A family of endoglucanases belonging to glycoside hydrolase family (GHF) 45 have been isolated from the pine wood nematode *Bursaphelenchus xylophilus*. Here we describe the purification and characterization of the recombinant enzymes, named Bx-ENG-1, 2, and 3, expressed in *Pichia pastoris*. The respective molecular masses of purified Bx-ENG-1, 2, and 3 were estimated to be 18, 33–39, and 100–140 kDa by SDS–PAGE, and 18, 67, and 252 kDa by gel filtration, suggesting that Bx-ENG-1 existed in an unglycosylated monomeric form and Bx-ENG-2 and Bx-ENG-3 in a glycosylated dimeric form. The enzymatic properties of the recombinant enzymes were similar to each other: optimal activity at 60°C at about pH 6.0, like other endoglucanases of GHF45. The recombinant enzymes displayed the highest activity toward lichenan, and lower activities were observed on carboxymethyl cellulose and amorphous cellulose. Nematode enzymes also hydrolyzed glucomannan, the most abundant hemicellulose in the cell walls of softwood. These substrate specificities suggest that *B. xylophilus* endoglucanases acted on the cellulose-hemicellulose complex in the cell walls, resulting in a weakening of the mechanical strength of the cell walls to facilitate the nematode’s feeding on plant cells.

**Key words:** *Bursaphelenchus xylophilus*; cellulase; endoglucanase; nematode; glycoside hydrolase family 45

Cellulose, the major polysaccharide component of plant cell-walls, is a polymer of β-1,4-linked glucose molecules. Individual cellulose molecules associate to form cellulose microfibrils through numerous intra- and intermolecular hydrogen bonds, providing rigidity to the plant cell-walls.

Endoglucanases are widely distributed among bacterial and fungal plant pathogens. They are used to degrade host cell-walls in order to allow penetration and colonization. Plant-parasitic cyst nematodes and root-knot nematodes are known to secrete endoglucanases. Genes encoding endoglucanases have been cloned from several species of plant-parasitic nematodes, including *Heterodera*, *Globodera* (cyst nematode), and *Meloidogyne* (root-knot nematode) species. These endoglucanases are produced within the esophageal gland cells of these nematodes and are secreted through the nematode stylet into plant tissues. They are thought to play an important role in the infection and parasitism of plants.

The pine wood nematode, *Bursaphelenchus xylophilus*, is the causal agent of pine wilt disease. Most *Bursaphelenchus* spp. are solely fungal feeders, and are transmitted by vector insects only to dead or dying trees during oviposition. *B. xylophilus* and a few other pathogenic species are unique in their capacity to feed on live trees as well as fungi. In the *B. xylophilus* pathogenic life cycle, dauer larvae (dispersal 4th stage juveniles) of the nematodes are transmitted to live trees as adult *Monochamus* beetles emerge from dead wood and fly to live trees to feed. The nematodes enter the tree through maturation feeding wounds caused by the vector beetle. The nematodes feed on cells in the tree and migrate through the tissues, spreading through the tree. This causes cell destruction, leading to wilting symptoms that result in the death of the tree within one year of infection.5) Pine wilt disease is the most serious forest disease in Japan and East Asia generally.

In order to identify the molecular mechanisms underlying parasitism in *B. xylophilus*, we undertook an expressed sequence tag project on this nematode.6) To date, three endoglucanase genes (Bx-eng-1, Bx-eng-2, and Bx-eng-3) have been cloned from *B. xylophilus*.

These endoglucanases are produced within the esophageal gland cells of the nematodes and are secreted through the nematode stylet into plant tissues. While the other nematode endoglucanases identified have all been classified into the glycoside hydrolase family (GHF) 5, the catalytic module of *B. xylophilus* enzymes have been classified into GHF45 according to sequence-based homology.8) Phylogenetic analysis showed that...
B. xylophilus sequences were most similar to fungal GHF45 endoglucanases. This similarity and the absence of such genes from other nematodes has led to the suggestion that they were acquired by horizontal gene transfer from fungi.7)

Here we describe the expression of the cloned endoglucanase genes in Pichia pastoris, and characterize some properties of the recombinant enzymes from B. xylophilus. This study made possible a comparison of their enzymatic properties with those of GHF45 enzymes from microbes and from other origins. Moreover, biochemical characterization of B. xylophilus endoglucanases provides a better understanding of their role in nematode parasitism.

Materials and Methods

Enzyme production and purification. An endoglucanase coding region without the putative signal sequence was amplified by PCR from a Bx-eng1 cDNA clone (clone ID: Bx_K1_02F06) that was derived from the Bursaphelenchus EST project using primers Bxeng1-sf-Xho (5'C CGGCTCAGAGCGATACCGGACAAA-CCACG 3') and Bxeng1-or-NotI (5' TTTTCTTTTTCCGCCCCAGCATGTCGGAACGGATGCA 3' for Bx-eng-1. For the Bx-eng-2 and Bx-eng-3 primers, Bxeng2-sf-Xho (5'C CGGCTCGAGAGAAGCTCGAAAACGAGAAAG 3') or Bxeng3-sf-Xho (5'C CCGTCTGGAAGACCTGTGGGCCCTTGACC 3') and Bxeng23-or-NotI (5' ATAGTTTATGGCGCCGATCGTCCGCA-CGGATGCAATT 3') were used in the amplification from each EST clone (clone ID: Bx_K1_1A02 and Bx_K1_1B07). The resulting PCR products were ligated directly into the pGAPZα vector (Invitrogen Carlsbad, CA) and then transformed into Pichia pastoris X-33 (Invitrogen). Recombinant P. pastoris was shake-cultured at 28°C in 150 ml of YPD medium (1% yeast extract, 2% peptone, 2% glucose) in a 500 ml flask. The culture supernatant (140 ml) was harvested by centrifugation after 96 h of growth. The supernatant was concentrated with an ultrafiltration membrane, BIOMAX-5 (Millipore, Billerica, MA), and equilibrated with 20 mM sodium acetate, pH 3.8, containing 150 mM NaCl. The active fractions were collected and stored at 4°C.

Polysaccharide substrate. Carboxymethyl cellulose (CMC) and glucomannan from konjac were purchased from Wako (Tokyo). Lichenan from Cetraria islandica, laminarin from Laminaria digitata, and galactomannan from Ceratonia siliqua were purchased from Sigma (St. Louis, MO). Glucomannan from larch (Larix leptolepis) was prepared from wood meal, as described previously.9) The ratio of glucose to mannose of the larch glucomannan was 1:3. Pustlan from Umbilicaria papullosa and xylanoglucon from tamarind were from Calbiochem (La Jolla, CA) and Megazyme (Wicklow, Ireland) respectively. Soluble xylan was prepared from birchwood xylan (Fluka, Buchs, Switzerland). Microcrystalline cellulose (MCC; ceolus KG-802) was kindly provided by Asahi Kasei (Tokyo). Ball-milled cellulose (BMC) and phosphoric acid swollen cellulose (PASC) were prepared from MCC following Wood.10)

Protein and enzyme assays. The concentration of the enzyme was determined by the absorbance at 280 nm, using a molar extinction coefficient of 52,325 M⁻¹ cm⁻¹ for Bx-ENG-1 and one of 51,295 M⁻¹ cm⁻¹ for Bx-ENG-2 and Bx-ENG-3, calculated from the amino acid sequences of the recombinant enzymes.

The standard enzyme assay was performed with CMC, 0.5% (w/v) in 0.5 ml of 50 mM sodium acetate buffer (pH 5.8) at 40°C, unless otherwise stated. Microcentrifuge tubes (1.5-ml) containing 450 μl of reaction mixture was incubated with 50 μl (50 pmol) of purified endoglucanase in the presence of 0.1% (w/v) BSA at 40°C for 10 min. Formation of reducing sugars was determined by a method modified from Lever.11) Samples (100 μl) were mixed with 1 ml of fresh 1% p-hydroxybenzoic acid hydrazide (PAHBAH) in 0.5 M NaOH, and heated in a heat block at 100°C for 5 min to stop enzyme activity and to label the enzyme product. Insoluble materials were removed by centrifugation, and the reducing sugar level absorbance was determined at 410 nm. The concentration of reducing sugar was determined using d-glucose as the standard. One unit of enzyme activity was defined as the amount of the enzyme needed to liberate 1 μmol of reducing sugar per min. Hydrolytic activity with soluble polysaccharide as substrate was assayed using the method described above.

The hydrolytic activity of BMC and PASC as substrate was assayed using the standard method, except that the reaction was carried out in 5-ml vials with rotary shaking at 150 rpm. Aliquots of the reaction mixture were taken at different times during hydrolysis, insoluble residues were removed by centrifugation, and the amount of sugar released in the supernatant was determined by PAHBAH assay.

The hydrolytic activity of MCC as substrate was assayed using a reaction mixture containing 100 pmol of...
enzyme and 10 mg of substrate in 1.0 ml of a 50 mM acetate buffer (pH 5.8) in the presence of 0.1% (w/v) BSA. The reaction mixture was incubated at 40 °C for 1 h with rotary shaking at 150 rpm. The concentration of reducing sugar was determined in the same manner as when BMC and PASC were used as substrates.

**Temperature and pH optima, and thermal stability.** The optimum temperature was determined by running the standard assay at temperatures from 10 to 80 °C. The optimum pH of the enzyme was determined by running the standard assay at 40 °C using 0.1 M citric acid/0.2 M Na₂HPO₄ and 0.1 M KH₂PO₄/0.05 M Na₂B₄O₇ buffers in pH ranges of 2.6–6.4 and 7.1–8.9 respectively.

**Cellulose-binding experiment.** The adsorption to MCC (1.0%, w/v) was studied with enzyme concentrations of 5.0 μg/ml in 50 mM acetate buffer at pH 5.8. BSA was included at 0.1% (w/v) to stabilize the enzyme and to reduce unspecific adsorption. The adsorption experiments were performed at 4 °C with rotary shaking of 150 rpm. The adsorption time was between 3 min and 45 min. The total reaction volume was 1.0 ml in 5.0-ml vials. The substrate with adsorbed enzyme was removed by centrifugation, and the amount of the enzyme activity remaining in the supernatant was analyzed by CMC. The activity was determined by PAHBAH assay.

**Detection of hydrolytic products.** The hydrolytic products of lichenan, CMC, and konjac glucomannan were determined by GPC. Soluble polysaccharide (12 mg) was digested with 4.0 μg of enzyme in 2.4 ml of 50 mM acetate buffer (pH 5.8) at 40 °C. Aliquots of the reaction mixture were taken at different times of hydrolysis, and the reaction was stopped by incubation at 96 °C for 5 min. The hydrolytic products were separated at 40 °C in the same buffer using a TSKgel G3000PWXL column (0.78 × 30 cm, Tosoh, Tokyo) and the eluate was analyzed with refractive index detector RI-8010 (Tosoh).

**Results**

**Modular architecture of Bx-ENG-1, 2, and 3**

Figure 1A schematically summarizes the structures of three B. xylophilus endoglucanases, encoded by the **Bx-eng-1**, **2**, and **3** genes. The Bx-eng-1 gene encoded a GHF45 enzyme consisting of a single catalytic module, whereas Bx-eng-2 and Bx-eng-3 encoded a GHF45 catalytic module and N-terminal extensions of 56 and 128 amino acid residues respectively. The catalytic modules of the three enzymes were highly homologous, especially as between Bx-ENG-2 and Bx-ENG-3, which shared 97% identity. Bx-ENG-1 shared 73 and 74% identity with the catalytic modules of Bx-ENG-2 and Bx-ENG-3 respectively.

The alignment of the N-terminal extensions of Bx-ENG-2 and Bx-ENG-3 is shown in Fig. 1B. The sequences are rich in alanine, serine, and threonine, and have a repeated unit, (S/T)(T/K)A(S/T)AAP occurring in the repeated segment.
Physico-chemical properties of the recombinant enzymes

To determine the biochemical properties of *B. xylophilus* endoglucanases Bx-ENG-1, 2, and 3, Bx-eng-1, 2, and 3 genes were expressed in *P. pastoris* under the control of a GAP promoter. *P. pastoris* cells carrying pGAP-C11-Bx-eng-1, 2, or 3 were cultured, and the recombinant enzymes were purified by steps involving Mono S, HiTrap Phenyl HP, and Superdex 200 HR column chromatography. These enzyme preparations were essentially homogeneous, each showing a single band on SDS–PAGE (Fig. 2A).

The respective molecular masses of Bx-ENG-1, 2, and 3 were estimated to be 18, 33–39, and 100–140 kDa by SDS–PAGE (Fig. 2A), and 18, 67, and 252 kDa by gel filtration on a Superdex 200 HR column chromatography. These enzyme preparations were essentially homogeneous, each showing a single band on SDS–PAGE (Fig. 2A).

The molecular mass of recombinant Bx-ENG-1, 18 kDa, was smaller than the calculated molecular weight of Bx-ENG-1, 25,383. This was probably due to disulfide bridges in the Bx-ENG-1 molecule, because Bx-ENG-1 showed a single band with a molecular size of 25 kDa when 2-mercaptoethanol was added to the SDS–PAGE sample.

The apparent molecular masses of recombinant Bx-ENG-2 and Bx-ENG-3 were much higher than the deduced amino acid sequences (30,293 and 36,439 respectively). The differences between the apparent and the expected molecular masses can be explained by glycosylation. Glycoprotein staining of the recombinant enzymes on SDS–PAGE revealed that Bx-ENG-2 and Bx-ENG-3 were glycosylated (Fig. 2A). Bx-ENG-3 appeared to be hyperglycosylated, and it might be thought that the carbohydrate moieties of the recombinant enzyme affect the migration of the protein on SDS–PAGE.

Effect of pH and temperature on enzymatic activity

The effect of pH on hydrolysis was studied for the purified recombinant enzymes with soluble cellulosic polymer CMC during a 10-min hydrolysis (Fig. 3A). Bx-ENG-1 showed maximal activity at pH 5.8. Under pH 5.0, the activity dropped rapidly and no activity was detected at pH 2.6 or 3.3. For Bx-ENG-2, the highest activity was obtained at pH 6.4. The maximum activity range was broad, with 70% of maximum activity occurring between pH 4.2 and 8.3. Bx-ENG-3 had maximum activity at pH 5.8. At acidic pH values, Bx-ENG-3 showed higher activity than the other enzymes; 25% of the maximal activity was retained at pH 2.6, while no activity was detected for Bx-ENG-1 or Bx-ENG-2 at this pH.

The effect of temperature on the hydrolysis of CMC at pH 5.8 was determined for Bx-ENG-1, 2, and 3 (Fig. 3B). The incubation time for each enzyme was 10 min. All enzymes had maximal activity at 60°C, but Bx-ENG-1 was less stable than the other enzymes. Half of its maximal activity was lost at 70°C, while more than 80% of maximal activity was observed for Bx-ENG-2 and Bx-ENG-3 at the same temperature.

Adsorption to MCC

The adsorption of Bx-ENG-1, 2, and 3 to MCC was studied with Cel5A (EG II) from *Trichoderma reesei* (Fig. 4). Adsorption was observed at 4°C to minimize substrate change due to enzymatic activity. Furthermore, BSA (1 mg/ml) was added to reduce unspecific adsorption of the enzymes.
The strongest adsorption to MCC was shown by Cel5A, which has a cellulose binding module at the N-terminal. More than 65% of Cel5A adsorbed to MCC within 3 min, and about 85% of Cel5A adsorbed after 45 min. Moderate adsorption was shown by Bx-ENG-2: about 20% of it adsorbed to MCC after 45 min. No desorption of these enzymes was detected up to 24 h. Weaker adsorption was shown by Bx-ENG-3: about 10% of it adsorbed to MCC after 45 min. Almost no Bx-ENG-1 was adsorbed to MCC. More than 95% of the initial activity was detected in the supernatant over time.

**Substrate specificity**

The substrate specificity of the purified recombinant enzymes toward polysaccharides is summarized in Table 1. All enzymes displayed the highest activity toward lichenan (β-1,3-1,4 linked glucan), and lower activities were observed on CMC, amorphous cellulose (PASC and BMC), and glucomannan. These enzymes showed very low activity toward MCC, and no activity toward laminarin (β-1,3 linked glucan), pustulan (β-1,3-1,6 linked glucan), galactomannan, xylan, or xyloglucan. These results indicate that B. xylophilus enzymes acted on the substrates containing β-1,4-glucoside linkages.

The respective modes of action of Bx-ENG-1, 2, and 3 were then examined by analyzing the hydrolysis products by GPC (Fig. 5). The initial peak of lichenan was widened in the course of the reaction, and its Mr decreased to around 23 kDa after 16 h of hydrolysis. Similar patterns of elution profiles were also observed in CMC and konjac glucomannan (data not shown). These results confirm that Bx-ENG-1, 2, and 3 hydrolyzed the substrate with the endo-depolymerase mode of action.

**Discussion**

In this paper, we have described the functional expression, purification, and characterization of a family
of GHF45 endoglucanases from the pine wood nematode *B. xylophilus*. These are the first GHF45 enzymes identified from any nematode species.

Zymogram analysis of the nematode homogenate showed that three CMC-degrading activity bands appeared, at approximately 19, 38, and 80–220 kDa respectively, after renaturation of the samples, which were loaded on an SDS–PAGE gel containing CMC (data not shown). These sizes are in agreement with the molecular masses of the recombinant enzymes by SDS–PAGE (Fig. 2), suggesting that the post-translational modification of the recombinant enzymes expressed by *P. pastoris* were similar to that of the native enzymes produced by *B. xylophilus*.

The molecular forms of the purified recombinant enzymes were quite different from each other. For instance, Bx-ENG-1 was unglycosylated and existed in a monomeric form. By contrast, Bx-ENG-2 and Bx-ENG-3 probably existed in a dimeric form with glycosylation, a form whose level of Bx-ENG-2 was more moderate than that of Bx-ENG-3 (Fig. 2). This is the first example of GHF45 enzymes that form a subunit structure. It is possible that Bx-ENG-2 and Bx-ENG-3 form a hetero subunit under the native condition, since the catalytic modules of these enzymes are almost identical.

The enzymatic properties of recombinant Bx-ENG-1, 2, and 3 were similar to those of the endoglucanases from GHF45, previously reported. Optimum activity of the recombinant enzymes was observed at 60 °C at about pH 6.0 (Fig. 3). Many of the GHF45 endoglucanases display optimal temperature between 50 and 60 °C and optimum pH between 6.0 and 7.0. *B. xylophilus* endoglucanases appear to have been particularly active at temperatures much higher than those found in the normal environmental conditions that the nematodes encounter. This is also the case for other cell-wall enzymes such as β-1,3-glucanase and pectate lyase from *B. xylophilus* and several invertebrate GHF45 endoglucanases.

Bx-ENGs displayed the highest activity toward lichenan and did not hydrolyze β-1,3-1,6 linked glucan (pustran), β-1,3 linked glucan (laminarin), galactomannan, xylan, or xyloglucan as substrates. Similar results (strict specificity toward β-(1,4)-glucoside linkage) have been obtained for other GHF45 enzymes. The lack of activity toward xyloglucan suggests that the high level of branching interfered with access to the catalytic cleft of the GHF45 enzyme.

The lower activity toward CMC (modified β-(1,4)-glucan) as compared to lichenan is consistent with previous studies of GHF45 endoglucanases, probably because CMC is highly substituted with the carboxymethyl group, which can interfere with enzyme activity. Bx-ENGs might also have released reducing sugars from insoluble cellulosic substrates BMC and PASC to a degree comparable to CMC, but they displayed almost no hydrolytic activity towards MCC, suggesting that these enzymes prefer the disordered and amorphous regions of cellulose to highly crystalline cellulose.

Bx-ENGs showed considerable activity toward glucomannan, although most GHF45 enzymes have not been studied as to their roles in the hydrolysis of glucomannan as a substrate. To our knowledge, *Trichoderma reesei* Cel45A (EG V) is the only GHF45 enzyme known to show hydrolysis of glucomannan, so Bx-

---

Fig. 5. Analysis of Lichenan Hydrolytic Products by GPC.

The endoglucanase (1.7 μg/ml) was incubated at 40 °C in 50 mM acetate buffer, pH 5.8. The hydrolytic products were separated on a TSKgel G3000PWXL column (Tosoh, Tokyo), and the eluate was analyzed with a refractive index detector (RI-8010; Tosoh). A, Bx-ENG-1; B, Bx-ENG-2; C, Bx-ENG-3. Hydrolysis times: 1, 0 min; 2, 2 min; 3, 1 h; 4, 16 h; 5, 136 h.
ENGs are the second example of GHF45 enzymes hydrolyzing it.

The ability of Bx-ENGs to hydrolyze glucomannan might be important in pine wood nematode parasitism, because glucomannan is the most abundant hemicellulose in the cell walls of softwood. B. xylophilus migrates primarily through the resin canals of trees and feeds on parenchyma cells surrounding the canals. The walls of the parenchyma cells consist of a primary wall, and there is no development of a secondary wall having and a thick and rigid structure. In primary walls, cellulose exists as elementary fibrils that form a complex with hemicellulose. Hence the results of the aforementioned substrate specificity study suggest that Bx-ENGs act on the cellulose-hemicellulose complex in cell walls, resulting in a weakening of their mechanical strength.

In a previous study, we found that the nematode also secretes pectate lyase, which degrades the pectin present in the primary cell walls of cambium and parenchyma cells. A mixture of these cell-wall-degrading enzymes might play an important role in softening the cell walls to facilitate the nematode’s feeding on plant cells in a tree.

The wood of pine wilt trees has a blue stain caused by Ophiostoma species. As the fungi increase, nematodes begin to feed on them. Interestingly, β-1,4-glucan-containing molecules were detected in several species of Ophiostoma in hyphal walls, although β-1,4-glucan has not been detected in most fungal cell walls. It is thus possible that the nematode uses its endoglucanases to degrade the cell walls of the fungus.

B. xylophilus secretes a mixture of at least three GHF45 endoglucanases. The soybean cyst nematode H. glycines has been found to have at least six endoglucanases, and their production was found to be developmentally regulated. The differential expression of the genes suggests different roles for the various endoglucanases during parasitism. Although it is not clear whether the endoglucanases are differentially expressed in B. xylophilus during parasitism, they might play specific roles for various enzymes during the parasitic stages, similarly to the enzyme of the cyst nematode. For instance, although most Bursaphelenchus spp. are solely fungal feeders, B. xylophilus is unique in its capacity to feed on live trees as well as fungi. Thus one possible explanation of the roles of the enzymes is that the nematode differentially uses its endoglucanases to degrade the cell walls of wood and fungi.

Bx-ENG-2 and Bx-ENG-3 have a functionally unknown sequence that is rich in alanine, serine, and threonine, and has a repeated unit, (S/T)(T/K)A(S/T)AAP (Fig. 1B). These structural features are similar to the linker region of fungal cellulases. The catalytic cores of fungal cellulases are linked to the carbohydrate binding modules via the Ser/Thr/Pro-rich linker regions. The linker of T. reesei Cel7A (cellobiohydrolase I) is heavily O-glycosylated, and it appears that the N-terminal sequences of Bx-ENG-2 and Bx-ENG-3 are so too. Generally, it has been reported that glycosylation protects proteins from proteolysis. In plant pathogens, protection of secreted enzymes from proteolysis might play an especially important role in the success of pathogen development in planta. Another possible role of the N-terminal sequences of Bx-ENG-2 and Bx-ENG-3 is to contribute to forming a subunit, since Bx-ENG-2 and Bx-ENG-3 existed in a dimeric form, whereas Bx-ENG-1 without the extra sequence existed in a monomeric form.

Interestingly, a similar domain structure that consists of the catalytic module and an additional functionally unknown sequence has been observed in the EGI of the phytopathogenic fungus Ustilago maydis in GHF45 endoglucanases. The additional sequence of EGI is located at its C-terminus, and is highly enriched in glycine, serine, and alanine. Furthermore, endoglucanases with similar domain structures have also been also reported in cyst nematodes, viz., Gr-ENG-2 from G. rostochiensis, Gt-ENG-2 from G. tabacum, and Hg-ENG-4 from H. glycines. These enzymes contain a GH5 catalytic module and a functionally unknown sequence having a repeated unit. These additional sequences might have a role in plant-pathogen interaction, since endoglucanases with such a domain structure are frequently found in plant pathogens. Further study, as on sequence deletion, is necessary in order to arrive at a biological interpretation of this information.

**Acknowledgments**

We thank Dr. T. Shimokawa (FFPRI) for help with the enzyme assays. We also thank Dr. N. Hayashi (FFPRI) for valuable suggestions. This work was funded by a Grant-in-Aid for the Encouragement of Young Scientists (B) 18780032 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

**References**

4. Goellner, M., Smant, G., de Boer, J. M., Baum, T. J., and Davis, E. L., Isolation of beta-1,4-endoglucanase genes from Globodera tabacum and their expression during...