constructs and plant transformation

Fragments of *Sl-cel7* (360 bp) and *Sl-cel8* (379 bp) were amplified from a cDNA library using primers that incorporate an *attB* recombination sites (*Sl-cel7*-C and *Sl-cel8*-C; Tab. 1). The *att*B-flanked PCR products were introduced into a Gateway (Invitrogen)-compatible vector (pk7GWIWG2(I) (Barthels, *et al.*, 1997). Constructs were introduced into *A. tumefaciens* strain LBA4404 by electroporation, and positive clones were used for potato transformation. Stem segments (0.5-1 cm) from *in vitro* plants were transformed with pk7GWIWG2::*Sl-cel7* or pk7GWIWG2::*Sl-cel8*, and regenerated (Engelen, *et al.*, 1994). Primer pairs *Sl-cel7* and *Sl-cel8* were used to check the silencing efficiency in individual transgenic potato lines, and a cluster specific primer set for a constitutively expressed LRR-containing potato gene was used to normalize band intensities (Bakker, *et al.*, 2003). Transgenic potato plants and empty vector controls were *in vitro* inoculated with nematodes as described above.

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Chapter 5

Identification and genomic organisation of an auxin-inducible and a CBM-harbouring cellulase from potato

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Manuscript in preparation

Abstract

Recently, we have shown that the potato cyst nematode *Globodera rostochiensis* specifically recruits two endo-β-1,4-D-glucanases - *Sl-cel7* and *Sl-cel8* - for feeding site development in host plant roots (this thesis, Chapter 4). In potato, the major host of potato cyst nematodes, virtually nothing is known about the cellulase gene family. Here, we present the cloning and the basic characterisation of two genomic sequences encoding the orthologues of *Sl-cel7* and *Sl-cel8a* in potato (our data suggest multiple isoforms of *cel8* in potato). The corresponding proteins consist solely of a catalytic core domain (St-CEL7) or of a catalytic domain linked to a carbohydrate binding module (CBM) (St-CEL8a). Recently, the CBM domain of Sl-CEL8a was shown to belong to CBM family 2 (Urbanowicz, *et al*,. 2005), and on the basis of shared characteristics between CBM domains in Sl-CEL8 and St-CEL8a we postulate that St-CEL8a harbours a cellulose binding domain as well. Potato cellulase sequences were used to mine EST databases and this information was included for the generation of a phylogenetic tree of the solanaceous endo-β-1,4-D-glucanase gene family. Our findings imply that the potato cellulases recruited by the potato cyst nematode are able to hydrolyse both xyloglucan (CEL7) and crystalline cellulose (CEL8), and thus provides a first explanation why nematodes recruit exactly these two members of the cellulase family.

Introduction

Plant cells are surrounded by a cell wall and the habitus of plants is directly related to the mechanical and tensile strength of this structure. Plant cell walls determine both the size and shape of protoplasts during plant growth and development. Prevailing models of the plant cell wall describe it as a complex composite of cellulose microfibrils and cross-linking glycans and xylans embedded in a matrix of pectin polymers and structural glycoproteins. This composition makes the plant cell wall water-insoluble, chemically stable and physically robust. As such, the plant cell wall also functions as a physical barrier between the plant and its potentially hostile biotic environment. Plant growth and development depend on the cell wall modification either by the modification of the existing components or by the incorporation of new components in order to accommodate changes in shape, size and function. In each of those processes, cell wall modifying enzymes are essential. During fruit ripening, for example, pectins are degraded by polygalacturonidases (Hadfield, *et al*,. 1998), hemicelluloses are depolymerised by multiple enzymes including endo-β-1,4-D-glucanases (Lashbrook, *et al.*, 1994). In parallel, plant pathogens have evolved ways to overcome this structural barrier. Many pathogens produces various types of cell wall-degrading enzymes (Rosso, *et al.*, 1999), and/or recruit enzymes produced by host plant (Goellner, *et al.*, 2001; Zhou, *et al.*, 2005).

Endo-β-1,4-D-glucanases (EGases) are enzymes that catalyze the hydrolysis of internal β-1,4-glucan linkages present in *e.g.* cellulose and in the backbone of xyloglucan, a major hemicellulosic polysaccharide in plant cell walls (Wong, *et al.*, 1977). EGases are widespread among prokaryotes en eukaryotes, and are divided into different groups based on amino acid homology in the catalytic core (Beguin, 1990). Remarkably, plant cellulases all belong to a single glycoside hydrolase (GH) family, namely GH 9 (Hayashi, *et al.*, 2005). For the limited number of plant species studied in detail so far, cellulases appear to be encoded by multi-gene families. *Arabidopsis* harbours 25 GH9 family members, rice is thought to contain at least 20 GH9 genes, whereas so far eight members were discovered in tomato. The plant and bacterial EGases within the GH9 family share a number of discrete amino acid domains (Brummell, *et al*,. 1994). Although EGases may include cellulose-binding domains (CBD) (*Arabidopsis*, 3; rice, 3) or membrane-anchored domains (*Arabidopsis*, 3; rice, 3), the majority of the plant cellulases solely comprises a catalytic domain. Little is known about the effect of the presence of a CBD moiety on the activity and specificity of plant cellulases. CELI, a cellulase from *Clostridium thermocellum,* belongs to GH family 9 and harbours a CBD domain. This cellulase was shown to be efficient in the degradation of insoluble cellulose substrates. Truncated forms of this enzyme lacking the CBD domain showed a highly reduced activity towards such substrates. In parallel, plant cellulases with a CBD

domain are hypothesized to bind more efficiently to cellulose microfibrils (Trainotti, *et al,.* 1999).

Cyst nematodes (members of the genera *Globodera* and *Heterodera*) are sedentary obligatory endoparasites that have complicated and intimate relationships with their host plants. Within the host root tissue, the infective juvenile induces a specialized feeding structure - a syncytium - that passes nutrients from the root's vascular system to the developing nematode. For growth and reproduction, the nematode completely depends on this structure (Golinowski, *et al.*, 1997). Feeding site development is accompanied by local cell wall dissolution, the formation of cell wall ingrowths in places near to the xylem vessels and cell wall stretching in the tissues surrounding the growing syncytium. Enzymes involved in reorganization of cell wall during syncytium formation most likely originate from the host plant. This assumption is supported by the fact that plant cell wall-modifying agents such as EGases, pectinmethylesterases, expansins were shown to be induced in developing syncytia (Niebel, *et al.*, 1993; Vercauteren, *et al.*, 2002; Gal, *et al.*, 2006).
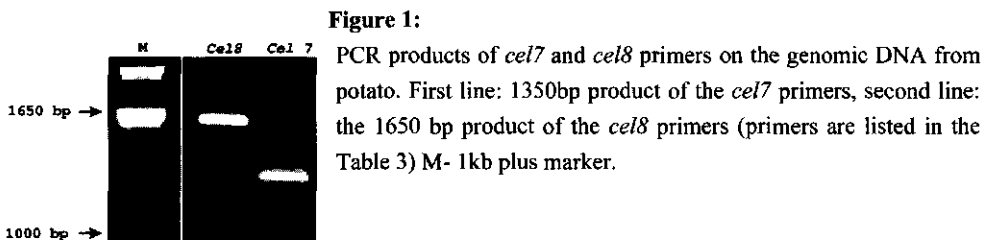
Here we present the cloning and characterization of two novel potato endo-ß-1.4-D-glucanases, *St-cel7* and *St-cel8a*, both indispensable for the potato cyst nematode development (this thesis Chapter 4). Both genes show high similarity to their homologues in tomato and tobacco (up to 95%), and were shown to be upregulated during syncytium development (Goellner, *et al.*, this thesis Chapter 4). In addition we screened the cDNA databases in order to *in silico* identify other family members in tomato, tobacco and potato. On the basis of an alignment of Solanaceous homologues of cellulase 7 and 8, their relatedness was assessed.
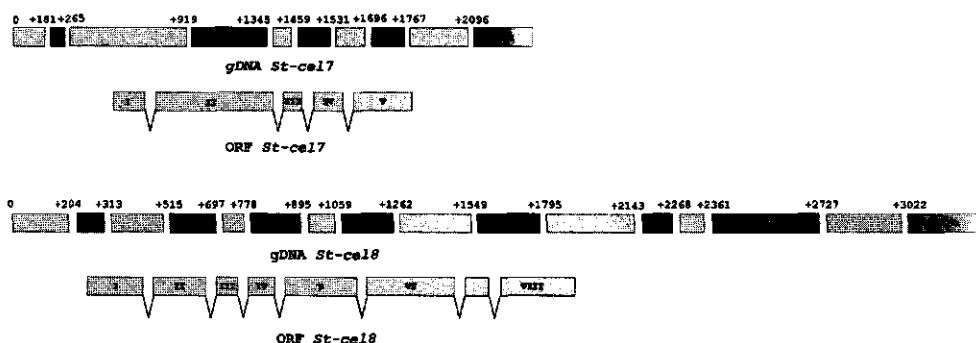
RESULTS

Identification of *St-cel7* and *St-cel8*

In tomato (*Solanum lycopersicum*), a close relative of potato (*S. tubersosum*), the cellulase family comprises at least eight members, including the nematode up-regulated *Sl-cel7* and *Sl-cel8* (Chapter 4 of this thesis). In order to check whether homologues of *Sl-cel7* and *Sl-cel8* were likely to be present in potato, the potato EST data base was screened with *Sl-cel7* and *Sl-cel8*. This resulted in the identification of two homologous unigenes, (SGN-286389, SGN-U272121) and on the basis of this information it was decided to investigate the genomic organization of *St-cel7* and *St-cel8*. To this end, a so-called genome walking strategy was followed (Siebert, *et al.*, 1995). In this strategy, selection of adaptor-ligated genomic DNA fragments from potato depends on the availability of target-specific primers. Therefore, two fragments of the putative *cel7* and *cel8* genes were amplified from potato gDNA with primers designed on the basis of corresponding tomato and tobacco cDNA sequences for *cel7* and for *cel8* (*cel7* F&R and *cel8* F&R, see Table 3). The amplification products with a size of respectively 1350 and 1650 bp were cloned and sequenced (Fig 1). A nucleotide-nucleotide BLAST search (blastn) with the potato genomic DNA fragments revealed highest homology with cDNA sequences of the tomato *cel7* and *cel8*. Gene-specific primers (8GSP F/R and 7GSPF/R, see Table 3), in combination with so-called adaptor primers (AP) needed to select genomic fragments from the four genomic DNA "libraries", were designed on the basis of these sequences.

Relevant potato genomic fragments were selected from four different "libraries" (see Materials and Methods) with two sets of primers developed specifically for *cel7* and *cel8*. Each set comprised of the primary (x) and a nested primer (y=x+1) in forward and reverse orientation (Table 3, nested primers were used to increase the specificity). Those secondary product(s) were subsequently cloned and sequenced. The new sequence information was used to generate a new set of GSP primers that allowed the selection of new genomic fragments more up or more downstream as compared to the secondary product. This procedure was repeated four times for *cel7* and three times for *cel8*, finally resulting in two contigs each including four (*cel7*) or five (*cel8*) partially overlapping potato genomic DNA fragments. The intron/exon structure of *St-cel7* (complete sequence) and *St-cel8* (nearly complete sequence) is presented in Fig 2.

Figure 1:

PCR products of *cel7* and *cel8* primers on the genomic DNA from potato. First line: 1350bp product of the *cel7* primers, second line: the 1650 bp product of the *cel8* primers (primers are listed in the Table 3) M- 1kb plus marker.

gDNA St-cel7

ORF St-cel7

gDNA St-cel8

ORF St-cel8

Figure 2:

The schematic representation of the genomic structure of the *St-cel 7* and the *St-cel 8* genes. The exons are depicted as grey bars, whereas introns are black.

Data base number	Nature, source and length	E-value	Identity/Gene
CK248726	EST (GenBank); 966 bp	1e-194	96% to *St-cel7*
CK248727	EST; (GenBank; 426 bp)	7e-84	95% to *St-cel7*
BF052961	EST(GenBank; 385 bp)	2e-68	99% to *St-cel7*
BE343210	EST(GenBank; 639 bp)	7e-65	99% to *St-cel8*
TC113342	12 ESTs,(TiIGR 2,453 bp)	9e-236	78,6% to *St-cel8*

Table 1:

Potato cellulase sequences identified from the TIGR database (http://tigrblast.tigr.org/tgi/).Blaxtn was used to select for most identical sequences.

Gene structure of *St-cel7* and *St-cel8*

Comparison of *in silico* spliced genomic sequences of *St-cel7* and *St-cel8* with known cDNA sequences of solanaceous endo-β-1,4-glucanases revealed highest similarities with the tomato homologue *Sl-cel7* in case of *St-cel7* (95,5% identity) and with the tobacco homologue *Nt-cel8* in case of *St-cel8* (88,7% identity). To confirm the correctness of *in silico* determined cDNA sequences of *St-cel7* and *St-cel8*, these conceptual cDNAs were used to interrogate the TIGR potato ESTs database. As a result four ESTs (CK248726 96 %, CK248727 95% BF052961 99% identity to *St-cel7* and BE343210-99% identity to *St-cel8*) and one unigene (TC113342 77,8% identity to *St-cel8*) were identified (Table 1).

Comparison of the *in silico* spliced *cel8* genomic DNA sequence from potato with its homologue in tobacco revealed a similarity of 88,7%, whereas the degrees of identity with the potato *cel8* unigene (TC113342) and the *Sl-cel8* cDNA were relatively low (78,6%, and 78%, respectively). Such a discrepancy can not be attributed to the error rate of DNA polymerase or sequencing mistakes. TC113342 consists of 12 ESTs assembled into a 2453 bp long open reading frame. The existence of two isoforms of *St-cel8* could explain this discrepancy. Hence, we postulate that potato harbours *St-cel8a* (with the highest identity to

Nt-cel8) and *St-cel8b* (TC113342, with highest identity to *Sl-cel8*). The *St-cel8* open reading frame (ORF) spans 1,656 nucleotides, which is significantly shorter than the ORFs of *Sl-cel8* (1,872 nt) and *Nt-cel8* (1,888 nt). This observation in combination with the lack of a starting codon suggest the missing of a relatively small fragment ($\approx$ 200 bp) at the 5' end of the genomic *St-cel8* sequence. Currently, the topology of *St-cel8* is characterized by eight exons interspersed by seven introns (Fig. 2).

The *St-cel7* gene encodes a mature protein of 479 amino acids, with a calculated molecular weight of 53,5 kDa. This protein was predicted to be preceded by a signal peptide for secretion with a cleavage site between amino acid residues alanine and glycine, 16 amino acids from the hydrophobic core. Both, St-CEL7 and St-CEL8 harbour a conserved tripeptide HHR (St-CEL7: 396-398aa, and St-CEL8: 346-348aa) typical for glycoside hydrolase family 9 (Brummell, *et al.*, 1994). Both potato cellulases contain also two highly conserved amino acid stretches (GGYYDAGDNVK and DELYWGAA) that are thought to be involved in their catalytic activity (Fig 3). In the case of St-CEL8 the catalytic and the cellulose binding domain (CBD) are separated by a proline-rich interdomain linker with a length of approximately 20 amino acid residues. The cyclic structure of proline provides some degree of stiffness to the linker; similar linkers are present in Sl-CEL8 and Nt-CEL8. The potato *cel8* CBD, shares 72,2% similarity to the CBD domain of tomato *cel8,* which was recently shown to belong to CBM family 2, and to have the ability to bind crystalline cellulose (Urbanowicz, *et al.* 2005) (Fig. 4).
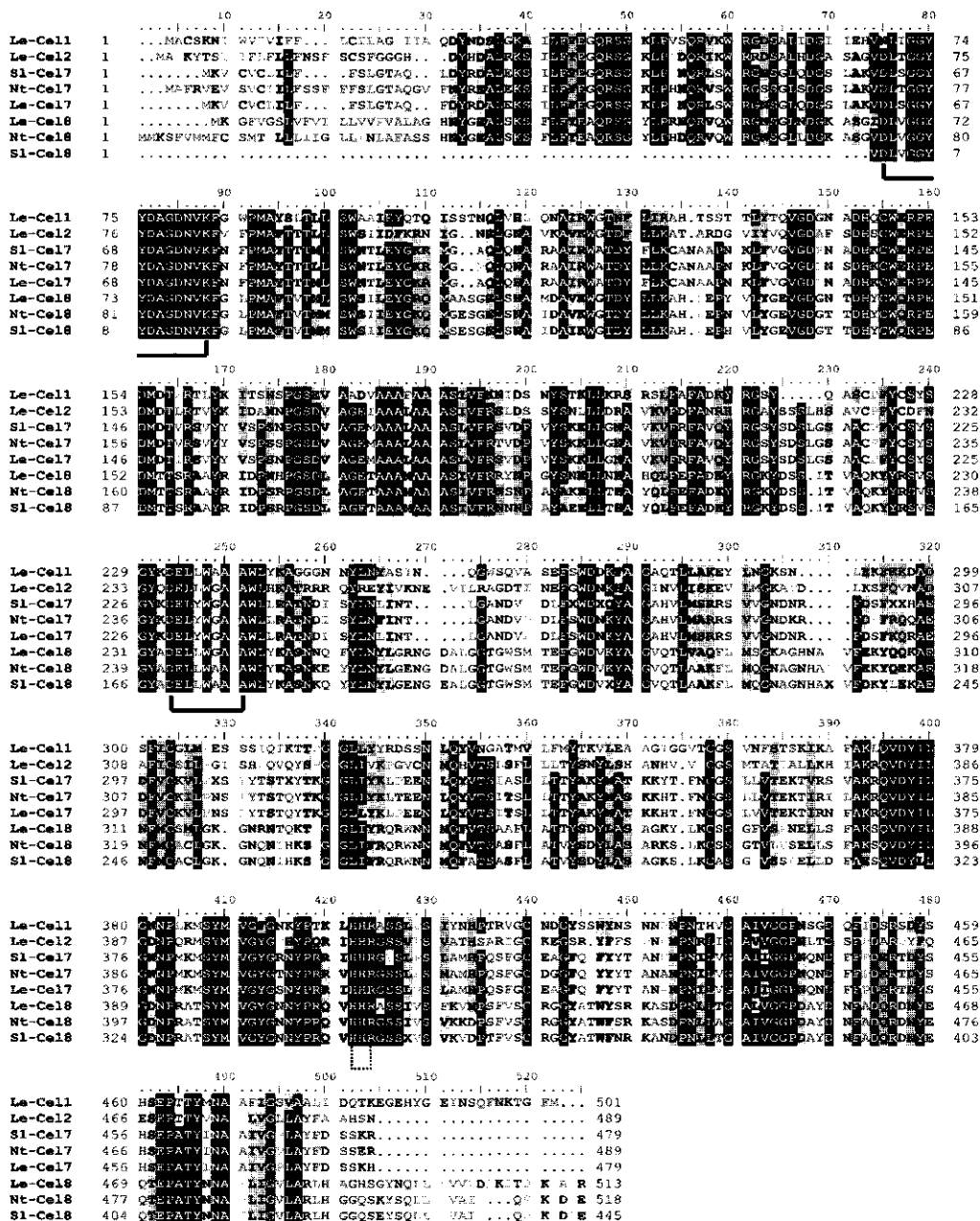
Figure 3:

Sequence alignment of deduced amino acid sequences of potato *cel7* and *cel8* with their tomato and tobacco orthologues. Black boxes show identities and conservative residues are marked with the grey boxes. Residues postulated to form the catalytic domain of plant EGases are underlined by the black line. Conserved residues HHR that comprise the signature of the EGases from the GH9 family are mark with the dashed line.

```
              10                20                30
-----------------+-----------------+-----------------+
D I A I E Q K E T T S █ V R E G K T Y Y R Y S A I V T █ K S     StCEL8-CBM
H V T I Q Q R A T S S █ A L N G K T Y Y R Y S A V V T █ K S     SlCEL8-CBM
D I T I E Q K E T A S █ V P K G K T Y Y R Y S V I V T █ K S     NtCEL8-CBM
P I T I S Q K M T N S █ K N E G K V Y Y R Y S T I L T █ R S     AtCEL8-CBM
- - - V T V V S T N T █ N G G G Q V N F E V K █ T G S T T L     GrCEL1-CBM
- - - █ E L R - - D K █ G S G A N F I L S F K █ N G N S K A     McCBP

              40                50                60
-----------------+-----------------+-----------------+
S K T L K N L K L S V S Q L Y G S L █ G L S K Y G - D S Y M     StCEL8-CBM
G K T V K N L K L S I V K L Y G P L █ G L T K Y G - N S F I     SlCEL8-CBM
A M T M K N L K L S I Y Q L Y G S L █ G L S K Y G - D S Y V     NtCEL8-CBM
T K T L K I L K I S I T K L Y G P I █ G V T K T G - N S F S     AtCEL8-CBM
█ G V K F S V T L P A G T T V A G S █ M N A A G S N E Y T       GrCEL1-CBM
█ A V K F Q L E L A S G Q T I Q S I █ V Q K Q S E N I Y T       McCBP

              70                80                90
-----------------+-----------------+-----------------+
F P A █ I N S L P - - A █ K S L E F V Y I H T A N S P A V V     StCEL8-CBM
F P A █ L N S L P - - A █ K S L E F V Y I H T A - S P A I V     SlCEL8-CBM
F P A █ L N S L P - - A █ K T L E F V Y V H S A - S P A T V     NtCEL8-CBM
F P S █ M Q S L P - - S █ K S M E F V Y I H S A - S P A D V     AtCEL8-CBM
L P S Y I N I K A K E A N K D A G M T L N █ S - G K P T A K     GrCEL1-CBM
L P N Y M S L N A G E E N K N A G L V V N █ P P T L P K I T     McCBP

              100
-----------------+
S V S S Y T L D     StCEL8-CBM
S V S S Y T L V     SlCEL8-CBM
S I S S Y T L V     NtCEL8-CBM
L V S N Y S L E     AtCEL8-CBM
V L S T T A █ S G   GrCEL1-CBM
L L G Q G A █ T P Y McCBP
```

Cellulomonas fimi (= archetype CBM family 2)
```
TG█SVTATRAEE█SDRF█VTYSVSGSSA█TVNLALNGSQTIQAS█ANVT█SGS
TRTVTPNGSGNTFGVTVMKNGSSTTPAAT█
```

W = exposed tryptophan residues (involved in cellulose binding)

Schematic representation of family 2 CBMs from bacteria (source: InterPro, IPR001919)
```
     +---------------------------------------------------+
     |                                                   |
     xCxxxxWxxxxxNxxxWxxxxxxxxWxxxxxxxxWNxxxxxGxxxxxxxxxxCx
```

'C': conserved cysteine involved in a disulphide bond.

Figure 4:
Alignment of family 2 CBM from plant and nematode cellulases (St, *Solanum tuberosum*; Sl, *Solanum lycopersicon*; At, *Arabidopsis thaliana* and Gr, *Globodera rostochiensis*; Mc, *Meloidogyne chitwoodi*). Within this family two (exposed) tryptophan residues are involved in cellulose binding.

Cluster analysis of the *Solanaceae* cellulase gene family

In the screening of potato EST and unigene databases with the cDNA sequences of all eight tomato endo-β-1.4-D-glucanases (*cel1-cel8*), 18 unigenes EST were identified with a high homology (Table 2) to tomato EGases. In order to identify all publicly available *Solanaceous* family members, tomato and tobacco databases were searched. The Unigenes were clustered in groups according to their origin and their nucleotide similarity score (E-value) with characterized cellulase genes.

The homologous sequences of the three related *Solanaceae* species, with a total of 31 sequences (summarized in Table 2) were used to construct a dendrogram (Fig. 5) applying the maximum likelihood algorithm. Within this tree, a number of clusters can be distinguished. Each cluster represents distinct a family *cel 2, 3, 6, 7 and 8*. The *cel 1,4* and *5* were represented by single tomato sequences. The overall identity score of homologous EGase gene sequences with characterized cellulase gene family members was very high (between 0 to 4e-7), indicating that the EGase family as a whole is conserved within the *Solanaceae*. The most distant groups were *cel3, cel6* and *cel8*, suggesting a distinctive role for these family members (Fig. 6). The similarity between individual subfamily members was very high.

Species	Name	Database Number	Origin	(cel1/8)
Sl	*Sl-cel1*	LEU13054	cDNA	*cel1*
Sl	*Sl-cel2*	LEU13055	cDNA	*cel2*
Sl	*Sl_U214839*	SGN-U214839	unigene	*cel3*
Sl	*Sl-cel3*	LEU78526	cDNA	*cel3*
Sl	*Sl-cel4*	SLU20590	cDNA	*cel4*
Sl	*Sl-cel5*	AF077339	cDNA	*cel5*
Sl	*Sl-cel7*	LEE14BDGL	cDNA	*cel7*
Sl	*Sl_U220961*	SGN-U220961	unigene	*cel8*
Sl	*Sl_U228696*	SGN-U228696	unigene	*cel8*
Sl	*Sl-cel8*	AF098292	cDNA	*cel8*
Sl	*Sl_BT013085*	BT013085	cDNA	*cel6*
Sl	*Sl_U220005*	SGN-U220005	unigene	*cel6*
Sl	*Sl_E321418*	SGN-E321418	unigene	*cel6*
Sl	*Sl_C120180*	SGN-C120180	unigene	*cel6*
Nt	*Nt-cel2*	AF362948	cDNA	*cel2*
Nt	*Nt-cel7*	AF362947	cDNA	*cel7*
Nt	*Nt-cel8*	AF362949	cDNA	*cel8*
St	*St_U254919*	SGN-U254919	unigene	*cel2*
St	*St_U243715*	SGN-U243715	unigene	*cel3*
St	*St_U243714*	SGN-U243714	unigene	*cel3*
St	*St_U248332*	SGN-U248332	unigene	*cel3*
St	*St_U259226*	SGN-U259226	unigene	*cel3*
St	*St_U259225*	SGN-U259225	unigene	*cel3*
St	*St_U265011*	SGN-U265011	unigene	*cel4*
St	*St_U265587*	SGN-U265587	unigene	*cel5*
St	*St_U256735*	SGN-U256735	unigene	*cel8*
St	*St-cel7*	This work	*in silico* cDNA	*cel7*
St	*St_U286389*	SGN-U286389	unigene	*cel8*
St	*St_U272121*	SGN-U272121	unigene	*cel8*
St	*St-cel8*	This work	*in silico* cDNA	*cel8*
St	*St_U250209*	SGN-U250209	unigene	

Table: 2

The most significant blastn result for mining of the SOL (Solanacea Genomics Network) with the tomato (*Sl*), potato (*St*) and tobacco (*Nt*) cellulases cDNA sequences obtained from the NCBI data base.
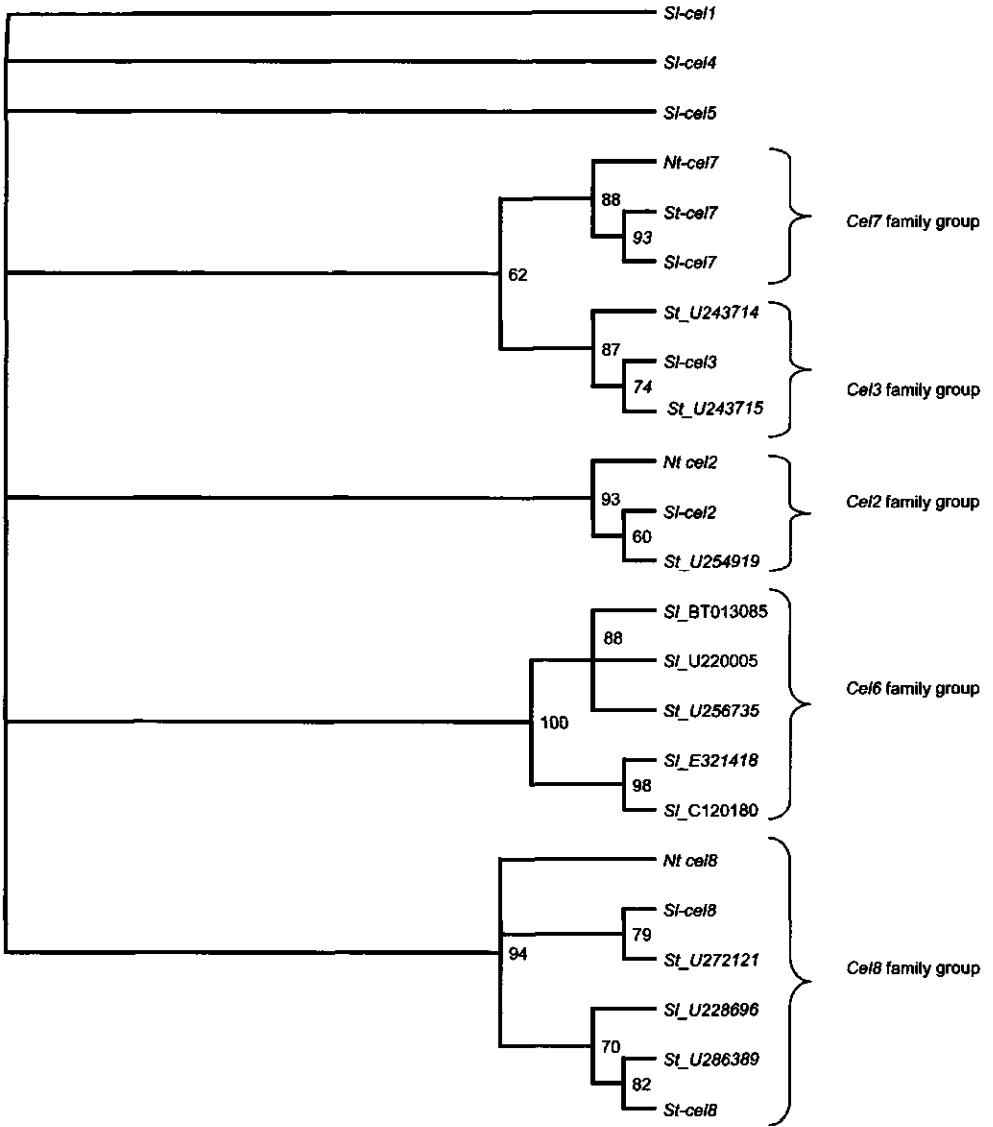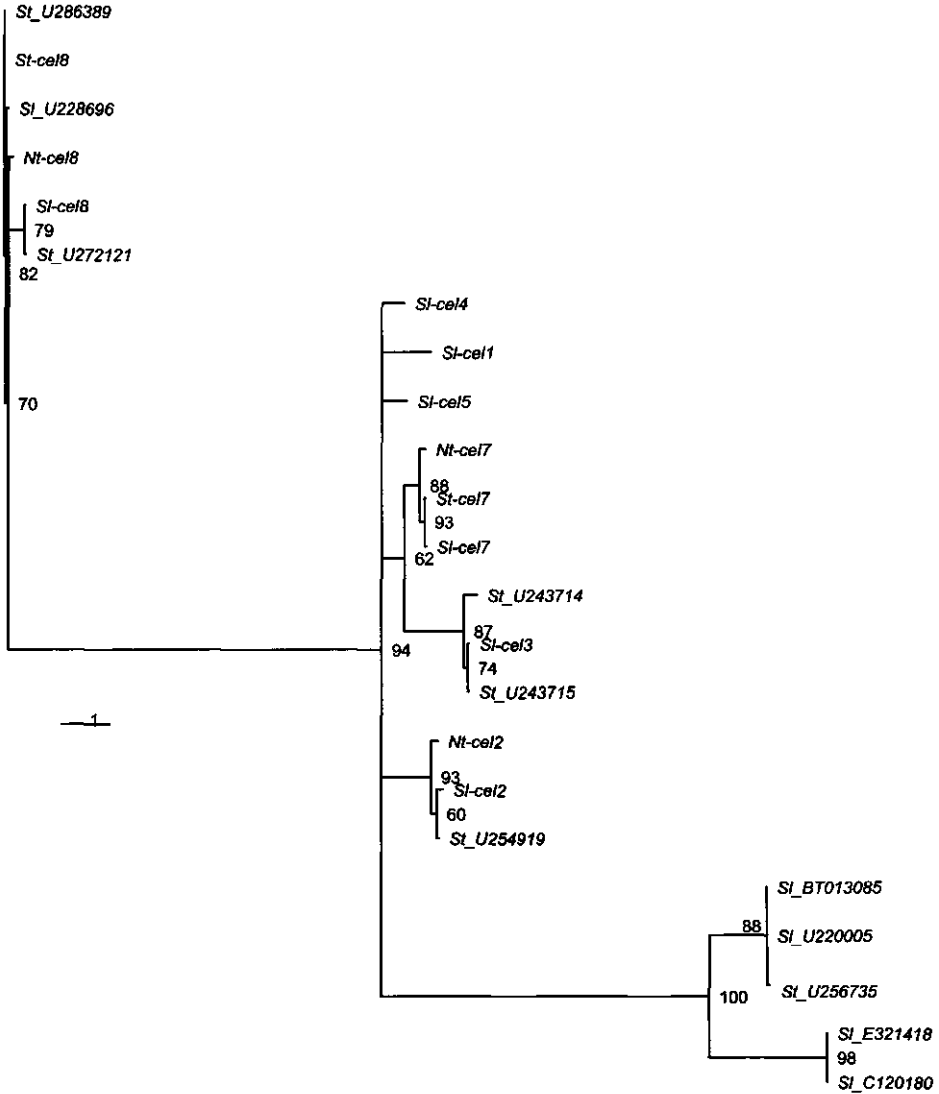
Figure 5:
Phylogenetic relationship of the 23 EGases from the *Solanaceae* family, *Solanum tuberosum* (St), *Solanum lycopersicon (Sl)* and *Nicotiana tabacum* (Nt). The sequences are annotated according to the Table 3. The bootstrap values are represented above every branch.

Figure 6:
Phylogenetic distance tree of the of cellulase genes of three species of the *Solanaceae* family, *Solanum tuberosum* (St), *Solanum lycopersicon* (Sl) and *Nicotiana tabacum* (Nt). The sequences were annotated according to table 2. The bootstrap values are represented above every branch. The length of the branches represents the phylogenetic distance between the sequences.

Discussion

Plant endo-β-1.4-glucanase (EGases) participate in the break-down of 1,4-β-glucosidic linkages, such as those that make up the backbone of xyloglucan, one of the major cell wall hemicellulosic polysaccharides. EGases are well characterized and encoded by multigene families in many plant species, including tomato and tobacco (Brummell, *et al.* 1994; Goellner, *et al.*, 2001). However, in potato (*Solanum tube*rosum), a very close relative of tobacco and tomato, the endo-β-1.4-glucanase gene family remains poorly characterized. In this paper we described the cloning of genomic sequence of two endo-β-1.4-D-glucanases, *St-cel7* and *St-cel8a*, by applying the genome walking technique. These cellulase family members are of special interest because they were shown to be recruited by cyst nematodes during feeding site formation (this thesis, Chapter 4). The sequences were analysed and the intron/exon structures as well as the predicted amino acid sequences were determined. Screening of the TIGR and SGH databases revealed several other, putative family members, and an integrated dendrogram of *Solanaceous* endo-β-1,4-D-glucanases is presented.

Expression of the tomato *cel7* is regulated by auxin and its mRNA accumulation increased in rapidly growing vegetative tissue and in the developing fruit (Catala, *et al.*, 1997; Catala, *et al.*, 2000). In *Arabidopsis* feeding site induction by plant parasitic nematodes was tightly correlated with a local increase in auxin level (Goverse, *et al.*, 2000; Karczmarek, *et al.*, 2004). Interestingly, increased expression of auxin-inducible genes such as *Nt-cel7* and *Sl-cel7* was also observed for other plant parasitic nematode-host plant combinations (Chapter 4, this thesis; Goellner, *et al.*, 2001). The silencing of the potato *cel7* severely hampered nematode development (up to 90% reduction) in *in vitro* experiments (this thesis, Chapter 4), and this observation underlines the importance of *cel7* up-regulation for proper feeding site development. The potato *cel7* is highly similar to the tomato and tobacco *cel7* (> 90% identity). Hence, the local recruitment of this auxin-inducible cellulase appears to be a common phenomenon in the interaction between cyst nematodes and *Solanaceous* host and it is anticipated that silencing of *cel7* orthologues in for instance tobacco and tomato would have similar effects on cyst nematode development.

The putative potato *cel8a* is a two domain cellulase with an overall identity of 88.7% with tobacco *cel8*. Interestingly, the *in silico* search revealed a potato unigene sharing the highest identity (93.6%) with the tomato *cel8*. Additionally new tomato ESTs have been identified with high homology to the *cel8* subfamily. This suggests that the *cel8* family group is more divergent than previously predicted and possibly potato harbours more than one isoform of *cel8*. In tomato eight cellulases have been described so far, and only CEL8 has a carbohydrate binding module (CBM). Recently Urbanowicz (Urbanowicz, *et al.*, 2005) showed that this GH9 family was linked to a family 2 CBM, and in domain-swapping experiments with cellulases from *Thermobifidia fusca* the CEL8 CBM was shown to bind to

crystalline cellulose. CBM family 2, actually cellulose binding domains, has so far only been found in bacteria and plant parasitic nematodes (Smant, *et al.*, 1998; Kudla, *et al.*, 2005). This family is characterized by two exposed tryptophan residues that are involved in cellulose binding, and alignment of CEL8 from potato, tomato and tobacco, and CBMs from cyst and root knot nematodes indeed reveals two conserved tryptophan residues (Fig 4). If we – on the basis of sequence similarity with Sl-CEL8- presume that St-CEL8 is harbouring a family 2 CBM, this would imply that St-CEL8 can degrade crystalline cellulose. The recruitment by cyst nematodes of at least two different cellulases that - in combination - can cleave 1,4-β-glucosidic bonds in both xyloglucan and cellulose suggests that syncytium proliferation involves the local depolymerisation of both types of polysaccharides.

The alignment of the catalytic core of *Solanaceae* cellulase gene sequences found in databases yielded an integrated dendrogram tree. Within this tree, five distinct cellulase gene subfamilies groups can be distinguished: *cel2, cel3, cel6, cel7* and *cel8*. The relatively high similarity between family group members is supported by the bootstrap values (Fig. 6) represented in the tree. The most distant groups were *cel3, cel6* and *cel8*, which may resemble their distinct functions and characteristics among the plant cellulases. Apart from the catalytic core domain, members of *cel3* and *cel8* families harbour a functionally distinct, additional domain. *cel8* contains a CBM domain that is presumably able to bind a crystalline cellulose, whereas *cel3* family members show high similarity to the *KORRIGAN* cellulase gene from *Arabidopsis* (Campillo, *et al.*, 1991). This group of endo-ß-1,4-D-glucanases, characterised by membrane-associated localisation, plays a role in the assembly of the cellulose-hemicellulose network in the expanding cell walls (Robert, *et al.*, 2005). The *cel6* was constructed on the basis of four unigenes and one cDNA clone; (see Tab. 3) and not much is known about the function of EGases from this subfamily.

This study provides insight in the basic characteristics of cellulases from potato, and these insights allow for a better understanding of mechanisms underlying plant gene recruitment by obligatory plant parasitic nematodes.

Material and methods

Plant material and genomic DNA extraction

Genomic DNA was extracted from *in vitro* cultured potato plantlets (line V; (Horsman, *et al.*, 2001) using the DNeasy Plant DNA isolation system (Qiagene, USA) according the manufacturer's instructions. DNA was precipitated by addition of 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volume of 95% EtOH. After 2 h of incubation at -80°C samples were centrifuged for 20 min at maximum speed. The pellet was air dried and redissolved in sterile water.

Isolation of the genomic sequences

Isolation of the genomic sequences was carried out with the help of the Universal GenomeWalker Kit (BD Biosciences Clontech, USA) according to manufacturer's protocol (Siebert, *et al.*, 1995). Two rounds of PCR were performed using the Advantage Genomic Polymerase mix (BD Biosciences Clontech). The primary PCR was performed using Adaptor Primer 1 (AP1) and a Gene-Specific Primer (GSPx, where x is odd number), followed by a second PCR with a nested adaptor-related primer (AP2) and a nested gene-specific primer (GSPy, where y is even number (y=x+1), so each pair x and x+1 are used together)). This procedure was repeated four times, each time with new, more distantly localized GSPs. Primer sequences are shown in Table 3. The parameters used during the primary PCR reactions were as follows: seven cycles [94°C, 2s; 72°C, 3 min] and 32 cycles [94°C, 2s; 65°C, 3 min]; within the last cycle, the PCR samples were incubated for an additional 4 min at 65°C. For the nested PCR, the same profiles were used, however, the number of cycles was different: five cycles with the first profile and 25 cycles with the second profile. The PCR fragments were analyzed on a 1% (w/v) agarose gel and cloned into the TOPO2.1-vector for transformation of TOP10 One Shot Chemically Component *Escherichia coli* cells as described in the manufacturer protocol (Invitrogen, USA). For each transformation, 10 positive colonies were grown at 37°C in 1ml of LB medium supplemented with 50μg/ml kanamycin for selection. Two μl of fresh overnight culture was diluted in 8μl of sterile water, heated at 94°C for 10 min and used as a template to amplify the cloned PCR product(s) with the same primer pair as used before. PCR reaction was performed in a total volume of 25 μl (2.5μl 10xPCR buffer, 2μl dNTPs (2.5mM), 1.5μl of each primer (10uM), 0.25μl *Taq* DNA polymerase, 7.25 MilliQ water and 10μl of bacterial template). The following PCR cycle file was applied: 5 min 95°C followed by 20 s 94°C, 30 s 60°C, 45 s 72°C for 25 cycles and finally 2 min elongation at 72°C. The DNA fragments were separated on 1% agarose gel in TAE buffer at 100V. From positive clones plasmid DNA was isolated using the Wizard®plus Midipreps DNA purification system (Promega, USA) as described in manufacturer's protocol. The DNA was further purified by alcohol precipitation as described above. The colonies were stored in a 15% (v/v) glycerol at -80C°.

	Primers sequence 5' -> 3'
Cel 8F	ACCAGAGAGTTCAATGGAGGGGTAATTC
Cel 8R	TCCACTGACAGATCGGTAGTACTTCTGGG
Cel 7F	CCTATATTGGGGAGCAGCATGGCTATTGAG
Cel 7R	GTACGTTCATCTGGGAAGAAATCATTTTGA

1ˢᵗ Walk	
7 GSP1F	GAGGACCATCTTTACCTTCTTTGGCAATGC
7 GSP2F	GGAGAAACTACCGCGAAGAATTCACCATA
7 GSP1R	CATTTATTAAATATCGGATCGACGGATAAGATTGC
7 GSP2R	CACCACGTGTCCCTATTTAGTCTTCCATTAGAGTG
8 GSP1F	AGTTTGCGGACAAGTACAGAGGCAAGTATGATAG
8 GSP2F	GTGGCCCAGAAGTACTACCGATCTGTCAGT
8 GSP1R	GTGGTCATGTCCTCTGGTCTTTGCCAACAGTA
8 GSP2R	GTCTGTGGTACCATCTCCAACCTACACATTATTTAC

2ⁿᵈ Walk	
7 GSP3R	GCAAGCTGCAGAGCCAAGTGAATCACTATAAGAC
7 GSP4R	CCATGCTGCTCCCCAATATAGGCTCATCCTTATA
8 GSP3F	GGCATCGGCTGGAAAATCCCTCAAATGTGCGTCCGG
8 GSP4F	CCCGAGCTCCTGGACTTTGCCGAGTCACAAG

3ʳᵈ Walk	
7 GSP3F	GGAGAAACTACCGCGAAGAATTCACCATAGAGG
7 GSP4F	GGCAATGCATCCACAATCATTTGGTTGTGAAGCTGG
7GSP4´F	CCCTAACATATTAGTTGGAGCCATTATCGGAGGTCC
8 GSP7F	CCACAGAGAGTTCATAGATGGAAGTCTTCATTTCC
8 GSP8F	CCTTGCAAAATGTTCCCATGGCGTAAGCTCTAC

4ᵗʰ Walk	
7 GSP7F	GCCTGCTACTTACATTAATGCTGCTATTGTTGG
7GSP8F	GTTGTTGTCCAGCTAAAAGGGCAAAGCTGATGC

Sequencing primers	
M13R	CAGGAAACAGCTATGAC
M13F	GTAAAACGACGGCCAG

Table 3:

List of primers.

Sequencing and sequence analysis

DNA sequencing was carried out by Greenomics (Plant Research International, Wageningen, The Netherlands). Sequencing was performed using approximately 1μg DNA template and 100 ng of each standard sequencing primer. DNA sequence analysis, manipulations and alignments were performed using the Vector NTI Advance program package (InforMax, USA). The obtained genomic sequences were compared with the non-redundant NCBI database with the blastn/blastp alignment programs. For the *in silico* identification of putative splice sites in potato genomic DNA, FSPLICE was used while

Nicotiana   tabacum   was   selected   as   reference   organism (http://www.softberry.com/berry.phtml). Conceptual cDNA sequences and selected potato ESTs were compared with the following homologues from tobacco and tomato: *Nt-cel7* (AF362947), *Nt-cel8* (AF362949), *Sl-cel7* (Y11268) and *Sl-cel8* (AF098292). For phylogenetic tree construction, other homologous sequences were obtained from the SOL Genomic   Network   (http://www.sgn.cornell.edu/index.pl)   and   the   TIGR   Database (http://tigrblast.tigr.org/ tgi/). The ClustalW algorithm as embedded in the BioEdit software package (Hall, 1999) was used to align cellulase sequences.

Similarity dendrogram of the *Solanaceae* cellulase gene family

On basis of the sequence alignment, a similarity dendrogram was created by the Modeltest 3.06 and PAUP*,4.0b10 programs (Posada and Crandall 1998; Rogers and Swofford 1998; Swofford 1998). The Modeltest was used to calculate the appropriate substitution model and parameters to be used in the dendrogram construction. Using the result from the Modeltest, a dendrogram was constructed with the program PAUP*v.4.0b10 using the maximum likelihood algorithm and implementing the GTR model (Rodriguez, Oliver, *et al.* 1990), with a gamma parameter value. The dendrogram was bootstrapped 100 times and bootstrap-values of 70 or higher were considered reliable, according to Hillis and Brull (1993).

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Chapter 6

Discussion and Final conclusions

Plants and microorganisms have been co-evolving since the period of the Upper Silurian and Lower Devonian approximately 400-425 millions years ago, when the first vascular plants emerged. Ever since plants have developed (sophisticated) defensive mechanisms, and the plant parasites, on the other hand, have tried to counteract or circumvent the host's adaptive changes. Sedentary endoparasitic nematodes such as cyst (CN; *Globodera* spp and *Heterodera* spp) and root-knot (RKN; *Meloidogyne* spp) nematodes are the perfect examples of highly adapted plant parasites. Once these nematodes have established a feeding site, they have a more or less guaranteed access to food and water from their host plant.

Cloning and the characterization of CWDEs of nematode origin

Both for cyst and root-knot nematodes, parasitism can be divided into two major steps: (1) invasion and migration through the host root tissue and (2) establishment of a feeding site. During the penetration and migration a nematode has to overcome a protective barrier: the plant cell wall. The plant cell wall is a rigid structure composed of cellulose, hemicellulosic and pectic polysaccharides with smaller amounts of glycoproteins and phenolic compounds. Some of the polymers that make up the plant cell wall are among the most complex structures known (Northcote, 1972). Plant cells are at the forefront of plant-pathogen interactions. This is the first barrier encountered by the pathogen, which must be overcome in order to access the host plant. In addition to a stylet, a protrusible puncturing device, nematodes use a mixture of polysaccharide-degrading enzymes for the successful invasion of the host root tissue. Unlike the most of other herbivores, plant parasitic nematodes do not depend on symbiotic microorganisms for the production of cell wall-degrading or modifying proteins. Support for this assumption was first provided by the detection of the endogenous production of the cellulases by cyst and root-knot nematodes (Ding, *et al.*, 1998; Smant, *et al.*, 1998). Cellulase transcripts were detected predominantly in the subventral glands of the mobile pre-parasitic J2 stage (De Boer, *et al.*, 1999; Rosso, *et al.*, 1999) supporting the hypothesis that both cyst and root-knot nematodes produce cellulolytic enzymes to facilitate the migration though the host tissue. Moreover, the potato cyst nematode *Globodera rostochiensis* was the first animal shown to produce a functional pectate lyase enzyme. These cell wall-degrading enzymes (CWDE) are secreted by infective second stage juveniles, and assist the migration through the host roots tissue (Popeijus, *et al.*, 2000). Cyst nematodes were also shown to produce and secrete expansins; proteins without enzymatic activity that make the main wall polymers more accessible to CWDEs (Qin, *et al.*, 2004). Expansins are a relatively new group of proteins and were till recently thought to be exclusively produced by the terrestrial plants (Cosgrove, 2000).

Plant endo-β-1,4-D-glucanases: their roles in plant development and nematode parasitism

The primary cell wall of dicotyledons consists of cellulose microfibrils embedded in a pectic and hemicellulosic matrix. The major hemicellulose in dicots is xyloglucan. Xyloglucan has a backbone composed of 1,4-linked β-D-glucose-pyranosyl residues and coats the surface of cellulose microfibrils, limits their aggregation and connects them via tethers that directly or indirectly regulate the mechanical properties of the cell wall. The cell wall is a dynamic structure whose composition and structure must constantly respond to the changes in growth, differentiation and the environment of the cell (Northcote, 1972). Those dynamic changes require the activity of multiple cell wall modifying enzymes. Plant endo-β-1,4-D-glucanases catalyze the endo hydrolysis of β-1,4 linked glucan residues, such as those that make up the xyloglucan backbone (Brummell, *et al.*, 1994) and therefore breaks the xyloglucan backbone.

Primary sequence comparison and hydrophobic cluster analysis have revealed that the catalytic core of bacterial, fungal and plant endo-β-1,4-glucanases can be grouped into families that share catalytic properties. Plant cellulases identified so far all belong to glycoside hydrolase family 9 (GH9, formerly known as family E), a family comprising cellulases from bacteria, plants, fungi and animals. Unlike bacterial, fungal and animal endo-β-1,4-D-glucanases, most plant cellulases solely consist of a catalytic core and lack a cellulose-binding domain. However, plant genome sequencing projects showed several carbohydrate binding module (CBM)-harbouring cellulases in *Arabidopsis* and rice. Among *Solanaceous* plants, tomato Cel8 was the first cellulase with a CBM, and recently a few more representatives have been cloned and annotated in the databases (Catal and Bennett, 1998; Trainotti, *et al.*, 1999). This particular group was postulated to degrade crystalline cellulose, a non-degradable substrate for "normal" plant cellulases.

The activity of plant cellulases has been detected in association with several developmental processes where changes in cell wall components occur such as cell expansion, abscission and fruit ripening (Brummell, *et al.*, 1999; Ferrarese, *et al.*, 1995; Inouhe, *et al.*, 2000). In general three categories of plant endo-β-1,4-D-glucanases can be distinguished. One group is found mainly in rapidly-growing tissue and is induced by auxin. For example the tomato *Cel7* was induced to high levels in growing plants upon application of auxin (Catala, *et al.*, 1997). Using the ethylene-insensitive tomato mutants Nr and *dgt* (*diageotropica*), an auxin-insensitive tomato mutant, it was shown that *Cel7* transcript accumulation by exogenous auxin application was auxin-specific, and not caused by ethylene production. The second group of plant endo-β-1,4-D-glucanases is found in aging tissues such as abscission zones and fruit and is induced by ethylene. The increase in EGase activity

has been related to abscission zones in avocado. The levels of avocado Cel1 increased more than 50-fold during fruit ripening (Cass, *et al.*, 1990). The growth of *Arabidopsis thaliana* with a mutation in the member of the third group of plant EGases, KORRIGAN (KOR) gene was stunted in most organs, plants had irregular patterns of cell expansion (Nicol, *et al.*, 1998). More detailed studies revealed that this group of EGases is hormone insensitive, membrane associated and is presence is required for the synthesis of the cellulose microfibrils (Brummell, *et al.*, 1997; Robert, *et al.*, 2005).

The second step in plant parasitism by nematodes is the induction and maintenance of the feeding structure. Cyst nematodes induce a so-called syncytium which is an expanding multinuclear complex characterised by progressive cell wall breakdown. The syncytium expands towards the vascular bundle and from there it expands along the xylem vessels. Up to 200 cells can be integrated into expanding syncytium (Golinowski, *et al.*, 1997). Root-knot nematodes, however, turn single plant cells into a multinuclear structure called giant cells by triggering repeated rounds of mitosis without cytokinesis (Jones and Northcote, 1972). Despite different ontogeny and highly dissimilar structure, both structures have several characteristics in common multiple: nuclei, proliferation of cellular organelles, enlarged nuclei and increased cytoplasmic density indicating high metabolic activity.

The proliferation of the nematode-induced feeding cells coincides with extensive modifications of the plant cell wall(s). The uniform structure of the syncytia suggests that the process is highly organised and precisely controlled. The enzymes involved in this extensive re-arrangement of syncytial walls might originate either from the nematode or from the host plant. However, the highly coordinated and selective incorporation of plant root cells make it unlikely that nematode cell wall-modifying proteins are used for syncytium proliferation. If the nematode would have injected cellulases into the initial syncytial cell, cell wall dissolution would occur in all directions and this is not observed. Cell wall dissolution starts preferentially at the specific sites in the cell, in the vicinity of plasmodesmata (Grundler, *et al.*, 1998). Unlike the syncytia, giant cell induced by the root-knot nematodes are not formed by the cell wall dissolution of the adjacent cells. However, in order to accommodate in size and shape, giant cells and the surrounding cell walls have to be extensively modified.

In this thesis, we show that the plant cyst nematode *Globodera rostochiensis* - apart from producing endo-β-1,4-glucanases that facilitate its migration (De Boer, *et al.*, 1999; Smant, *et al.*, 1998) - is employing plant genes in order to establish successful feeding structure. The tomato cellulase family consists of at least eight members. RT-PCR experiments, hybridisation experiments and immunolocalisation studies revealed that only two isoforms, *Sl-Cel7* and *Sl-Cel8*, are consistently up-regulated in syncytia induced by *Globodera rostochiensis* (this thesis, Chapter 4). Transgenic potato plants carrying hpRNA-silencing constructs for either *cel7* or *cel8* were *de facto* resistant to nematodes, showing that those enzymes are indispensable for syncytium establishment and development. Interestingly,

tobacco *cel7* and *cel8* were also shown to be up-regulated in feeding sites induced both by cyst (*Globodera tabacum*) and root-knot nematodes. These findings suggest that similar mechanisms are involved in the syncytium and giant cell proliferation. On the other hand, the promoter of *Arabidopsis thaliana CEL1* was exclusively induced in giant cells of *M. incognita*. *Cel1*-driven *gus* expression was detectable at the very onset at parasitism, within the developing giant-cell and no expression was found in syncytia at any time point (Goellner, *et al.*, 2004). Apparently, there are *cis*-elements in the *At-cel1* promoter that are up-regulated exclusively by root-knot nematodes. In tobacco, both root-knot and cyst nematode infection resulted in the local accumulation of transcripts for five EGases, including the tobacco equivalents of *Sl-cel7* and *Sl-cel8*. Apparently, the promoters of *cel7* and *cel8* in tobacco possess *cis* elements that are activated by both cyst and root-knot nematodes (Goellner *et al.*, 2001) Hence, the pattern of plant cellulase recruitment by cyst and root knot nematodes could provide us with a lead towards the identification of promoter elements that are responsive to cyst and root knot nematodes, and to elements that are specifically activated by *Meloidogyne* spp.

EGases are not the only plant cell wall-modifying proteins involved in the feeding site development. Cyst and root knot nematodes also use α and β expansins. *Sl-Exp5* α expansin was up-regulated in the gall cells adjacent to the feeding cell of *M. incognita* (Gal, *et al.*, 2006) and in the syncytial cells of *G. rostochiensis* (Sobaczak, unpublished). Furthermore the expression of β expansin *Sl-EXPB1*, *Sl-EXPB2* and *Sl-EXLA1* and expansin-like genes is up-regulated between 3 and 14 days post infection which parallels the expansion of the feeding site (Kudła, PhD thesis 2006). Additionally pectinmethylesterases were found to be up-regulated in and around both feeding structures (Vercauteren, *et al.*, 2002). Based on those findings it is reasonable to assume that so far identified proteins are just a small fraction of the complex group of cell wall modifying agents that are employed by plant parasitic nematodes.

The regulation of cell wall remodelling in morphogenesis of feeding cell

The intriguing question remains how nematodes direct the extensive re-modeling of the plant cell walls during induction of their feeding structure. Both root knot and cyst nematodes select a certain plant cell as a starting point for the feeding site development. However, up till now, the presumed signal transduction pathways that lead to the re-differentiation of the root cell into a feeding cell remains poorly understood. It is believed that proteins produced in the dorsal esophageal gland of infective second stage juveniles trigger the observed changes. The presence of auxins or auxin analogs in nematode saliva of both cyst and root-knot nematodes has been suggested in a number of papers, but the data published so far are inconclusive (reviewed by Goverse, 2000b). Chorismate mutase (CM), a protein that could

potentially affect IAA biosynthesis, was cloned from *M. javanica* (Lambert, *et al.*, 1999). MjCM-1 was produced in the esophageal glands and secreted into the host plant at the onset of parasitism (Doyle and Lambert, 2003). Transgenic expression of MjCM-1 in soybean hairy roots resulted in a phenotype that could be rescued by the application of IAA. The authors suggested that a local reduction in IAA biosynthesis could underlie feeding site induction. This hypothesis is apparently contradictory with various lines of evidence that point at a local accumulation of auxin in early feeding cell development (Goverse, *et al.*, 2000b; Karczmarek, *et al.*, 2004). The expression of auxin-inducible genes such as the *Arabidopsis* ribosomal protein S5 (Goverse, *et al.*, 2000b; Weijers, *et al.*, 2001) in early syncytium development, and the observation that the auxin insensitive tomato mutant *diageotropica (dgt)* is *de facto* resistant to both the potato cyst nematode *G. rostochiensis* (Goverse, *et al.*, 2000b) and the root-knot nematode *M. incognita* (Richardson & Price, 1984) suggest a role for auxin in feeding cell development. In Chapter 2 we showed that the auxin responsive DR5 element is activated, at the very onset of parasitism, by cyst and root-knot nematodes. DR5 activity was observed not only in the feeding site but also in the surrounding cells, suggesting that high auxin levels preconditions cells prior to the incorporation into the syncytium. In case of giant cells, surrounding cells are not incorporated in the nematode feeding structure, however high activity of the DR5 element could be observed in developing giant cells. Interestingly, tomato *cel7* is involved in the re-modification of the cell walls in both nematode feeding structures and is also induced upon application of auxin (Catala, *et al.*, 1997). The expression of *Cel8* is also up-regulated in both structures. Unfortunately little is know about the transcriptional and hormonal regulation of the *cel8* expression. Regulation of α and β expansin expression by the phytohormone auxin has been reported (Downes, *et al.*, 2001; Hutchison *et al.*, 1999). Interestingly, also these cell wall loosening proteins proved to be present in both syncytia and giant-cells (Kudła, unpublished, Wieczorek, unpublished). This prompted me to assume that both cyst and root knot nematode-induced cell wall modifications are, at least partly, mediated by a local increase of the auxin level.

Ethylene is another plant hormone that may play a significant role in nematode-plant interaction. Ethylene-overproducing *A. thaliana* mutants were shown to be hypersensitive for cyst nematode infections (Wubben, *et al.*, 2001). Ethylene activates the cell wall break down in the aging tissues by activation of cell wall degrading enzymes (Jiang and Fu, 2000). Moreover, ethylene can induce DNA endoreduplication and can modify the cell shape via changes in the cytoskeleton (Shibaoka, 1994). Interestingly, 1-aminocyclopropane-1-carboxylate (ACC) oxidase (ACO) and syntase (ACS), the key enzymes in ethylene biosynthesis (Fluhr & Mattoo, 1996), were found to be up-regulated in the nematode induced syncytium (Pierzgalska, unpublished). Auxin induced the expression of some of those enzymes (Bekman, *et al.*, 2000; Saito, *et al.*, 2005; Shiomi, *et al.*, 1998; Woltering, *et al.*, 2005), therefore syncytium development is probably accompanied by auxin-induced ethylene

production. Numerous studies have shown that small peptides signaling play an important role in the various aspects of plant growth and development. Those peptides are mostly secretory and anticipate in the cell-to cell signaling (Matsubayashi and Sakagami, 2006). In tomato, the lateral root primordia ar flanked by regions of the vascular bundle expressing *Sl-ENOD40* (Vleghels, *et al.,* 2003). Interestingly, in many endoparasitic nematode – host plant interactions, feeding cell establishment is associated with lateral root formation (Goverse, *et al.*, 2000a and papers cited therein). In the interaction between *Medicago truncatula* and the root knot nematode *Meloidogyne incognita*, *ENOD40* expression was found in (Koltai, *et al.*, 2001) or exclusively around (Favery, *et al.*, 2002) giant cells, and a possible overlap in plant signal transduction pathways underlying nodule formation and nematode-induced feeding site formation was suggested. However, when tomato roots were infected with either root knot or cyst nematodes such a correlation could not be found. The expression of tomato *Sl-ENOD40* was unaffected by cyst or root-knot nematodes infections, and this held both for the nematode feeding structure and for the tissues immediately surrounding it. A possible explanation for this discrepancy could be the difference in regulation of *ENOD40* expression in legume and non-legume plants.

Resistance

In an attempt to control the populations of the plant parasitic nematodes researchers focused on cloning and characterisation of the naturally occurring resistance gene(s). The first introduced resistance gene into cultivars of tomato was the *Mi* gene, conferring the resistance against root-knot nematodes *M. incognita*, *M. arenaria* and *M. javanica*. This gene was introduced in 1940s and is used up to now in the commercially available tomato cultivars (Smith, 1944). In 1992 the Mi-1.2 gene was finally mapped and cloned and the coding region was analysed by the transformation of the susceptible tomato line. The transfer of the coding DNA sequence of the Mi gene conferred a resistance in the analysed tomato plants (Ho *et al.*, 1992). This raised the possibility to transfer the *Mi* gene to another plant species, however when tobacco plants - fairly close relatives of tomato - were transformed with the tomato *Mi* gene, no resistance was found. The hypersensitive response in tomato is triggered by an extensive signal-transduction pathway and presumably the *Mi*-encoded protein will only trigger the desired resistance response in case the subsequent pathway is highly similar to the one in tomato. In other words, the cloning of the *Mi* gene did not open the door for a massive introduction of root knot nematode-resistance in other crops. Hence, proteins encoded by natural host plant resistance genes are only the starting point of a non-conserved signal-transduction pathway finally leading to a hypersensitive response. Consequently successful transfer of host plant resistance genes will be restricted to (very) closely related host plants.

In engineering of artificial resistance there are essentially two targets: the nematode itself or its feeding structure. Up till now, a range of different anti-nematode components have been tested, however the most successful attempt has been through the expression of protease inhibitors (Atkinson, *et al.*, 2003). Cysteines, however had been showed to have an toxic effect on non-target invertebrates (Cowgill, *et al.*, 2002) and therefore this approach raised serious concerns about its environmental safety.

Another possibility is to target the nematode feeding cell. It can be achieved in two different ways: by the expression of toxic components (Jung and Wyss, 1999) or by hampering the recruitment of plant genes indispensable for the feeding site induction and development (De Almeida Engler, *et al.*, 2005). Feeding site is the only nutrition source for the developing parasite, and the reproduction success of nematode depends on fully developed feeding site. A substantial part of genes that are essential for feeding site development is also essential for the host plant. Hence, manipulation of such genes will most likely give unpredictable negative effects on plant development. However, manipulation of susceptibility genes that are members of gene families could have minor or no effects to plant fitness. In Chapter 4 we explore the possibility of controlling the population of cyst nematodes by the silencing of two genes, *St-cel7* and *St-cel8* that are essential for syncytium induction in the absence of an obvious aberrant plant phenotype. Interestingly, in the parasitism of tobacco cyst nematode (*G. tabacum) Nt-cel7* and *Nt-cel8* are induced in the infected roots (Goellner, *et al.*, 2001). Syncytia share the same morphological characteristics regardless the nematode-host combination. It is then reasonable to state that this approach could be applicable as well for the controlling of other cyst nematodes such as the soybean cyst nematode *Heterodera glycines* and the beet cyst nematode *Heterodera schachtii*. More interestingly, tobacco roots infected with root-knot nematode *M. incognita* showed also an increased expression of c*el7* and *cel8 (*Goellner, *et al., 2001)*. Hence, it would be worthwhile to investigate the effect of cellulase silencing on the *M. incognita* development. If distinct nematode species recruit similar sets of genes it is reasonable to speculate that by knocking out of such a gene(s) it is possible to engineer plants resistant to a wide spectrum of different parasitic nematodes. In order to accommodate a set of giant cells the surrounding cell walls have to be extensively modified. The other advantage of this system is that it does not require any detailed knowledge about the gene function as long as the gene is indispensable for feeding site development and silencing has no, or a negligible effect on plant fitness.

Despite the fact that dsRNA technology still requires some improvements and additional tests, it seems to be the most promising approach to engineer safe and durable artificial resistance. One of the improvements could be the use of feeding-site specific promoters that could deliver dsRNA species in a restricted and directed manner. That would minimize the chance of undesirable effects of silencing on the plant fitness. The rolD promoter from *Agrobacterium rhizogenes* (Elmayan and Tepfer, 1995) that was tested in

tomato by Grichko *et al.,* (2005) could be a reasonable alternative. Recently, more specific promoter elements were identified in *Medicago truncatula* and in *M. sativa,* namely *MtPT1* and *MsPRP2 (*Winicov, *et al.,* 2004; Xiao, *et al.,* 2005*).* Properly targeted prevention of the recruitment of susceptibility factors by a pathogen could be a highly specific and relatively bio-safe approach for pathogen and pest management.

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Podsumowanie

Nicienie są bezkręgowymi robakami obłymi, które stanowią jedną z najliczniejszych grup zwierząt komórkowych. Większość nicieni są to wolnożyjące organizmy, aczkolwiek niewielka grupa przystosowała się do pasożyniczego trybu życia. Nicienie, z rodzaju mątwików i guzaków są przykładem pasożytów roślin, które stanowią poważne zagrożenie w uprawach. Celem niniejszej pracy badawczej było scharakteryzowanie oraz lepsze zrozumienie interakcji zachodzących pomiędzy pasożytniczymi nicieniami, mątwikiem ziemniaczanym (*Globodera rostochiensis*) i guzakiem południowym (*Melodogyne incognita*), a ich roślinnymi żywicielami z rodziny psiankowatych (*Solanaceae*). Mątwik ziemniaczany poraża tylko i wyłącznie rośliny z rodzaju psiankowatych, *Solanum lycopresicon* (pomidor) oraz *Solanum tuberosum* (ziemniak), guzak jest natomiast typowym pasożytem poliksenicznym, może porażać wiele roślin z różnych niespokrewnionych ze sobą rzędów.

Mątwiki (nicienie cystowe) są bardzo poważnym zagrożeniem w uprawach, w skali światowej wyrządzają szkody, które ocenia się na około 400 miliardów złotych. Mątwik ziemniaczany jest organizmem kwarantannowym i może spowodować straty plonu nawet do 80%. Cykl życiowy mątwika ziemniaczego rozpoczyna się w glebie, gdzie pod wpływem substancji wydzielanych z korzenia roślin psiankowatych, z cyst wykluwają się larwy infekcyjne. Larwa infekcyjna zwana J2 migruje w kierunku korzenia rośliny żywicielskiej i następnie wewnątrz korzenia przemieszcza się w kierunku wiązki przewodzącej. Podczas tej wewnątrzkomórkowej wędrówki pasożyt musi pokonać barierę, jaką stanowi ściana komórkowa. W tym celu tym larwa infekcyjna jest wyposażona w sztywny przypominający igłę iniekcyjną, sztylet. Prócz sztyletu, który umożliwia mechaniczną perforację ściany komórkowej, nicienie produkują enzymy, które depolimeryzują ścianę komórkową. Po dotarciu do wiązki przewodzącej, larwa wybiera jedną komórkę, którą przekształci w strukturę odżywczą zwaną syncytium. Syncytium jest jedynym źródłem pokarmu dla rozwijającego się pasożyta, dlatego rozwój i sukces reprodukcyjny mątwika jest całkowicie uzależniony od prawidłowego funkcjonowania tejże struktury. Syncytium jest tworem wielokomórkowym, który powstaje w wyniku zlewania się protoplastów sąsiadujących komórek, za pomocą otworów powstających w drodze rozpuszczania ścian komórkowych. Proces ten jest ściśle kontrolowany, syncytium rozszerza się w kierunku wiązek przewodzących i około 200 komórek zostaje wchłonięte w rozwijające się syncytium. Ponadto rozwój, syncytium związany jest ze zmianami histologicznymi w budowie komórek do niego włączonych: system wakuolarny ulega zupełnej przebudowie, następuje proliferacja mitochondriów, rybosomów, plastydów i retikulum endoplazmatycznego oraz kondensacja cytplazmy, co jest oznaką wysokiej aktywności metabolicznej tej struktury. Następnie, po kilkukrotnym linieniu larwy przekształcają się bądź w samce, (jeśli syncytium nie dostarczyło wystarczającej ilości pożywienia) bądź w samice. Samce odrywają się od rośliny i zapładniają samice, które przytwierdzone są do korzenia za pomocą aparatu gębowego. Po zapłodnieniu samica składa jaja do wewnątrz, następnie obumiera i przekształca się w ochronną cystę o cytrynkowatym kształcie. Cysty te są niesamowicie odporne na działanie czynników zewnętrznych i mogą przetrwać w stanie uśpienia wiele lat.

Cykl życiowy guzaków przypomina cykl, który przechodzą nicienie cystowe, aczkolwiek nie można zapomnieć o kilku bardzo ważnych różnicach. Larwy guzaków wylęgają się z wyrośli, które wytworzone są przez partenogenetycznie rozmnażające się samice. Wyrośla te stanowią galaretowatą masę, w której zawieszone są jaja. Po wylęgnięciu z jaja, larwa inwazyjna migruje w kierunku rośliny żywicielskiej. Podobnie jak w przypadku mątwików, larwa wyposażona jest w sztylet. Korzystając z mechanicznej siły szyletu i enzymatycznej depolimeryzacji ścian komórkowych, larwa wędruje wewnątrz korzenia. Następnie wybiera odpowiednią komórkę, która przekształca się w tak zwaną komórkę ogromną. Komórka ogromna powstaje wskutek powtarzających się cyklicznie podziałów komórkowych, w których nie dochodzi do cytokinezy. Rezultatem jest, więc komórka zawierająca liczne jądra i jak w przypadku syncytiów charakteryzująca się zwiększoną kondensacją cytoplazmy i proliferacja komórkowych organelli. Następnie, po kilku linieniach, larwa przekształca się w dojrzałą samicę, która produkuje galaretowatą masę zawierającą setki jaj. W ten sposób cykl się zamyka.

Auksyna jest jednym z podstawowych hormonów roślinnych. Wiele prac naukowych wskazuje na potencjalną rolę tego hormonu w inicjacji i rozwoju syncytium oraz komórek ogromnych. Aczkolwiek większość z tych obserwacji to wyłącznie obserwacje pośrednie. W związku z tym, badania przedstawione w rozdziale drugim niniejszego opracowania naukowego, mają na celu uzyskanie bezpośredniego dowodu eksperymentalnego na zaangażowanie auksyny w rozwój struktury odżywiającej. W celu tym użyto promotora DR5, którego ekspresja monitorowana była za pomocą genu reporterowego *gus*. Ogólna zasada działania genu markerowego polega na tym, że syntetyzowane jest białko, którego efekty działania można bezpośrednio obserwować. W tym przypadku zastosowano GUS, który jest enzymem przekształcającym bezbarwne związki chemiczne w produkty o łatwo zauważalnej barwie niebieskiej. DR5 jest promotorem, który aktywuje ekspresję genów w obecności śladowych ilości auksyn. Nawet najmniejsze zmiany w koncentracji tego hormonu są rejestrowane w postaci zmiennej aktywacji tego promotora, w tym przypadku monitorowane jako różne natężenie niebieskiego koloru w komórkach. W ten sposób można uzyskać szczegółowe informacje na temat miejscowo-czasowej aktywności promotora DR5. Transgeniczne rośliny *Arabidopsis thaliana* zawierające konstrukt DR5 promotor::*gusA*, zostały porażone mątwikami i guzakami. W obydwu przypadkach zaobserwowano aktywację promotora już w 18 godzin po infekcji roślin pasożytami. Ta wysoka ekspresja zauważalna była przez następne 3 do 5 dni. Obserwacja ultra cienkich skrawków z zainfekowanych roślin potwierdziła obserwacje makroskopowe. Niebieska barwa była obserwowana w komórkach inicjalnych obydwu struktur. Dodatkowo w przypadku mątwikow aktywacja promotora była zauważalna również w komórkach, które zostaną wchłonięte w rozrastające się syncytium. Ta specyficzna aktywacja promotora DR5 może być dwojako wytłumaczona: pod wpływem infekcji nicieniami auksyna jest akumulowana, bądź wzrasta wrażliwość komórek na auksynę. Aczkolwiek bazując na doświadczeniach opisanych w literaturze, akumulacja auksyny wydaje się być bardziej prawdopodobna.

W rozdziale trzecim przedstawiono dokładna analizę ekspresji *ENOD40* genu w korzeniach roślin niemotylkowych porażonych mątwikami i guzakami. *ENOD40* gen należy

do rodziny genów, które aktywowane są w roślinach motylkowych podczas symbiozy z bakteriami brodawkowymi. Aczkolwiek, dokładne badania na roślinach niemotylkowych ujawniły, że gen ten występuje również u roślin niemotylkowych. W obydwu przypadkach, ekspresja *ENOD40* zlokalizowana jest w tkance przewodzącej. Rozwój struktur odżywczych nicieni jest również związany ze zmianami histologicznymi w obrębie tkanki przewodzącej, więc ekspresja tego genu może być potencjalnie aktywowana w korzeniach roślin porażonych nicieniami. I rzeczywiście, eksperymenty wykonane z użyciem guzaków i roślin motylkowych potwierdziły te przypuszczenia. W pomidorze (roślina niemotylkowa), użytym w doświadczeniu opisanym w tym rozdziale, nie zanotowano jednakże żadnych zmian w ekspresji genu *ENOD40*, zarówno w korzeniach porażonych mątwikami jak i w korzeniach porażonych guzakami. Ponadto aktywacja promotora genu *ENOD40*, monitorowana za pomocą genu reporterowego gus, obserwowana była wyłącznie w miejscach charakterystycznych dla endogennej ekspresji. Obserwacje potwierdzono mikroskopowo, za pomocą analizy ultra cienkich skrawków z zainfekowanych korzeni pomidora. Podobne rezultaty uzyskano obserwując aktywację promotora soi, rośliny motylkowej (*Gm-ENOD40*) w porażonych transgenicznych pomidorach. Dodatkowo w odróżnieniu do eksperymentów przeprowadzonych na roślinach motylkowych, nadekspresja promotora *Gm-ENOD40* w *A. thaliana*, nie miała żadnego wpływu na rozwój nicieni. Doświadczenia przedstawione w tym rozdziale sugerują, że w przypadku roślin niemotylkowych, ekspresja genu *ENOD40* nie ulega zmianie podczas infekcji pasożytami. Te zaskakujące wyniki być może są spowodowane różną regulacją ekspresji genu *ENOD40* w roślinach motylkowych i nie-motylkowych.

W morfogenezie syncytium, strukturze odżywczej mąwików, aktywowane są enzymy roślinne, które katalizują lokalny rozkład ścian komórkowych. Dzięki temu powstają otwory między sąsiadującymi komórkami, które w ten sposób zostają włączone w rozwijające się syncytium. Za ten proces odpowiedzialne są enzymy degradujące ścianę komórkowa, między innymi, endo-1,4-ß-glukanazy (celulazy). Analiza ekspresji tych genów z użyciem RT-PCRu, hybrydyzacji *in situ* oraz immunolokalizacji (przy użyciu specyficznych przeciwciał) w korzeniach pomidora porażonego przez mątwika ziemniaczanego, wykazała specyficzną indukcję dwóch izoform, *Sl-cel7* i *Sl-cel8*. Następnym krokiem było wytworzenie roślin transgenicznych, posiadających antysensowne konstrukty genowe przeciwko *cel7* i *cel8*. Tak przygotowane rośliny (ziemniaki), zostały następnie porażone mątwikami. Analiza danych wykazała zahamowanie rozwoju mątwików w wyciszonych roślinach ziemniaka. To doświadczenie udowodniło, że mątwiki w bardzo specyficzny sposób wykorzystują geny żywiciela roślinnego w celu ukształtowania w pełni funkcjonalnego syncytium. Ponadto, doświadczenie to pokazało, że dokładna wiedza na temat rozwoju syncytium może być przydatna w uzyskiwaniu roślin odpornych na nicienie.

W piątym rozdziale opisana jest indentyfikacja celulaz produkowanych przez ziemniaka. Badania skoncentrowane były na sklonowaniu *cel7* i *cel8*, ponieważ te dwie izoformy są aktywowane podczas procesu infekcji. Pierwszym krokiem była analiza bazy danych roślin psiankowatych, która umożliwiła identyfikację dwóch sekwencji charakteryzujących się dużą homologią do sekwencji pomidora, *Sl-cel7* i *Sl-cel8*. Sklonowane

pełne sekwencje genomowe zostały zanalizowane i analiza tychże sekwencji sugeruje obecność kilku (co najmniej dwóch) izoform *cel8* w genomie ziemniaka, w związku z tym, sklonowana sekwencja została nazwana *cel8a*. Następnie pełne ramki odczytu zostały przetłumaczone na sekwencję aminokwasową. Enzymy te składają się z części katalitycznej, CEL8a dodatkowo zawiera CBM (ang. carbohydrate binding module) domenę, która umożliwia hydrolizę krystalicznej celulozy. Dane te wskazują na to, że roślinne celulazy, które są wykorzystywane przez mątwiki, są w stanie hydrolizować ksyloglukan i cellulozę, w związku z tym tłumaczą, dlaczego te specyficzne izoformy są aktywowane podczas inicjacji syncytium. Ponadto sekwencje celulaz z pomidorów, tytoniu i ziemniaka użyte zostały do przeszukiwania bazy danych roślin psiankowych, co pozwoliło identyfikację wielu nowych, potencjalnych sekwencji celulazowych i umożliwiło stworzenie drzewa filogenetycznego, ukazującego relacje międzygatunkami

Samenvatting

Nematoden - "aaltjes" in gewoon Nederlands - zijn relatief kleine wormvormige organismen die in zeer grote aantallen in vrijwel iedere bodem voorkomen. "Het grote publiek" is nauwelijks op de hoogte van het bestaan van deze dieren, omdat ze behalve klein (veelal kleiner dan 1 mm), ook transparant en min of meer kleurloos zijn. Dit is niet verwonderlijk want deze organismen leven in de bodem, een habitat waarin licht geen rol van betekenis speelt. Dit betekent dat het hebben van een kleur geen functie heeft; voor de goede orde, nematoden hebben ook geen ogen (hoewel er soorten bekend zijn die waarschijnlijk licht kunnen waarnemen). Nematoden nemen – door hun aantal en door hun alomtegenwoordigheid – een belangrijke plaats in het bodemvoedselweb. De meeste nematoden zijn bacterie-eters, maar daarnaast zijn er ook schimmel-eters, algen-eters en "carnivoren" - nematoden die leven van andere nematoden. Parasitaire nematoden vormen een minderheid binnen de aaltjesgemeenschap. Desondanks geniet deze groep de grootste bekendheid. Zowel mensen als dieren kunnen last hebben van nematoden. Aarswormen (*Enterobius vermicularis*) zijn een vrij bekend verschijnsel dat jaarlijks voorkomt bij duizenden (veelal) kinderen in Nederland. De nematoden lijken op stukjes wit garen in de uitwerpselen en zijn hinderlijk (je krijgt er jeuk van), maar vrijwel onschadelijk. In de tropen komen een aantal nematodensoorten voor die veel kwalijker gevolgen hebben voor de gezondheid.

Naast dierparasieten hebben zich in de loop van de evolutie ook plantenparasitaire nematoden ontwikkeld. Deze nematoden zijn in z'n algemeenheid te herkennen aan een spijkerachtig structuur in de kopregio. Deze stekel, die kan worden uitgestoken en weer worden ingetrokken, wordt gebruikt om de plantencelwand te doorboren. Men dient zich hierbij te realiseren dat voor een klein organisme als een nematode de stugge plantencelwand een grote barrière is. Mede dankzij onderzoek dat gedaan is door het WU - Laboratorium voor Nematologie weten wij dat het binnendringen van de plant mogelijk gemaakt wordt door het prikken van een serie gaatjes in de plantencelwand (te vergelijken met "prikken", een activiteit die vroeger populair was op kleuterscholen) in combinatie met de activiteit van celwandafbrekende enzymen die door de nematode wordt uitgescheiden.

Aardappelcystenaaltjes, een term waarmee twee soorten aangeduid worden, *Globodera rostochiensis* en *G. pallida*, zijn in Nederland vooral bekend als veroorzakers van aardappelmoeheid. Aardappelmoeheid is een interessante term omdat het aangeeft hoe deze parasiet te werk gaat: de waardplant wordt niet gedood, maar langzaam uitgeput. Als resultaat hiervan groeit de plant matig en is ie erg gevoelig voor stress. Echter, infectie door een aardappelcystenaaltje zal 'an sich' niet leiden tot de dood van de waardplant.

Wat doet het aardappelcystenaaltje in de waardplant? Nadat de infectieuze larve de wortel is binnengedrongen gaat "het" - het juveniel is op dat moment noch een mannetje,

noch een vrouwtje - op zoek naar een geschikte cel van waar uit een voedingscel gevormd kan worden. Dit is opmerkelijk en biologisch gezien een bijzonder gegeven: de parasiet induceert de vorming van zijn/haar eigen voedingsbron. Na selectie van een initiële voedingscel activeert de nematode plantenenzymen en die zorgen ervoor dat er gaten ontstaan tussen de initiële voedingscel en aangrenzende cellen. Zodoende ontstaat er een geheel van enige honderden plantencellen. Opmerkelijk hierbij is dat plantencellen die zich in een soort rusttoestand bevonden geactiveerd worden; de centrale vacuole verdwijnt, er komen meer organellen en het cytoplasma wordt dichter – kortom de voedingswaarde van deze gefuseerde cellen, dat ook wel aangeduid wordt met de term "syncytium", wordt onder invloed van de nematode enorm vergroot. Hierbij moet opgemerkt worden dat de voedingsstructuur verbonden worden met het vaatsysteem van de plant, en op deze manier kan de nematode nog meer voedsel aan de plant onttrekken. Als de nematode erin slaagt om een goed functionerende voedingscel te induceren zal de larve uitgroeien tot een vrouwtje, maar als de voedingscel kleiner is en/of minder goed functioneert zal het zich ontwikkelen tot een mannetje.

Dit proefschrift gaat over plantenfactoren die betrokken zijn bij de vorming van de voedingscel door de nematode. In hoofdstuk 2 wordt de betrokkenheid van het plantenhormoon auxine (indolazijnzuur) nader onderzocht. Auxine wordt voor het overgrote deel niet in de wortels gemaakt, maar in de bovengrondse delen. Voor het transport wordt, in tegenstelling tot wat je zou kunnen denken, niet het vaatweefsel gebruikt; auxine wordt van cel tot cel getransporteerd. Eerder onderzoek heeft uitgewezen dat manipulatie van dit hormoon een essentiële gebeurtenis is in de cascade die uiteindelijk leidt tot voedingscelvorming. Het direct monitoren van auxine in de plant is technisch erg lastig, het betreft hier een klein molecuul dat in zeer lage concentraties actief is. Daarom is in dit proefschrift ervoor gekozen om een promotor (een stuk DNA dat bepaalt of een gen wordt afgelezen) te gebruiken die heel specifiek geactiveerd wordt door auxine; namelijk de DR5 promoter. Als je deze promoter gebruikt om een kleurvormend enzym te activeren kan je aan de hand van de aanwezigheid van een kleurstof bepalen waar auxine accumuleert. Dit is gedaan in plantenwortels die geïnfecteerd werden met cystenaaltjes of met wortelknobbelaaltjes en DR5 bleek zeer snel (enige uren) na infectie geactiveerd te zijn. Deze activering was ook nog eens zeer lokaal, in eerste instantie troffen we alleen keurstof aan in de initiële voedingscel. Deze vinding maakt het waarschijnlijk dat auxine accumulatie een van de eerste gebeurtenissen is van een cascade die uiteindelijk leidt tot voedingscelvorming. We denken dat de nematoden de export van auxine uit de cel blokkeert. Als de import van auxine doorgaat leidt dat automatisch tot een lokale accumulatie van dit plantenhormoon. De lokale auxine ophoping is een belangrijk element in het leren begrijpen van fysiologische veranderingen die uiteindelijk leiden tot voedingscelvorming.

ENOD40 ("early nodulation"40) is een klein eiwit met hormoonachtige karakteristieken dat een belangrijke rol speelt bij wortelknolvorming in leguminosen. Ook

leguminosen zijn vatbaar voor plantenparasitaire nematoden en onderzoeksgroepen in Frankrijk en de Verenigde Staten hadden vastgesteld dat *ENOD40* ook een rol speelde bij voedingscelvorming het wortelknobbelaaltje. Opmerkelijk genoeg komt *ENOD40* niet alleen voor bij leguminosen, maar ook bij niet-stikstofbinders zoals bijvoorbeeld tomaat. In hoofdstuk 3 hebben we in samenwerking met het Laboratorium voor Moleculaire Biologie (WU) de activering van de tomatenpromoter van *ENOD40* getest op tomatenplanten die geïnfecteerd werden met cystenaaltjes of wortelknobbelaaltjes. Hierbij bleek dat er geen correlatie was tussen nematodeninfectie en *ENOD40* activering. Hiermee lijkt er een opmerkelijk en vooralsnog onverklaard verschil te zijn tussen de infectie door nematoden van leguminosen en niet-wortelknobbelvormden plantensoorten.

Nadat het cystenaaltje een initiële voedingscel heeft geselecteerd vindt er op een zeer gecoördineerde manier celfusie plaats tussen cellen. Voedingscelvorming volgt niet het patroon van een zich uitbreidende olievlek, en dat is een van de redenen waarom het onaannemelijk is dat celwandafbrekende enzymen van de nematode zelf hiervoor verantwoordelijk zijn. In hoofdstuk 4 wordt nagegaan in hoeverre plantencellulases betrokken zijn bij gecoördineerde celwandafbraak ten behoeve van syncytiumvorming. Dit is bekeken in tomaat geïnfecteerd met aardappelcystenaaltjes. Tomaat heeft op z'n minst 8 verschillende cellulasen en wij hebben laten zien dat twee daarvan – *cel7* en *cel8* – geactiveerd worden door de nematode. In samenwerking met de Landbouwuniversiteit van Warschau (Polen) is gekeken naar veranderingen in de ruimtelijke distributie van transcripten van *cel7* en *cel8* gedurende het infectieproces. Daarbij kwam naar voren dat *cel7* vanaf één dag na infectie geactiveerd werd, en het hoogste transcriptieniveau werd aangetroffen na vijf dagen. *Cel8* komt wat later tot expressie; vanaf drie dagen na infectie van de wortels met infectieuze juvenielen. Vervolgens is deze activering ook op eiwitniveau bekeken. Specifieke kippenantilichamen maakten het mogelijk om CEL7 en CEL8 eiwitten te monitoren in dwarsdoorsneden van de plantenwortel. Ook hier kwam weer hetzelfde beeld naar voren, waarbij verhoogde aanwezigheid van CEL8 werd voorafgegaan door CEL7. Een logische, volgende vraag is hoe belangrijk de rekrutering van deze plantenenzymen is voor de ontwikkeling van de nematode. Als de nematode er niet in slaagt om deze plantengenen voor zichzelf te laten werken, kan de nematode zich dan – wellicht wat langzamer – toch ontwikkelen, of is deze activering essentieel voor de verdere ontwikkeling van dit pathogeen? Deze vraag is beantwoord door de betreffende genen één voor één uit te schakelen. Dit is gebeurd in aardappel - een plant die in genetisch opzicht enorm veel lijkt op tomaat – door middel van dubbelstrengs RNA (dsRNA). Met behulp van dsRNA werden *cel7* en *cel8* transcripten specifiek gedestabiliseerd. Opmerkelijk genoeg had dit weinig effect op de aardappelplanten zelf, de nematoden daarentegen hadden de grootst mogelijk moeite om een syncytium te vormen. Als ze er al in slaagden om zich te vestigen op de transgene aardappelwortel dan waren de volwassen vrouwtjes min of meer transparant. Dit houdt in eieren niet of nauwelijks tot ontwikkeling waren gekomen. De resultaten waren min of meer

gelijk voor *cel7* en *cel8* en daarmee is bewezen dat de rekrutering van plantencellulases essentieel is voor het aardappelcystenaaltjes, en dat zowel *cel7* als *cel8* geactiveerd moeten worden. Kennelijk hebben deze leden van de cellulase-familie een andere functie.

Is het nu toeval dat het aardappelcystenaaltje juist *cel7* en *cel8* rekruteert, en geen andere leden van deze genfamilie? Waarschijnlijk niet: een typische eigenschap van *cel7* is dat het door auxine geïnduceerd wordt. Eerder hadden we in hoofdstuk 2 gezien dat auxine accumuleert in de initiële syncytiumcel, het lijkt dus niet onlogisch dat *cel7* juist daar in hoge mate tot expressie komt. Van de expressie van *cel8* is erg weinig bekend, maar het is wel de enige tomatencellulase met een cellulose bindend domein. Anders dan men wellicht zou vermoeden, kan 'gewoon' cellulase niet of nauwelijks cellulose afbreken; daarvoor is een cellulose bindend domein nodig. Het tweetal dat door de nematode geactiveerd wordt is dus in staat om xyloglucan (*cel7*) en cellulose (*cel8*) te depolymeriseren. Uit het huidige onderzoek blijkt dat beide typen enzymen nodig zijn voor de vorming van een goed functionerend syncytium.

In hoofdstuk 5 wordt de genetische organisatie van *cel7* en *cel8* in aardappel onderzocht. In tegenstelling tot tomaat waar met name cellulases die een rol spelen bij de rijping van de tomatenvrucht uitgebreid onderzocht zijn, zijn cellulasen uit de aardappel nauwelijks gekarakteriseerd. Een opmerkelijke vinding hierbij is dat er waarschijnlijk meer dan één variant van *cel8* in aardappel aanwezig is. Dit zou een mogelijke verklaring zijn voor het feit dat uitschakeling van een *cel8* geen grote gevolgen heeft voor het fenotype van de aardappelplant. Dit was in eerste instantie nogal opmerkelijk omdat *cel8* nu juist de enige cellulase is met een cellulose bindend domein. Parallelle pogingen om in samenwerking met het "Department für Angewandte Pflanzenwissenschaften und Pflanzenbiotechnologie" van de Universität für Bodenkultur (Wenen) *cel8* uit te schakelen in tomaat zijn gestrand, en dit zou kunnen liggen aan de afwezigheid van een tweede isovorm van *cel8* in tomaat.

Dit proefschrift geeft ons een aanzienlijk aantal nieuwe inzichten in de intieme en elegante interactie tussen het aardappelcystenaaltje en de waardplant, en biedt daarnaast handvatten voor de ontwikkeling van milieuvriendelijke en specifieke waardplantresistenties tegen cystenaaltjes.

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My adventure in Wageningen started in April 1999, when I came to the Netherlands for the very first time, to start my undergraduate project. Only for four months, so I thought. The four months changed into eight months, eight months into 4 years. That would not have been possible without the kind assistance of prof. Władyslaw Golinowski, who gave me idea to come here and who recommended me to dr. Johannes Helder. Dear prof. Golinowski, your dedication and scientific enthusiasm have been always inspiring for me. You are an outstanding mentor, and I truly hope that in the future we will have the opportunity to work together. With those words I would like to express my deepest gratitude to my promotor prof. Jaap Bakker, who put trust in me and gave me the opportunity to conduct the research with his excellent group. I wish to extend my gratitude to dr. Aska Goverse for the guidance during all those years that I have spent in Wageningen. Aska, you are not only a great scientist but also extraordinary supervisor. Your doors were always open and you walked with me hand in hand and helped me to attain all of this. You are a great example to follow. My profound gratitude goes also to dr. Johannes Helder for the bright scientific insight, constructive criticism and the patience in going through this manuscript. Your contribution to this thesis was essential. Hans I have learned a lot form you and I hope to benefit from this knowledge during the rest of my scientific career.

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remember your bright smile! Lisette I wish to thank you for the great support with all of the bureaucratic matters. You have been my first Dutch teacher, and helped me a lot with overcoming the fear of speaking Dutch☺. Liesbeth you have taught me everything about plant transformation, it was a great pleasure to work with you! Jan without your help it would not be possible to finish the cloning of the potato *cel7* and *cel8*: thanks a lot for your contribution and intensive help to accomplish the full sequences of *cel8*. Your help was indispensable! Martijn, thanks for helping with the 'trees'. Jan van Bezoojen and Hanny van Megen, I wish to thank for your unspeakable kindness. I will always remember your charming smile! Sven you were the best (and only!) singing lab-mate I have ever had! Ik mis je gezang! Ania, Patric, Aga, Olga, Eric, Erwin, Saijd, Martijn, Nikkie, Kamila, Dieuwertje I wish you a lot of strength in finishing yours PhDs! Wiellemien and Hans, I wish you both a lot of success with yours new carriers! Geert, Ling and Erin thanks for the en-lightening discussions and creative suggestions during our lab meetings: your support contributed immensely to the success of my experiments. I cannot forget my MSc students that I had the pleasure to supervise. Dieuwertje Alex and Gosia. Thanks! And everybody, thanks for all fun in and outside lab!

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Working abroad is not always easy, you feel alone, homesick and strange. But friends make it easy! When I arrived here, I was placed in the very best corridor 5b in Dijkgraaf. My dear 'corridor' friends, it was great to have you around! Thanks to your great cooking I gained 6 kg ☺ I really had the time of my life and I will never forget those crazy parties, talking about everything until the early morning hours, watching 'Friends' together and cooking and cooking… Marcus and Nati, I hope we will stay in touch!

Poland is a big country with a population in excess of 40 million citizens. How big is the chance, to meet few of them in Wageningen and with all of them make friends for a lifetime? I'm really lucky one! I want to thank you my dear friends for making my life sparkle, richer, happier and above all much funnier! Pisek, what I would do without you? You make me laugh, when there is absolutely nothing to laugh about. Your optimism is contagious, your sense of humour is excellent, and your easy going-character makes you irresistible. Pisku thank you for your friendship, all the glasses of wine we drank together,

About the author

Aneta Karczmarek, was born in Warsaw, Poland, on the 19th of September, 1976. In 1995, she obtained her diploma at the XXXVIII lyceum, Warsaw, where she took Chemistry and Biology as her main subjects. The same year, she started her study at the Warsaw Agriculture University, where she did the Interfaculty Studies of Biotechnology. The second year of study, until graduation, she received the merit scholarship. In October1998, she started her first undergraduate project, entitled "Hepatocyte Transplantation: early graft failure, liver stem cells" at the Medical Research Centre Polish Academy of Sciences, Department of Surgical Research and Transplantology. In April, 1999 she visited Wageningen where she worked for at the Laboratory of Nematology. This eight month research resulted in the Thesis entitled "Functional analysis of nematode secretions using potato virus X expression vector". She was very enthusiastic about the plant- parasitic nematodes interactions and in May 2000, she obtained her Master Diploma, few months later she left Warsaw to start the advanced studies at the same laboratory. For four and a half years, she worked under the supervision of dr. Aska Goverse, on a project aiming at the better understanding of compatible nematode-host interaction. This dissertation comprises of the obtained results.

At this moment she works as a post-doc at the department of Molecular Cytology, at the University of Amsterdam, in the group of dr. Tanneke den Blaauwen. The project she works on focuses on the elucidation of the molecular mechanisms underlying the longitudinal elongation of *Escherichia coli* cells.

Education Statement of the Graduated School Experimental Plant Sciences (EPS)

1) Start-up phase

    First presentation of your project, Writing a Chapter 6 of this thesis

2) Scientific Exposure

    EPS PhD student days-2001/2003

    EPS theme symposia-2002-2004

    NWO Lunteren days and other National Platforms-2001-2003

    Seminars (series), workshops and symposia-2003

    International symposia and congresses- Nonema International Project Meetings-2000-2004; Fourth International Congress of Nematology, 2002; International Symosium of the European Society of Nematologists, 2004

    IAB interview-2003

    Presentations- Nonema International Project Meeting-2000-2004; Fourth International Congress of Nematology, 2002; International Symosium of the European Society of Nematologists, 2004; Discussieplatform Experimentele Planten Wetenschappen, 2003

3) In-Depth Studies

    EPS courses or other PhD courses- Interactions between Plants and Attacking Organisms, 2000, Environmental Signaling: Arabidopsis as a Model, 2001, Disease Resistance in Plants, 2002, Molecular Basis of Microbe-Plant Interactions, 2003

    Journal club, 2000-2005

4) Personal development

    Skill training courses: Project Management & Time Planing, 2003, English Scientific Writing (12 lessons) 20001/2002

    Organisation of PhD students day: PhD students day, Utrecht "A new dimension to networking", 2003

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plant parasitic nematodes