

森林総合研究所
交付金プロジェクト研究 成果集 14

マツノザイセンチュウのESTに
よる遺伝子解析

EST Analysis of the Pine Wood

Nematode *Bursaphelenchus xylophilus*

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Foreword

The first outbreak of pine wilt disease in Japan occurred in the far western end of the country near Nagasaki Bay in 1905. Since then, the pine wilt disease has expanded its distribution in Japan all the way to the northern part of Honshu Island, killing thousands of domestic pine trees (*Pinus thunbergii*, *P. densiflora*, *P. luchuensis*) in the process. A lack of accurate biological knowledge about the pathogen and its vector has delayed the implementation of suitable control measures. Today, the disease occurs not only in Japan, but also in China and Korea in Asia, and Portugal in Europe. People in these countries are currently working to bring it under control.

At first, the mortality of pine trees was thought to have been caused by mass attacks by insects, until the discovery of the true pathogen, later named the pine wood nematode, in 1969. Since the discovery, a huge amount of research has been done to clarify its mode of transmission, identify the pathogenesis of isolates, and formulate suitable control measures. But we still have not succeeded in controlling the disease because of the difficulties in disrupting the transmission of its widely distributed pathogen.

The research project "EST Analysis of the Pine Wood Nematode *Bursaphelenchus xylophilus*" was conducted from 2003 to 2005, with funding from the Forestry and Forest Products Research Institute. This was the first research project to analyze the systematic genome of the pine wood nematode. The report of the project describes some genes related to pathogenesis, and the origins of these genes. We are distributing this report for your reference. We hope that this report can contribute to the advancement of nematology and genomics, and to the improvement of control measures.

Motoaki Okuma
President
FFPRI

序 文

マツノザイセンチュウによるマツ材線虫病は、1905年頃長崎港周辺に発生したものがわが国では最古の記録といわれている。その後、この被害の原因や媒介機構が正確に把握されていなかったことから、被害域が拡大し、森林病害として類を見ない規模でアカマツやクロマツなど日本の在来種マツ樹の集団枯死を引き起こした。また、近年は、この病害は、わが国では本州北部に拡大すると共に、東アジア地域やヨーロッパのポルトガルにも侵入拡大し、各国で対策に苦慮している。

当初この被害の原因は昆虫類の集団加害によるものと考えられていたが、1969年に、後にマツノザイセンチュウと命名される線虫による病害であることが明らかにされた。わが国では、この病気の伝染生態や線虫の病原性、防除法などについて、精力的に研究が進められてきた。しかしながら、いったん定着した侵入病害は恐るべき勢いで拡大し、また防除の難しさから、この病害の制圧には至っていない。

本研究は2003年から2005年までの3年間、森林総合研究所の交付金プロジェクトとして実施された。マツノザイセンチュウの遺伝子に体系的に取り組んだ研究としては、最初のものである。このプロジェクトでは病原性に関連する遺伝子をはじめ、いくつかの興味ある知見が得られている。ここにその結果をとりまとめ、本報告書を作成した。この報告を足がかりに、新たな防除法の開発や、線虫学など科学の深化に寄与できれば幸いである。

平成19年3月

独立行政法人 森林総合研究所
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Contents

Project Overview.....	1
Chapter 1. EST analysis of the pine wood nematode <i>Bursaphelenchus xylophilus</i>	6
Chapter 2. Cloning and characterization of cell wall degrading enzymes from the pine wood nematode <i>Bursaphelenchus xylophilus</i>	
2-1. Cellulases	19
2-2. β -1,3-glucanases.....	33
2-3. Pectate lyases.....	51
プロジェクトの概要.....	73

Project Overview

I. Research term and budget

Term: 2003-2005 (3years)

Budget: FFPRI projects

II. Principal project researchers

Project Leader: Manabu Kusunoki

Final Report: Taisei Kikuchi

III. Research centers

FFPRI: Headquarters office

IV. Objectives

The pine wood nematode, *Bursaphelenchus xylophilus* is the causal agent of pine wilt disease, the most serious forest disease in Japan and East Asia. *B. xylophilus* is native to North America and is present in the USA, Canada and Mexico. Here it causes little or no damage, other than in non-indigenous species, presumably due to the evolution of tolerance or resistance in native tree populations. However, the nematode was introduced into Japan approximately 100 years ago. Here, where native trees have had no previous exposure to the pathogen, it causes enormous damage.

More than 30 years have passed since *B. xylophilus* was found to be the causal agent of pine wilt disease. Although the life history and some behavioral ecological aspects of this nematode are well characterised, little is known about the molecular basis of basic biology and host-parasite interactions in *B. xylophilus*.

Molecular biology has enabled the details of many aspects of nematode biology and of host-parasite interactions to be analysed at a far deeper level than would once have been thought possible. A technique – analysis of expressed sequence tags (ESTs) – has been of paramount importance in developing an understanding of the proteins produced by nematodes that allow them to parasitise plants. To date, more than 400,000 EST sequences from nematodes, including free-living species as well as animal and plant parasites, are available in databases. One of the most remarkable findings that has emerged from this work is the presence of genes within plant-parasitic nematodes that are absent from all other nematodes and most other animals studied to date. There is a strong argument that these genes have been acquired by horizontal gene transfer (HGT).

Previous studies on these parasitism genes have been largely restricted to cyst and

root-knot nematodes. There have been few studies on plant parasitism genes in other nematodes that are phylogenetically or ecologically distinct from cyst and root-knot nematodes. *B. xylophilus* is part of the same clade as cyst/root-knot nematodes. However, *Bursaphelenchus* spp. are not directly related to these nematodes but form a distinct grouping with other fungal feeding nematodes including *Aphelenchoides* spp. Studies on plant parasitism genes in nematodes other than cyst and root-knot nematodes will undoubtedly further our understanding of the origins of plant parasitism and may provide clues to the mechanism of inter-kingdom gene transfer. In order to address this, and in order to investigate molecular mechanisms underlying parasitism in *B. xylophilus*, we have performed an Expressed Sequence Tag (EST) project on this nematode.

III. Methodology

Chapter 1

Four cDNA libraries from *B. xylophilus* and one cDNA library from *B. mucronatus* were constructed. ESTs were generated by sequencing randomly selected clones from each library.

Chapter 2

We identified three distinct types of cell wall degrading enzyme genes from the EST dataset; cellulase, β -1,3-glucanase and pectate lyase. These genes were characterized by genomic Southern hybridization, in situ hybridization, immuno localarization, biochemical analysis using recombinant proteins and phylogenetic analyses.

VI. Results

Chapter 1

We present an analysis of over 13,000 ESTs from *B. xylophilus* and, by way of contrast, over 3,000 ESTs from a closely related species that does not as readily parasitise plants; *B. mucronatus*. Four libraries from *B. xylophilus* and one library from *B. mucronatus* were used to generate ESTs. 69% of the total *B. xylophilus* ESTs were from the mixed-stage library derived from the nematodes feeding on fungi, 11% were from the KP library made from nematodes feeding on plant material and 20% were from the two dauer larvae libraries. A variety of proteins potentially important in the parasitic process of *B. xylophilus* and *B. mucronatus*, including proteins important in fungal feeding as well as proteins that break down various components of the plant cell wall, were identified in the libraries. Additionally several gene candidates potentially involved in dauer entry or maintenance were also identified in the EST.

Chapter 2-1

Many plant parasitic nematodes produce endogenous plant cell wall degrading enzymes. The similarity of these genes to glycosyl hydrolase family (GHF) 5 endoglucanase genes from bacteria has led to the suggestion that they were originally acquired by horizontal gene transfer from bacteria. In this study, three GHF45 endoglucanase genes, *Bx-eng-1*, 2 and 3 were cloned from *B. xylophilus*. The enzymatic activity of Bx-ENG-1 was confirmed by heterologous expression in *Escherichia coli* and the endogenous nature of the genes was confirmed by Southern blotting. The presence of predicted signal peptide sequences at the N-termini of the proteins encoded by these genes coupled with the specific localization of the transcripts of *Bx-eng-1* imply that these cellulases could be secreted into plant tissues. Phylogenetic analysis showed that these cellulases are most similar to GHF45 cellulases from fungi. No similar genes have been described from any other nematode species. This suggests that horizontal gene transfer has occurred from fungi as well as bacteria within the Nematoda and that transfer of plant cell wall degrading enzymes has played a key role in the evolution of plant parasitism by nematodes on more than one occasion.

Chapter 2-2

We report the cloning and functional characterization of an endo- β -1,3-glucanase from the pine wood nematode *B. xylophilus* acquired by horizontal gene transfer from bacteria. This is the first gene of this type from any nematode species. We show that a similar cDNA is also present in another closely related species, *B. mucronatus* but that similar sequences are not present in any other nematodes studied to date. The *B. xylophilus* gene is expressed solely in the oesophageal gland cells of the nematode and the protein is present in the nematode's secretions. The deduced amino acid sequence of the gene is most similar to glycosyl hydrolase family 16 proteins. The recombinant protein, expressed in *Escherichia coli*, preferentially hydrolyzed the β -1,3-glucan laminarin, and had very low levels of activity on β -1,3-1,4-glucan, lichenan and barley β -glucan. Laminarin was degraded in an endoglucanase mode by the enzyme. The optimal temperature and pH for activity of the recombinant enzyme were 65°C and pH 4.9. The protein is likely to be important in allowing the nematodes to feed on fungi. Sequence comparisons suggest that the gene encoding the endo- β -1,3-glucanase was acquired by horizontal gene transfer from bacteria. *B. xylophilus* therefore contains genes that have been acquired by this process from both bacteria and fungi. These findings support the idea that multiple independent horizontal gene transfer events have

helped shape evolution of several different life strategies in nematodes.

Chapter 2-3

Two pectate lyase genes (*Bx-pel-1* and *Bx-pel-2*) were cloned from the pine wood nematode, *B. xylophilus*. The deduced amino acid sequences of these pectate lyases are most similar to polysaccharide lyase family 3 proteins. Recombinant BxPEL1 showed highest activity on polygalacturonic acid and lower activity on more highly methylated pectin. Recombinant BxPEL1 demonstrated full dependency on Ca^{2+} for activity and optimal activity at 55°C and pH 8-10 like other pectate lyases of polysaccharide lyase family 3. The protein sequences have predicted signal peptides at their N-termini and the genes are expressed solely in the oesophageal gland cells of the nematode, indicating that the pectate lyases could be secreted into plant tissues to help feeding and migration in the tree. This study suggests that pectate lyases are widely distributed in plant parasitic nematodes and play an important role in plant-nematode interactions.

VI. Conclusions (Suggestion for future research)

This project demonstrates that EST generation is an effective method for discovery of new genes in plant parasitic nematodes. Previous characterization of the *Bursaphelenchus* species genome has been limited to a very few sequences which were used only for phylogenetic analysis and diagnostic purposes. The EST sequences from this study will provide a solid base for future research to investigate the biology, the pathogenicity and the evolutionary history of this nematode.

One of the most remarkable findings of the EST analysis was the identification of genes which encode cell wall degrading enzymes that are likely to be important for plant parasitism. Three distinct types of cell wall degrading enzyme genes were identified from the EST dataset; cellulase, β -1,3-glucanase and pectate lyase. Molecular characterization of these genes showed that these are endogenous nematode genes and that the nematode uses a mixture of enzymes to attack the plant or fungal cell wall.

It is likely that horizontal gene transfer has played an important role in the evolution of plant parasitism in at least two major groups of plant-parasitic nematodes. Studies on other nematodes, particularly the less intensively studied ectoparasites such as *Trichodorus* and the fungal feeding nematodes belonging to the groups different from *Bursaphelenchus* such as *Aphelenchus* and *Tylencholaimus*, would be useful in order to determine whether the presence of cellulases or other cell wall degrading enzymes is a requirement for nematode parasitism of plants and whether HGT has driven the evolution of plant parasitism in other nematode groups. As more genome sequences

are obtained from a wider range of nematodes and as EST datasets are compared to one another and analysed in more detail it is possible that other horizontally acquired genes may be identified and the role that this process plays in the evolution of nematodes will be fully appreciated.

V. Publications

- Kikuchi, T., Jones, J.T., Aikawa, T., Kosaka, H., and Ogura, N. (2004) A family of glycosyl hydrolase family 45 cellulases from the pine wood nematode *Bursaphelenchus xylophilus*, FEBS Letters, **572**, 201-205.
- Kikuchi, T., Shibuya, H., and Jones, J.T. (2005) Molecular and biochemical characterization of an endo-beta-1,3-glucanase from the pinewood nematode *Bursaphelenchus xylophilus* acquired by horizontal gene transfer from bacteria, Biochemical Journal, **389**, 117-125.
- Kikuchi, T. (2005) Genomics in plant pathogenic microbes, Journal of the Japanese Forest Society, **87**, 251-260 (in Japanese).
- Kikuchi, T., and Ogura, N. (2005) Cellulases from the pine wood nematode, Green Age, 2005/3, 18-21 (in Japanese).
- Jones, J.T., Furlanetto, C., and Kikuchi, T. (2005) Horizontal gene transfer from bacteria and fungi as a driving force in the evolution of plant parasitism in nematodes., Nematology, **7**, 641-646.
- Kikuchi, T., Shibuya, H., Aikawa, T., and Jones, J.T. (2006) Cloning and characterization of pectate lyases expressed in the esophageal gland of the pine wood nematode *Bursaphelenchus xylophilus*, Mol. Plant-Microbe Interact., **19**, 280-287.

VI. Project members

Chapter 1: Taisei Kikuchi, Takuya Aikawa, Hajime Kosaka, Nobuo Ogura

Chapter 2: Taisei Kikuchi, Takuya Aikawa

XI. Project components

- I. Construction of cDNA libraries from the pinewood nematode *Bursaphelenchus xylophilus* (2003-2004).
- II. EST generation and analysis (2003-2005).

Chapter 1

EST analysis of the pine wood nematode *Bursaphelenchus xylophilus*

Objectives

The *C. elegans* genome sequence, was completed several years ago (The-Caenorhabditis-elegans-Sequencing-Consortium, 1998) and since then substantial annotation of the sequence has taken place. Although genome sequencing projects are well underway for several other nematode species including *C. briggsae*, *Haemonchus contortus* and *Brugia malayi* such resources are not available for many other nematodes. Consequently, many parasitic nematode genomes are being explored using Expressed Sequence Tags (ESTs). Analysis of ESTs by single pass random sequencing of cDNA libraries is a powerful tool for rapid and cost-effective gene discovery. High-throughput projects on more than 30 nematode species have generated nearly 300,000 ESTs from parasitic nematodes including datasets from animal parasites and plant parasites (Table 1-1) (Parkinson et al., 2003; Parkinson et al., 2004). Including the sequences from *C. elegans* and *C. briggsae*, there are currently over 640,000 nematode ESTs in the publicly accessible dbEST database. More than 120,000 ESTs have been sequenced from a variety of plant parasitic nematodes (Table 1-1). EST analysis has been a powerful tool for identification of plant parasitic nematode genes which have a possible role in parasitism. For example, genes encoding putative parasitism proteins including pectate lyase (Popeijus et al., 2000), cellulase (Rosso *et al.*, 1999; Smant *et al.*, 1998), polygalacturonase (Jaubert et al., 2002), expansin (Qin et al., 2004) and chorismate mutase (Jones *et al.*, 2003; Lambert *et al.*, 1999) were first identified in ESTs from cyst (*Globodera* and *Heterodera*) and root-knot (*Meloidogyne*) nematode species.

Most of this analysis has been performed on the economically important Tylenchid nematodes. Almost all plant parasitic nematodes in Table 1-1 are Tylenchid, with the exception of several groups of ectoparasitic nematodes such as *Xiphinema spp.*, *Trichodorus spp.* and *Longidorus spp.* which are found in clades I and II of the Phylum. By contrast, previous characterization of the *Bursaphelenchus* species genome has been limited to a very few sequences which were used only for phylogenetic analysis and diagnostic purposes.

B. xylophilus is a plant parasite and is part of the same clade (IVb) as Tylenchoid nematodes. However *B. xylophilus* is not directly related to the plant parasitic Tylenchoid (cyst and root-knot) nematodes but is more closely related to fungal feeding nematodes with which it shares some life cycle characteristics (De Ley and Blaxter,

2002). In order to investigate molecular mechanisms underlying parasitism in *B. xylophilus* and as an entrée to characterizing *B. xylophilus* genome, we performed an EST project on this nematode.

In this chapter, we present an analysis of over 13,000 ESTs from *B. xylophilus* and, by way of contrast, over 3,000 ESTs from a closely related species that does not as readily parasitise plants; *B. mucronatus*.

Methods

Nematodes and culture conditions

B. xylophilus used in this study was the Ka-4 isolate and *B. mucronatus* used was the Un1 isolate. To obtain mixed-stage nematodes, the nematodes were cultured on fungi (*Botrytis cinerea*) for 1 week at 25°C or on pine (*Pinus densiflora*) callus cell cultures and then extracted for 3h at 25°C using the Baermann funnel technique.

B. xylophilus dauer larvae (dispersal 4th stage larvae) were produced using the artificial pupal chamber method in a pine log as described in (Aikawa and Togashi, 1998). Dauer larvae which migrated into the vector beetles were separated by Baermann funnel extraction. Dauer larvae which remained in the logs were collected from the wood surface by hand using a platinum wire picker after the logs had been cut into pieces. Separated nematodes were washed with 0.1M NaCl and 30% sucrose (Lewis and Fleming, 1995) and stored at -80°C.

Construction of cDNA libraries

Poly(A)⁺ RNA was extracted from frozen nematodes using an RNeasy kit (Qiagen) and subsequently an oligotex-dT30 mRNA purification Kit (Takara, Japan) following the manufacturer's instructions. Analysis of the total RNA on a denaturing agarose gel showed a smear from 50 to 3,000 bp with two distinct bands of ribosomal RNA. Two of the cDNA libraries, K1 and U1, were constructed using the SuperScript Plasmid system (Invitrogen). Inserts were directionally cloned in the *NotI* and *SalI* sites of the pSPORT1 vector. The libraries KP, KDi and KDw were constructed using the SMART PCR cDNA amplification method (Clontech). SMART-amplified cDNAs were directionally ligated into the pDNR-lib vector. Each plasmid construct was transformed into *Escherichia coli* DH5 α by electroporation.

EST generation

Individual transformants from the cDNA libraries were picked into 96 well plates containing 0.5 ml of Plusgrow medium (Nacalai Tesque) and appropriate antibiotics. Plates were incubated overnight at 37°C. A small aliquot of each culture was stored at -80°C after being mixed with same volume of 25% glycerol in Plusgrow. Plasmid DNA was isolated and purified according to the glass beads method described in Dederich et al. (2002).

cDNA inserts were sequenced from the 5' end using the M13-T7 primer (5'-TAATACGACTCACTATAGGG-3') and BigDye terminator ver. 3.1 kit (Applied Biosystems) on an ABI 3100 DNA sequencer (Applied Biosystems).

Raw sequence trace data from the 3100 sequencer were processed in an automated pipeline, the trace2dbEST package. Before submitting them to the public database (DDBJ), sequences were processed to assess quality, remove vector sequence, remove contaminants and cloning artifacts and to identify BLAST similarities.

Clustering and sequence analysis

Clustering was performed using PartiGene, a software pipeline designed to analyze and organize EST data sets. Sequences were clustered into groups (putative genes) on the basis of sequence similarity using CLOBB (Parkinson et al., 2002). Clusters were assembled to yield consensus sequences using Phrap (P. Green, unpublished data). Then, each consensus sequence was subjected to BLAST analysis against the GenBank non-redundant protein and nucleotide databases.

Results and Discussion

cDNA libraries and EST generation

Four libraries from *B. xylophilus* and one library from *B. mucronatus* were made in order to generate ESTs (Table 1-2). Sixteen clones were randomly selected from each libraries and the length of their cDNA insert were measured after digestion with appropriate restriction enzymes. The average insert size in the K1, Kp, KDi, KDw and U1 libraries were 1.3kb, 1.2kb, 0.8kb, 0.9kb and 1.3kb respectively. Using these libraries, 13,327 ESTs from *B. xylophilus* and 3,193 ESTs from *B. mucronatus* were generated. 69% of the total *B. xylophilus* ESTs were from the mixed-stage library (K1) derived from the nematodes feeding on fungi, 11% were from the KP library made from nematodes feeding on plant material and 20% were from the two dauer larvae libraries

(KDi and KDw) (Table 1-3).

Cluster formation

To reduce data redundancy, improve base accuracy, increase transcript length and determine gene representation within the library, ESTs from the *B. xylophilus* and *B. mucronatus* libraries were grouped by identity into clusters (putative genes). The 13,327 *B. xylophilus* ESTs grouped into 6,487 clusters and the 3,193 *B. mucronatus* ESTs formed 2,219 clusters. Clusters of *B. xylophilus* varied in size from a single EST (4,377 cases) to 251 ESTs (1 case) (Fig. 1-1). *B. mucronatus* clusters varied from a single EST (1,794 cases) to 73 ESTs (1 case) (Fig. 1-2). In both species, the great majority of clusters contained 10 or fewer ESTs, demonstrating the representation of the libraries used for this analysis. Assuming 19-20,000 total genes as in *C. elegans*, these clusters are likely to represent 30% of all *B. xylophilus* genes and 11% of *B. mucronatus* genes.

Transcript abundance and highly represented genes

In *B. xylophilus* the 25 most abundantly represented clusters account for 10% of ESTs (Table 1-4). A high level of representation in a cDNA library generally correlates with a high level of expression in the original biological sample, although artificial bias can be introduced during library construction. Transcripts abundantly represented in the *B. xylophilus* libraries included genes encoding cytoskeleton proteins (such as actin, myosin, collagen) and proteins that carry out core eukaryotic energetic and metabolic processes (such as cytochrome C oxidase, ADP/ATP translocase, elongation factor protein) (Table 1-4). Similar genes were seen in the *B. mucronatus* abundant clusters (Table 1-5).

A variety of proteins potentially important in the parasitic process of *B. xylophilus* and *B. mucronatus* were present in the K1 and U1 libraries. These included genes that could encode proteins important in fungal feeding as well as proteins that break down various components of the plant cell wall. These sequences are analysed in more detail in chapters 2. This demonstrates that EST generation is an effective method for discovery of the new genes in plant parasitic nematodes.

Conclusion

This project demonstrates that EST generation is an effective method for discovery of new genes in plant parasitic nematodes. Previous characterization of the *Bursaphelenchus* species genome has been limited to a very few sequences which were

used only for phylogenetic analysis and diagnostic purposes. The EST sequences from this study will provide a solid base for future research to investigate the biology, the pathogenicity and the evolutionary history of this nematode. In addition further bioinformatic analysis, including functional categorization and a detailed comparative analysis of the ESTs, will provide useful information to investigate the dauer biology of *B. xylophilus*.

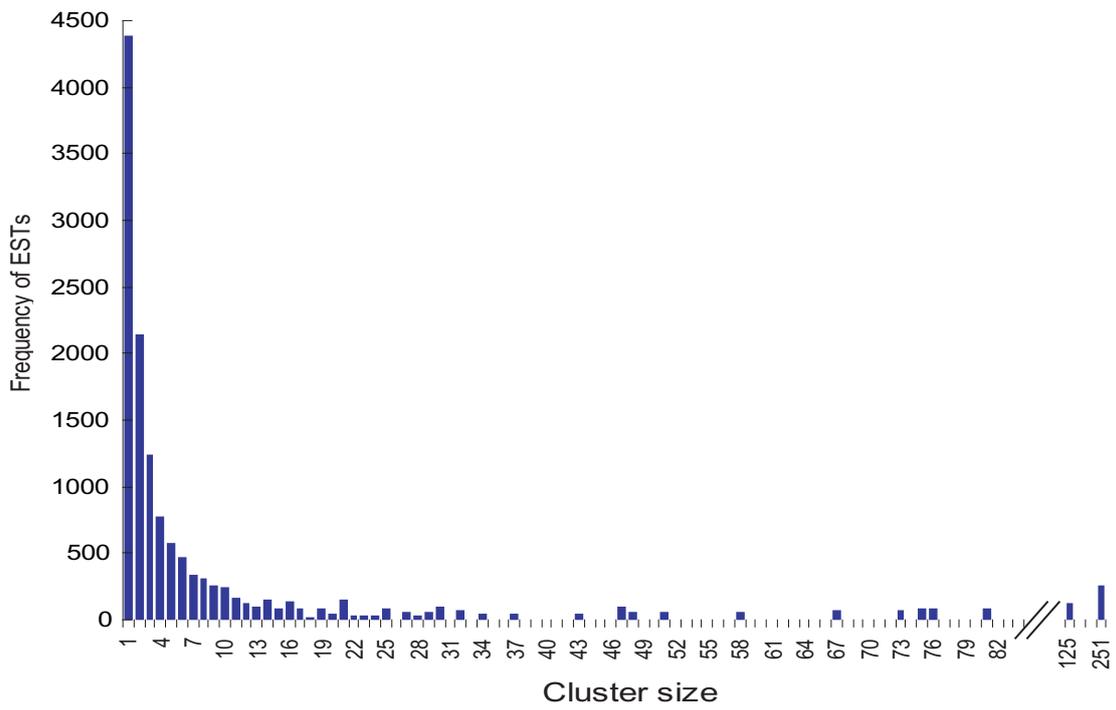


Fig. 1-1. Distribution of *B. xylophilus* ESTs by cluster size. For example, there were three cluster of size 25 containing a sum of 75 ESTs.

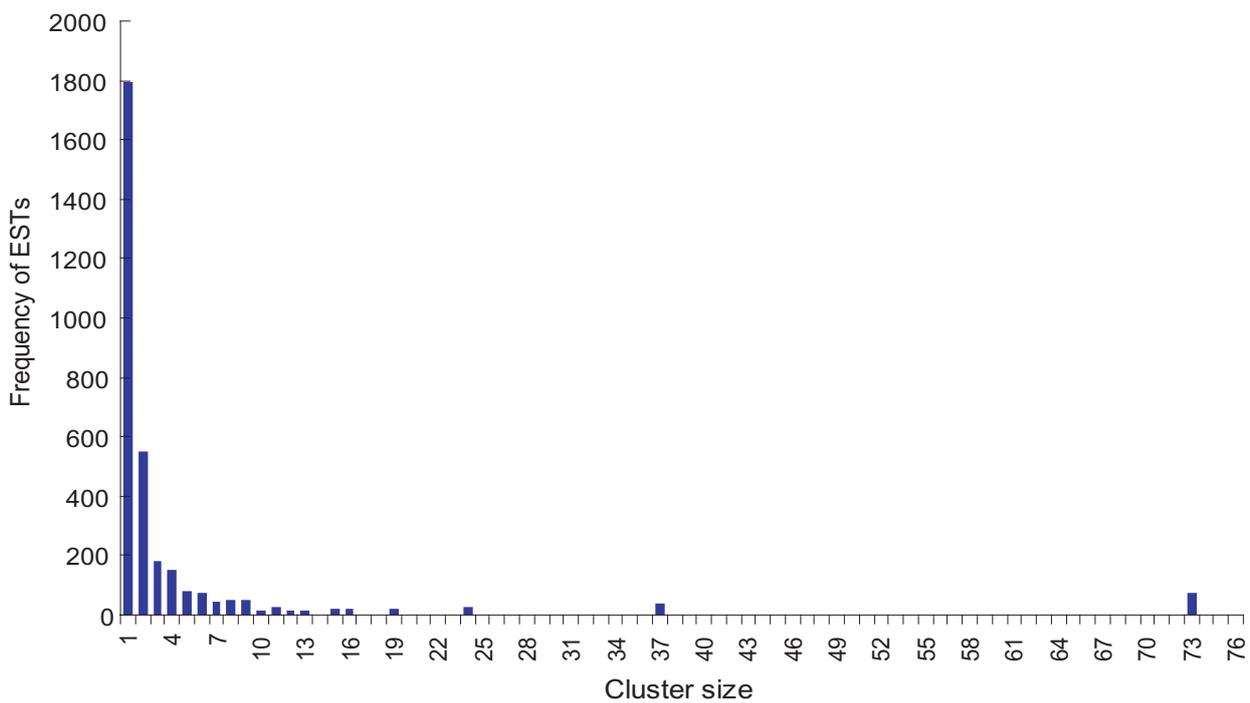


Fig. 1-2. Distribution of *B. mucronatus* ESTs by cluster size. For example, there were nine cluster of size 9 containing a sum of 45 ESTs.

Table 1-1. Nematode Expressed Sequence Tag Projects

Species	Description	Submitted ESTs ^a	Planned ESTs	Clades ^b	Sequencing center ^c
<i>Globodera pallida</i>	Potato cyst nematode	4,378	4,378	IVb	GSC, SCRI, WAG
<i>Globodera rostochiensis</i>	Potato cyst nematode	5,934	5,934	IVb	SCRI, WAG
<i>Heterodera glycines</i>	Soy bean cyst	24,438	11,500	IVb	GSC
<i>Heterodera schachtii</i>	Sugar beat cyst nematode	2,818	3,000	IVb	GSC
<i>Meloidogyne arenaria</i>	Root-knot nematode	5,171	91,500 (all mel sp.)	IVb	GSC
<i>Meloidogyne chitwoodi</i>	Root-knot nematode	12,218	---	IVb	GSC
<i>Meloidogyne hapla</i>	Root-knot nematode	24,452	---	IVb	GSC
<i>Meloidogyne incognita</i>	Root-knot nematode	19,934	---	IVb	GSC, WAG
<i>Meloidogyne javanica</i>	Root-knot nematode	7,587	---	IVb	GSC
<i>Meloidogyne paranaensis</i>	Root-knot nematode	3,710	---	IVb	GSC
<i>Pratylenchus penetrans</i>	Cobb's lesion nematode	2,048	2,048	IVb	GSC
<i>Pratylenchus vulnus</i>	Plant lesion nematode	2,485	unknown	IVb	GSC
<i>Rodopholus similis</i>	Migratory endoparasite	1,154	1,154	IVb	GSC
<i>Xiphinema index</i>	California dagger nematode	9,349	9,348	I	GSC, SCRI
<i>Ancylostoma caninum</i>	Dog hookworm	9,331	11,500	V	GSC
<i>Ancylostoma ceylanicum</i>	Human hookworm	10,651	6,000	V	GSC
<i>Ancylostoma duodenale</i>	Human hookworm	0	9,500	V	GSC
<i>Ascaris suum</i>	Swine gut parasite	39,242	25,000	III	GSC, ED, PSU
<i>Ascaris lumbricoides</i>	Human gut parasite	1,822	6,000	III	ED, PSU
<i>Brugia malayi</i>	Human lymphatic parasite	26,215	3,500	III	FGP, GSC
<i>Dirofilaria immitis</i>	Canine heart worm	4,005	5,000	III	GSC
<i>Haemonchus contortus</i>	Sheep gut parasite	21,967	5,000	V	GSC, ED, PSU
<i>Necator americanus</i>	Human hookworm	4,766	~ 16,000	V	ED, Sanger
<i>Onchocerca volvulus</i>	Human filarial parasite	14,974	1,230	III	FGP
<i>Ostertagia ostertagi</i>	Cattle gut parasite	7,009	10,000	V	GSC
<i>Parastrongyloides trichosuri</i>	Possum gut parasite	7,963	10,000	IVa	GSC
<i>Strongyloides ratti</i>	Rodent gut parasite	14,761	21,500	IVa	GSC
<i>Strongyloides stercoralis</i>	Human gut parasite	11,392	10,922	IVa	GSC
<i>Teladorsagia circumcincta</i>	Sheep gut parasite	4,313	~ 16,000	V	ED, Sanger
<i>Toxocara canis</i>	Canine gut parasite	4,889	5,000	III	GSC, ED
<i>Trichinella spiralis</i>	Human muscle parasite	10,767	23,000	I	GSC
<i>Trichuris muris</i>	Mouse threadworm	3,063	8,000	I	ED, Sanger
<i>Trichuris trichuria</i>	Human threadworm	0	8,000	I	ED, Sanger
<i>Trichuris vulpis</i>	Dog whipworm	3,063	3,000	I	GSC
<i>Wuchereria bancrofti</i>	Human bancroftian filariasis	4,847	unknown		GSC
<i>Caenorhabditis briggsae</i>	Free living	2,424	2,424	V	GSC
<i>Caenorhabditis elegans</i>	Free living	302,074	-	V	NIG, GSC, TIGR, Sanger
<i>Pristionchus pacificus</i>	Free living	8,818	12,096	V	GSC
<i>Zeldia punctata</i>	Free living	391	1,000	IVb	GSC
<i>Bursaphelenchu xylophilus</i>	Pine wood nematode	-	13,000	IVb	FFPRI
<i>Bursaphelenchu mucronatus</i>	Pine wood nematode	-	3,000	IVb	FFPRI
Total		644,423			

^a ESTs deposited as at 10 October 2004.

^b The phylum Nematoda has previously been defined into five clades (Dorris et al., 1999).

^c **GSC**, Genome Sequencing Center, Washington University, USA; **SCRI**, Scottish Crop Research Institute, UK; **WAG**, Wageningen University, the Netherlands; **ED**, University of Edinburgh, UK; **PSU**, Pathogen Sequencing Unit, Sanger Institute, UK; **FGP**, The Filarial Genome Network, UK; **NIG**, National Institute of Genetics, Japan; **TIGR**, The Institute for Genome Research, USA; **FFPRI**, Forestry and Forest Product Research Institute, Japan.

Table 1-2. cDNA Libraries Used for Generating ESTs

Species	Library name	Strain	Stage	Description	Average insert size
<i>B. xylophilus</i>	K1	Ka4	Mixed stage	Vigourously growing on fungi	1.3k
	Kp	Ka4	Mixed stage	Growing on plant	1.2k
	KDw	Ka4	Dauer larvae (JIV) ^a	Separated from wood	0.8k
	KDi	Ka4	Dauer larvae (JIV) ^a	Separated from insect	0.9k
<i>B. mucronatus</i>	Un1	U1	Mixed stage	Vigourously growing on fungi	1.3k

^a JIV, dispersal 4th stage juveniles.

Table 1-3. The Clones and ESTs Generated from the *Bursaphelenchus* Libraries

Species	Library	Clones ^a	ESTs ^b	success rate (%)	average length
<i>B. xylophilus</i>	K1	9792	9194	94	578
	Kp	1824	1476	81	550
	KDw	960	658	69	442
	KDi	3264	1999	61	455
	Bx total		15840	13327	84
<i>B. mucronatus</i>	U1	3456	3193	92	564

^a Clones were randomly picked up from each library to generate sequences. ^b Sequences containing more than 100 bases with PHRED quality =>20 after removing contaminants (vectors and bacterial sequences) were submitted to the public database as ESTs.

Table 1-4. The Most Abundantly Represented Transcripts in the *B. xylophilus* cDNA Libraries

NO.	Cluster ID	ESTs	ESTs from library			Non-redundant Genbank		
			K1	KP	KD	Best identity descriptor	Accession	E-value
1	BXC00007	251	218	9	24	cytochrome oxidase subunit I [Ancylostoma duodenale]	CAD10437	e-109
2	BXC00034	125	60	12	53	cytochrome oxidase subunit III [Necator americanus]	CAD88796	4e-69
3	BXC00032	81	76	2	3	ACT2, actin 2 [Onchocerca volvulus]	AAA29410	e-166
4	BXC00111	76	67	0	9	cytochrome b [Steinernema carpocapsae]	AAT00527	2e-72
5	BXC00695	75	18	10	47	novel		
6	BXC00145	73	69	2	2	elongation factor protein 3 [Caenorhabditis elegans]	AAA81688	e-160
7	BXC00237	67	47	16	4	putative cystein protease [Caenorhabditis elegans]	AAB65956	1e-84
8	BXC00760	58	45	2	11	cytochrome oxidase subunit II [Necator americanus]	CAD10450	9e-71
9	BXC00160	51	25	0	26	heat shock protein 16-1 [Caenorhabditis elegans]	AAA28066	4e-08
10	BXC02760	48	9	12	27	ADP/ATP translocase [Caenorhabditis elegans]	CAA53718	e-114
11	BXC00200	47	33	14	0	ADP/ATP translocase [Caenorhabditis elegans]	CAA53718	e-116
12	BXC00253	47	39	2	6	tropomyosin [Heterodera glycines]	AAQ12016	2e-83
13	BXC00074	43	26	0	17	heat shock protein 16.1 [Caenorhabditis elegans]	AAB04839	7e-09
14	BXC00002	37	27	0	10	ATPase subunit 6 [Necator americanus]	CAD88793	8e-60
15	BXC00392	34	21	4	9	translationally controlled tumor protein-like protein [Wuchereria bancrofti]	AAK71499	1e-74
16	BXC00133	32	23	4	5	ornithine decarboxylase antizyme [Onchocerca volvulus]	AAF68270	4e-17
17	BXC00686	32	18	4	10	hypothetical protein Y69E1A.1 [Caenorhabditis elegans]	CAA22256	3e-27
18	BXC00003	30	25	0	5	gex interacting protein 7 [Caenorhabditis elegans]	AAN84883	e-115
19	BXC00742	30	16	3	11	actin protein 4 [Caenorhabditis elegans]	AAT92068	e-131
20	BXC00966	30	10	13	7	ribosomal protein, large subunit 7A [Caenorhabditis elegans]	AAK84600	3e-75
21	BXC00103	29	23	0	6	heat shock protein 70 [Heterodera glycines]	AAG47839	e-108
22	BXC00446	29	27	2	0	glutamine synthetase (glutamate-ammonia ligase) (gln-3) [Caenorhabditis elegans]	CAB60321	e-135
23	BXC01658	28	28	0	0	cuticle preprocollagen [Meloidogyne incognita]	AAC48358	2e-27
24	BXC00126	27	26	1	0	mitochondrial ATP synthase alpha [Caenorhabditis briggsae]	CAE64108	e-125
25	BXC00643	27	14	2	11	actin protein 4 [Caenorhabditis elegans]	AAT92068	e-131

Table 1-5. The Most Abundantly Represented Transcripts in the *B. mucronatus* cDNA Library

NO.	Cluster ID	ESTs	Non-redundant Genbank		
			Best identity descriptor	Accession	E-value
1	BMC00042	73	cytochrome c oxidase subunit I [Steinernema carpocapsae]	AAT00530	0.0
2	BMC00049	37	elongation factor protein 3 [Caenorhabditis elegans]	AAA81688	e-156
3	BMC00151	24	actin 2 [Onchocerca volvulus]	AAA29410	e-160
4	BMC00090	19	heat shock protein 16-48a [Caenorhabditis elegans]	AAA28067	1e-13
5	BMC00239	16	heat shock protein 70 A2 [Panagrellus redivivus]	AAN84543	e-162
6	BMC00465	15	cytochrome oxidase subunit III [Necator americanus]	CAD88796	1e-68
7	BMC00312	13	glyceraldehyde-3-phosphate dehydrogenase [Ascaris suum]	BAB68543	e-146
8	BMC00371	12	cuticle preprocollagen [Meloidogyne incognita]	AAC48358	4e-27
9	BMC00032	11	ADP/ATP translocase [Caenorhabditis elegans]	CAA53718	e-177
10	BMC00124	11	cytochrome oxidase subunit II [Necator americanus]	CAD10450	4e-65
11	BMC00023	10	heat shock protein protein 17 [Caenorhabditis elegans]	AAQ62453	1e-10
12	BMC00013	9	alcohol dehydrogenase 4 [Saccharomyces kluyveri]	AAP51049	e-106
13	BMC00284	9	ATPase subunit 6 [Caenorhabditis elegans]	AAO16323	2e-61
14	BMC00296	9	heat shock protein 16-1 [Caenorhabditis elegans]	AAA28066	3e-8
15	BMC00324	9	aldehyde dehydrogenase protein 1[Caenorhabditis elegans]	AAA20615	e-164
16	BMC00552	9	cysteine proteinase PWCP1 [Paragonimus westermani]	AAF21461	2e-81
17	BMC00268	8	60S acidic ribosomal protein P0 [Ixodes scapularis]	AAV66850	e-108
18	BMC00316	8	tropomyosin [Heterodera glycines]	AAQ12016	3e-97
19	BMC00472	8	glutamine synthetase (gln-3) [Caenorhabditis elegans]	CAB60321	e-168
20	BMC00481	8	putative mitochondrial ATP synthase alpha	CAE64108	e-179
21	BMC00483	8	probable arginine kinase [Caenorhabditis elegans]	CAB00062	e-153
22	BMC00507	8	fatty Acid CoA Synthetase (acs-2)[Caenorhabditis elegans]	CAB03012	e-104
23	BMC00057	7	collagen family member (col-84) [Caenorhabditis elegans]	CAA88865	1e-22
24	BMC00153	7	major sperm protein 78 [Caenorhabditis elegans]	CAB03362	8e-42
25	BMC00174	7	heat shock protein 70 A [Heterodera glycines]	AAN78300	0.0
26	BMC00196	7	myosin Light Chain (mlc-3) [Caenorhabditis elegans]	AAA50701	3e-63
27	BMC00276	7	cathepsin F-like cysteine proteinase [Brugia malayi]	AAT07059	3e-54
28	BMC00781	7	aldolase [Heterodera glycines]	AAG47838	e-157

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Chapter 2

Cloning and characterization of cell wall degrading enzymes from the pine wood nematode *Bursaphelenchus xylophilus*

2-1. Cellulases

Objectives

The plant cell wall is the primary barrier faced by most plant pathogens and the production of enzymes able to degrade this cell wall is of critical importance for plant pathogens including plant parasitic nematodes. Cellulose is a major component of plant cell walls and consequently cellulases (β -1,4-endoglucanases) are produced by many plant pathogens including bacteria and fungi (Barras et al., 1994; Walton, 1994). Endogenous cellulase genes have also been identified from plant parasitic nematodes, including *Heterodera*, *Globodera* (cyst nematode) and *Meloidogyne* (root-knot nematode) species (Goellner et al., 2000; Rosso et al., 1999; Smant et al., 1998). These cellulases are produced within the esophageal gland cells of these nematodes and secreted through the nematode stylet into plant tissues (de Boer et al., 1999). They are therefore likely to facilitate penetration and migration of nematodes into root tissues during parasitism. The proteins encoded by these genes belong to glycosyl hydrolase family (GHF) 5 and are far more similar to bacterial than to eukaryotic cellulases. It has therefore been suggested that these genes have been acquired via horizontal gene transfer from bacteria (e.g. Yan et al., 1998). Other genes thought to be involved in host-parasite interactions in plant parasitic nematodes are also thought to have been acquired by horizontal gene transfer from bacteria (Jaubert et al., 2002; Jones et al., 2003; Popeijus et al., 2000). GHF5 cellulase genes have also been found in one migratory endoparasitic nematode *Pratylenchus penetrans* (Uehara et al., 2001) that is related to cyst and root-knot nematodes. By contrast, there have been few studies on plant parasitism genes in other nematodes that are phylogenetically or ecologically unrelated to cyst or root-knot nematodes.

Molecular phylogenetic analysis using small subunit ribosomal DNA sequences have shown that *B. xylophilus* is part of the same clade (IVb) as cyst/root-knot nematodes and *Pratylenchus* spp. (Blaxter et al., 1998; Dorris et al., 1999). However, *Bursaphelenchus* spp. are not directly related to these nematodes but form a distinct grouping with other fungal feeding nematodes including *Aphelenchoides* spp. (De Ley and Blaxter, 2002). In order to investigate molecular mechanisms underlying

parasitism in *B. xylophilus*, we performed an Expressed Sequence Tag (EST) project on this nematode (Chapter 1). In this sub-chapter, we present the cloning and characterization of a family of GHF45 cellulase genes from *B. xylophilus* identified during this EST project.

Methods

Biological Material

B. xylophilus used in this study was the Ka-4 isolate, which originated from Ibaraki Prefecture, Japan and was subsequently cultured on *Botrytis cinerea* grown on autoclaved barley grains for 1 week at 25°C before being subcultured or harvested for experiments. Nematodes for experiments were separated from *B. cinerea* hyphae on a Baermann funnel (Thorne, 1961) for 2 h at 25°C. The nematodes were then washed 5 times in M9 buffer (42.3 mM Na₂HPO₄, 22 mM KH₂PO₄, 85.6 mM NaCl, 1mM MgSO₄, pH 7.0) with centrifugation at 700 g for 2 min to remove any remaining *B. cinerea* mycelium.

Isolation of cDNA and gDNA Clones

A cDNA library was constructed using mRNA derived from mixed-stage *B. xylophilus* that were vigorously growing on *B. cinerea* at 25°C. The cellulase gene was identified during an EST project carried out using this library (Chapter 1). One full-length cDNA (clone 03BK1-02-C05) encoding a cellulase, designated *Bx-eng-1* was identified during BLAST analyses of sequences generated in this project. Further cDNA clones encoding related but different sequences (*Bx-eng-2* and *Bx-eng-3*) were subsequently identified in ESTs. The plasmid clones from which each of these sequences was obtained were identified and re-sequenced in both directions in order to obtain full-length cDNA sequences.

The *Bx-eng-1*, 2 and 3 genomic coding regions were obtained by polymerase chain reaction (PCR) amplification from *B. xylophilus* genomic DNA extracted as described below, using pairs of gene-specific primers flanking each open reading frame (ORF). Genomic DNA fragments of the 5' flanking regions of *Bx-eng-1*, 2 and 3 were obtained from *B. xylophilus* genomic DNA using the LA PCR in vitro Cloning Kit (Takara Bio, Japan) and gene specific primers following the manufacturers instructions. PCR products were cloned using the pGEM-T Easy vector (Promega) and sequenced.

Genomic Southern Hybridization

Approximately 0.2 ml of packed mixed-stage nematodes were frozen on liquid nitrogen and resuspended in 1 ml of Proteinase K solution (20 mM Tris, [pH 8.0], 50 mM EDTA, 1% SDS, 0.3 mg/ml proteinase K). Nematodes were lysed by adding glass beads (710 to 1180 μm in diameter; Sigma-Aldrich) and agitating at room temperature for 5 min. The nematodes were then incubated at 55°C for 3 h in order to digest proteins. The DNA was phenol/chloroform extracted twice and then precipitated with ethanol. The resuspended DNA was treated with RNase (0.1 mg/ml) at 37°C for 1 h. Five μg of nematode genomic DNA was used for Southern Blotting. *B. cinerea* genomic DNA was extracted from fresh mycelia using a DNeasy Plant kit following the manufacturer's instructions (Qiagen). Seven μg of fungal DNA was used for Southern blotting.

Nematode or *B. cinerea* genomic DNA was digested with *Eco*RI or *Hind*III, and the digestion products were separated on 1% (w/v) agarose gels, and then blotted onto Hybond-N+ Nylon membranes (Amersham bioscience) using standard protocols (Sambrook and Russell, 2001). Blots were hybridized overnight with the digoxigenin-labeled DNA probe described below at 42°C in DIG Easy Hyb buffer (Roche Diagnostics). After washing, chemiluminescent detection was performed with CDP-Star (Roche Diagnostics) according to the manufacturer's instructions. A probe specific to *Bx-eng-1* was made by PCR amplification of the full-length cDNA from the original plasmid with primers ENG00s (5' TCTAAAATGAAGTCTCTTGTG 3') and ENG00a (5' AGTCCTCTAAGCATCGTC 3'). The PCR product was gel purified using a Min elute gel extraction kit (Qiagen) and then labeled with digoxigenin d-UTP using the DIG-High Prime Labeling Kit (Roche Diagnostics).

Expression of Recombinant Protein

The Bx-ENG-1 coding region without the putative signal sequence was amplified by PCR from the original plasmid using primers ENG10s (5' CAGGATACCGGCAAAACCACG 3') and ENG00a with cycling parameters of 94°C (2 min); 30cycles of 94°C (45 s), 53°C (45 s), and 72°C (1 min). The resulting PCR product was ligated directly into the pQE-30UA vector (Qiagen) and then transformed into *Escherichia coli* M15[pREP4] (Qiagen). Plasmids with the insert in the correct reading frame and which showed no sequence changes during the PCR process were selected following sequencing of the plasmid clones.

Recombinant *E. coli* containing these constructs were grown at 25°C in 20 ml LB medium containing 100 mg/ml ampicillin and 25 mg/ml kanamycin until an OD₆₀₀ of 0.7 was reached. Isopropyl-1-thio- β -D-galactoside (IPTG) was added to a final

concentration of 0.1mM and incubation was continued for a further 20 h. The cells were harvested by centrifugation at 10,000 g for 15 min and resuspended in 1 ml of lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0). The cells were sonicated twice for 1 min, and cell debris was removed by centrifugation. The supernatant was used in enzyme assays. The enzymes were purified from the supernatant using HisTrap HP in accordance with the manufacturer's protocols (Amersham Biosciences) and used for raising antiserum described below.

CMC Plate Tests

Cellulase activity assays were performed as described by Mateos et al. (1992). Protein samples were spotted on CMC agarose plates [1% agarose, 0.2% carboxymethyl cellulose (Nacalai Tesque, Japan), in phosphate citric acid buffer (0.05 M K₂HPO₄, brought to pH 5.2 with 1 M citric acid)] and incubated at 40°C for 5 h. The plates were stained with 0.1% Congo red (Sigma-Aldrich) for 30 min and washed with 1 M NaCl solution.

Total homogenate and secretions from *B. xylophilus* were prepared for cellulase activity assays. Approximately 10⁵ nematodes were suspended in 10 ml M9 buffer and incubated for 5 h at 25°C to allow gut contents to be digested. The nematodes were then washed three times with M9 buffer by centrifugation. Half of the nematodes were homogenised and aliquots of the soluble fraction of this homogenate were used in CMC assays. The remaining nematodes were resuspended in 1ml M9 buffer and incubated for 2 days at 10°C. After the nematodes were removed by centrifugation, 20 µl of the resulting supernatant was used in CMC assays in order to test for endoglucanase activity in nematode secretions. Buffer without nematodes put through the same procedures were used as negative controls.

In situ Hybridization and immunolocalization

In situ hybridizations were performed essentially as described by de Boer et al. (1998). Briefly, mixed-stage *B. xylophilus* were washed in M9 buffer and fixed in 4% paraformaldehyde in M9 buffer overnight at 4°C, then for 5 h at room temperature. After randomly cutting the fixed nematodes on glass slides with a razor blade, the nematode sections were permeabilized with proteinase K, methanol, and acetone. The nematode sections were then hybridized overnight at 55°C with digoxigenin labelled sense or antisense probes generated using the ENG00s and ENG00a primers shown above, which were detected after washing using an anti-digoxigenin antibody conjugated to alkaline phosphatase. Bound probe was detected by overnight staining

with nitroblue tetrazolium and bromo-chloro-indolyl phosphate at 4°C. Specimens were examined with differential interference contrast microscopy.

An antiserum was raised against Bx-ENG-1 by injecting recombinant protein purified from *E. coli* into rabbits. Immunofluorescence labeling of *B. xylophilus* with antisera against Bx-ENG-1 was performed essentially as described previously (Goverse et al., 1994). Rabbit antisera raised against Bx-ENG-1 were diluted 1:100 in PBS containing 0.2% Tween 20 and 1% blocking reagent (Roche). The primary antiserum was detected using goat anti-rabbit antibodies conjugated to Alexa 488 (Molecular Probes) used at a final dilution of 1:500. Labeled nematodes were examined with a Nikon fluorescence microscope.

Phylogenetic Analysis

The deduced protein sequences of *B. xylophilus* cellulases were compared with protein sequences of GHF45 cellulases from bacteria, fungi, protists and animals in a phylogenetic analysis. For this analysis all signal peptides were removed. Sequence alignments were constructed using SOAP V1.1 software (Loytynoja and Milinkovitch, 2001). 35 different sets of alignment parameters (gap opening penalties from 6 to 14 in steps of 2, and extension penalties from 0.02 to 0.14 by steps of 0.02) and a criterion of 100% conservation across alignments for filtering out ambiguous alignment-sites were used. Tentative phylogenetic trees for the alignment were calculated with neighbour joining (NJ) and maximum parsimony (MP) methods using PAUP* v. 4.0b10 (Swofford, 2000). The tree topologies thus obtained were subjected to maximum likelihood (ML) analysis using PROTML in the MOLPHY v.2.3b3 package (Adachi and Hasegawa, 1996) with the local rearrangement and the JTT-F options of amino acid substitution model. Local bootstrap probability was estimated using the resampling of estimated log likelihood (RELL) method (Kishino et al., 1990) with the best tree. Two GHF45 sequences (CAC59695 from *Mytilus edulis* and CAA83846 from *Hypocrea jecorina*), which may belong to a subfamily of GHF45 cellulases were excluded from this analysis as their similarities with other GHF45 sequences were so low that stable alignments could not be obtained.

Results

A Family of Endogenous *B. xylophilus* GHF45 Cellulase cDNAs

During an EST project performed on a *B. xylophilus* cDNA library, a clone with

similarity to cellulases was identified and designated *Bx-eng-1*. The *Bx-eng-1* cDNA comprised 762 bp and contained an ORF of 672 bp. The cDNA contained a 6 bp 5' UTR and an 81 bp 3' UTR, which contained a polyadenylation signal (AATAAA) 13nt upstream of the polyA tail. This spacing is similar to that seen for many *Caenorhabditis elegans* genes (Blumenthal and Steward, 1997). The ORF could encode a 23,824 Da protein (Bx-ENG-1) with similarity to GHF45 cellulases from a variety of organisms (see below). A predicted signal peptide of 15 amino acids, which presumably targets the mature peptide for secretion from the cell, was identified at the N-terminal of deduced protein sequence using the Signal P program (Nielsen et al., 1997). Subsequent EST analysis led to the identification of two other cDNAs that could also encode proteins similar to GHF45 cellulases (Fig. 2-1-1) and which also had predicted signal peptides at their N-termini.

Analysis using BLASTX software (<http://www.ncbi.nlm.nih.gov/BLAST/>) showed that the amino acid sequences deduced from the three *B. xylophilus* cDNAs were highly similar to fungal GHF45 cellulases. For example, Bx-ENG-1 shared 66% identity with cellulases from *Scopulariopsis brevicaulis* and *Rhizopus oryzae* (GenBank accession number JC7308 and BAC53988, respectively). Sequence alignment revealed that conserved residues of the GHF45 cellulases were present in the proteins predicted by all three *B. xylophilus* cDNAs (Fig. 2-1-1).

Endogenous Origin of the *B. xylophilus* Endoglucanases

In order to confirm that the *B. xylophilus* cellulase genes were of nematode origin, analysis of genomic DNA was performed. A Southern blot containing genomic DNA from *B. xylophilus*, and from the fungus on which the nematodes were reared, (*B. cinerea*), as a negative control, was made and hybridized with a cDNA probe generated from the *Bx-eng-1* cDNA. The probe hybridized strongly to two fragments in the *Eco*RI and three fragments in the *Hind*III digested of DNA of *B. xylophilus* (Fig. 2-1-2). No signal was obtained from *B. cinerea* genomic DNA. In addition, PCR products were amplified from *B. xylophilus* gDNA for each of the cellulase clones. The gDNA corresponding to the *Bx-eng-1* cDNA contained no introns but the gDNA corresponding to the *Bx-eng-2* and *Bx-eng-3* clones each contained one intron at the site indicated in Fig. 2-1-1. These introns are bordered by canonical *cis*-splicing sequences, are small (98 bp and 132 bp respectively) and AT rich (61.2% and 65.9% respectively), all features commonly found in nematode introns (Blumenthal and Steward, 1997). The intron positions of *Bx-eng-2* and 3 were identical to each other (Fig. 2-1-1), but were different to those of a GHF45 cellulase gene from an insect, *Apriona germari*

(AY451326) (Fig. 2-1-1).

Protein Expression and Cellulase Activity Assays.

To confirm that *Bx-eng-1* encodes a functional cellulase, part of the *Bx-eng-1* ORF (encoding the mature peptide without the signal peptide) was cloned in frame into an expression vector and enzymatic activity of the recombinant protein was analysed. Hydrolysis of CMC was indicated by clear haloes after staining the gel with Congo red with the Bx-ENG-1 sample (Fig. 2-1-3B). No CMC hydrolysis was observed with *E. coli* containing the expression plasmid without *Bx-eng-1* (Fig. 2-1-3A). These data confirm Bx-ENG-1 as a functional cellulase. CMC hydrolysis was also observed with total homogenate (data not shown) and secretions (Fig. 2-1-3C) of *B. xylophilus* in the same assay, while no hydrolysis was detected with buffer controls (data not shown).

Spatial Localization of *Bx-eng-1* mRNA and protein

In situ mRNA hybridization showed that digoxigenin-labeled anti-sense probes generated from *Bx-eng-1* specifically hybridized with transcripts in the esophageal gland cells of *B. xylophilus* (Fig. 2-1-4A). No hybridization was observed in *B. xylophilus* with the control sense cDNA probes of *Bx-eng-1* (Fig. 2-1-4B). Hybridization signals were detected in female, male, and propagative larvae of *B. xylophilus*.

It was not possible to determine precisely which gland cell or cells were the sites of *Bx-eng-1* expression. This is because it is difficult to distinguish each gland cell as the three esophageal gland cells of *B. xylophilus* are dorsally overlapping and all connect to similar positions in the large median esophageal bulb (Nickle et al., 1981).

Immunolocalization studies with antibodies raised against the recombinant Bx-ENG-1 showed that the protein was present in the oesophageal glands of the nematodes. In addition, antibody binding was also observed on the exterior of nematode's head (Fig. 2-1-4C, 4D). No such binding was observed when using pre-immune serum in place of antiserum (not shown).

Phylogenetic Analysis

A phylogenetic tree generated using maximum likelihood analysis from an alignment of the Bx-ENG-1, 2 and 3 deduced proteins with GHF45 cellulases from bacteria, fungi, protists and animals is shown in Fig. 2-1-5. Similar analysis using neighbour joining generated trees with similar topology. This analysis showed that the Bx-ENG-1, 2 and 3 sequences clustered with fungal sequences and were not associated with GHF45

sequences reported from insects.

Discussion

In this sub-chapter, we describe a family of GHF45 cellulases from the pine wood nematode, *B. xylophilus*. In addition we have demonstrated the function of the protein encoded by one of these genes, *Bx-eng-1*. This is the first example of a functional GHF45 cellulase from any nematode. The presence of a 15 amino acid predicted signal peptide at the N-terminus of each of the *B. xylophilus eng* sequences and the localization of the *Bx-eng-1* mRNA in the esophageal glands suggest that Bx-ENG-1 is present in stylet secretions of the nematode. Localization of the protein by immunofluorescence confirms this. Cellulase activity has been reported in homogenates and secretions of *B. xylophilus* (Odani et al., 1985; Yamamoto et al., 1986) and was confirmed in this study. Moreover, close observations of pine tissues infected with *B. xylophilus* suggest that the destruction of pine cells might be a result of cell wall degrading enzymes such as cellulase (Ichihara *et al.*, 2000; Ishida and Hogetsu, 1997; Kusunoki, 1987). Bx-ENG-1, 2 and 3 could therefore be secreted through the nematode stylet into plant tissues, and may participate in the weakening of the cell walls, allowing nematodes to feed and migrate more easily in pine tissues. It is also possible that endoglucanases are used by the nematode to degrade the cell walls of fungi on which it feeds as cellulose, as well as chitin and other polysaccharides, has been shown to be present in the walls of some of the fungi on which *B. xylophilus* feed (Cherif *et al.*, 1993; Jewell, 1974).

The Bx-ENG-1, 2 and 3 predicted proteins showed strong similarity to fungal cellulases and these proteins are not usually present in animals. It was therefore important to confirm the nematode origin of these genes. Several lines of evidence supported this. First, the presence of a poly-A tail at the 3' end of the genes and of introns within the coding regions amplified from gDNA of two of the three cDNAs excluded a bacterial origin for these genes. In addition, the introns showed several features typical of nematode introns including canonical *cis*-splicing signals, a short length and AT rich sequence (Blumenthal and Steward, 1997). Second, in situ hybridization localized the transcripts of *Bx-eng-1* specifically to the esophageal glands of *B. xylophilus*, a region which, despite extensive electron microscope analysis, has never been shown to contain symbiotic bacteria or fungi. Third, the specific hybridization of *Bx-eng-1* probe with *B. xylophilus* genomic DNA in Southern blot analysis confirmed the nematode origin of *Bx-eng-1* and showed that these genes were

not present in the fungus on which the nematode was raised. These data strongly support a nematode origin for the *Bx-eng-1*, 2 and 3 genes.

Cellulases are found in 14 of the 91 known glycosyl hydrolase families (GHFs), which are classified on the basis of amino acid sequence similarity and hydrophobic cluster analysis (Henrissat and Bairoch, 1993) (see also: <http://afmb.cnrs-mrs.fr/CAZY/acc.html>). While other nematode cellulases identified to date are from GHF5, the *B. xylophilus* sequences are more similar to GHF45 cellulases and contain the active site and signature sequence of these proteins. GHF45 cellulases have been found from fungi, bacteria, protists, and a very small number of animals (<http://afmb.cnrs-mrs.fr/CAZY/acc.html>). Phylogenetic analysis showed that Bx-ENG-1, 2 and 3 are more similar to fungal cellulases than those from other organisms. The catalytic domain of the *B. xylophilus* cellulases shows 62-66% overall amino acid identity with cellulases from two fungi, *S. brevicaulis* and *R. oryzae*. This extremely high similarity between *B. xylophilus* cellulases and fungal cellulases, together with the absence of sequences resembling GHF45 cellulases from other nematodes, including *C. elegans* and *C. briggsae* for which full genome sequences are available, suggests that the *B. xylophilus* cellulases might have been acquired via horizontal gene transfer from fungi.

There are few documented examples of horizontal gene transfer from fungi to animals. Detailed bioinformatic analysis has suggested that genes acquired by horizontal transfer from fungi are present within the *C. elegans* genome (Parkinson and Blaxter, 2003), although these sequences are unrelated to those presented here. In addition, a pectinase gene is present in a weevil species (*Sitophilus oryzae*) that may have been acquired by transfer from fungi (Shen et al., 2002) and GHF45 cellulases have been reported from two insect species (*A. germari* and *P. cochleariae*). However the *B. xylophilus* sequences are more similar to those of fungi than the insect sequences. This, coupled with the fact that the intron positions in the insect and nematode genes are different, and the absence of GHF45 cellulases from other insect and nematode species suggests that the nematode and insect genes are unlikely to have been vertically inherited from a common ancestor.

Horizontal transfer of GHF5 cellulases from bacteria to other plant parasitic nematodes has been proposed and it has been suggested that this transfer occurred from a bacterium closely associated with an ancestor of these root-knot and cyst forming plant parasites (Bird and Koltai, 2000; Scholl et al., 2003). The ancestor of *B. xylophilus* was probably a fungal feeder (De Ley et al., 2002), and more closely associated with fungi than with bacteria. We have been unable to detect a GHF5

cellulase gene from *B. xylophilus* using PCR with degenerate primers designed from conserved regions of the amino acid sequence (Rosso et al., 1999; Uehara et al., 2001) (data not shown). Similarly, GHF5 cellulases are not present in the 5,000 ESTs we have analysed from *B. xylophilus* to date and GHF45 cellulases are absent from the extensive EST datasets derived from root-knot and cyst forming nematodes. Although *B. xylophilus* and cyst/root-knot nematodes belong to the same monophyletic group, clade IVb (Blaxter et al., 1998; Dorris et al., 1999), they are not directly related, with *Bursaphelenchus* clustered with other fungal feeders rather than with plant parasitic nematodes. This, coupled with the observation reported here that cyst and root-knot nematodes contain GHF5 cellulases while *B. xylophilus* has only GHF45 cellulases suggests that cyst/root-knot nematodes and *Bursaphelenchus* spp may have evolved both the ability to digest cellulose and the ability to parasitise plants independently and that horizontal gene transfer seems to have played a key role in this process on both occasions. The role of horizontal gene transfer in the acquisition of GHF5 cellulases by cyst and root-knot nematodes has recently been questioned (Lo et al., 2003). However, the presence of different cellulases in different nematode groups, as described here, strengthens the argument that horizontal gene transfer has played a key role in evolution of plant parasitism by nematodes and that this has been the case on more than one occasion.

```

BxENG1 16 -----*
BxENG2 54 -----TTAASSAGGNSADMSYSYVSGTSTGCTTRYWDCKKSCWPGKALIKSG-ETQCDVHDQPHNDGNTOSGCGSSAYSCTECPNAVNDLISGFAAKKLAGSSSSGCCAGC
BxENG3 121 TTKASSTAARTTQASGNSADMSYSYISGGTGGCTTRYWDCKKSCWPGKATLKSQ-ETQCDVHDQPHNDGNTOSGCGSSAYSCTECPNAVNDLISGFAAKKLAGSSSSGCCAGC
R.oryzae 100 KTTTTAKASTPNSSSSSSSCKYSAVSGGASGNGVTRYWDCKKASCWPGKANVSS--PWKSCNKDGVLTALSDSNAGSGCNGGNSYMCNDNCPNAVNDNLAYGFAAAISGGGSSRNCCSCF
S.C_Brev 20 -----QASGNGCTTRYWDCKKSCWPGKAPUSQ-PPMACDINDNPHDGGILWSSGCEPGGGAYMCSHSPNAVDDLALYGNAAWNIIGGQTESDWCNCAY
A.germ1 21 -----VNLNKVPYGISGSGCTTRYWDCKKSCWVENL-AMGCTPWACSAAGSTVAA-SVKSSCVGGTSYMCENQPKSVNSMFAALGVAASTFGADTNMCCACL
A.germ2 21 -----DHHVTPLVGGVSGTGGCTTRYWDCKKSCWKNLKSRSCKPVEACAADGKTVVKE-SVKSAKERGAGAYMCSDDQPKVNSMFAALGVAASTFGADTNMCCACL
P.coch 22 -----APDASPEIVPVDGGLSGNGCTTRYWDCKKSCWKNENITPTMTPEVQCATDQNTVVNA-SVSSGCI-GSSSYMCENQKAFVNSMFAALGVAASTFGADTNMCCACL
          AAAAAAAAAAAAAAAAAA
          *Int
BxENG1 107 ELLEFNDQPVAGKKEVQATNTGGDLGSDN---HFDLMLPGGGVGIFN-GCQAQWKSFAEGWQRYGGVS-SKADCATLPTALQPGCNQRFDFWFKNAQNPQMFFRQVKCAFELTAKSCTIISDDA
BxENG2 170 ELLEFNSGTVNGKKEVQATNTGGDLGSDN---HFDLALPGGGVGIFN-GCTAQWGAAPSAGWGAQYGGVS-SRSDCSULPAKLQPGCDWRFDWFGNSQNPQVTFKQVFCPKTLTDKSKCIKADD-
BxENG3 243 ELLEFNSGTVNGKKEVQATNTGGDLGSDN---HFDLALPGGGVGIFN-GCTAQWGAAPSAGWGSQYGGVS-SRSDCSQLPATLQPGCDWRFDWFGNSQNPQVTFKQVFCPKTLTDKSKCIKADD-
R.oryzae 221 ELLEFNSTVAGKKEVQVNTNTGGDLGSDN---HFDLALPGGGVGIFN-GCASSQWGAAPNDWGSRYGGVS-SASDCSSDPSALQAGCWRFPNWRNAQNPQMFFRQVKCAFELTAKSCTIISDDA
S.C_Brev 115 ELLEFNSTVAGKKEVQVNTNTGGDLGSDN---HFDLALPGGGVGIFN-GCDDWQWEPNPGWGRYGGVS-TRACDSFPEALKAGCWRFPNWRNAQNPQMFFRQVKCAFELTAKSCTIISDDA
A.germ1 123 KLEFQD-ALGKKEVQVNTNTGGDLGSDN---HFDLALPGGGVGIFN-GCASSQWGAAPNDWGSRYGGVS-SRSDCSQLPATLQPGCDWRFDWFGNSQNPQVTFKQVFCPKTLTDKSKCIKADD-
A.germ2 125 RLEKQD-DLGGKKEVQVNTNTGGDLGSDN---HFDLALPGGGVGIFN-GCASSQWGAAPNDWGSRYGGVS-SRSDCSQLPATLQPGCDWRFDWFGNSQNPQVTFKQVFCPKTLTDKSKCIKADD-
P.coch 129 LLEKQD-QLGGKKEVQVNTNTGGDLGSDN---SRIWFFPGGGVGIFN-GCHDQWTPRGAAGDQYGGV-SVEQCSDLPEVLRQGRFRFELNENVSQNPQVTFKQVFCPKTLTDKSKCIKADD-
          Int

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Fig. 2-1-1. Alignment of *B. xylophilus* endoglucanases with other GHF45 endoglucanases. Residues conserved in all proteins are marked in black, residues conserved in a proportion of the proteins marked in shades of grey, with greater levels of conservation indicated by darker shades of grey. The numbers to the left indicate the amino acid position of the respective proteins. Active site residues are marked with asterisks, GHF45 cellulase consensus sequence is underscored with arrowheads. Position of intron in *B. xylophilus* sequences 2 and 3 is indicated by "int" above the alignment, position of the introns in the *A. germari* sequence is indicated by "int" below the alignment. BxENG1, 2 and 3 - *B. xylophilus* sequences, R.oryzae - GHF45 endoglucanase from *Rhizopus oryzae* (fungus - BAC53956), S.C_Brev - *Scopulariopsis brevicaulis* (fungus - JC7308), A.germ1 - *Apriona germari* (insect - AAN78326), A.germ2 - *A.germari* (insect - AAR22385), P.coch - *Phaedon cochleariae* (insect - CAA76931). All signal peptides and N-terminal extensions of BxENG-2 and BxENG-3 that show no similarity to GHF45 sequences have been removed for clarity.

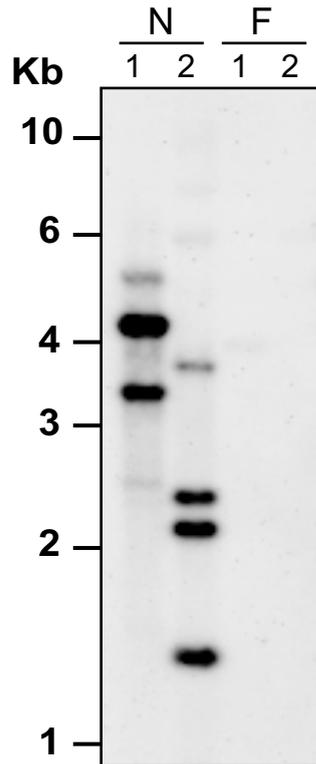


Fig. 2-1-2. Southern blot analysis of *Bx-eng-1*. Genomic DNA from nematode, *B. xylophilus* (N) and fungus, *B. cinerea* (F) were digested with *EcoRI* (1) or *HindIII* (2). The blot was hybridized with a probe generated from *Bx-eng-1* cDNA.

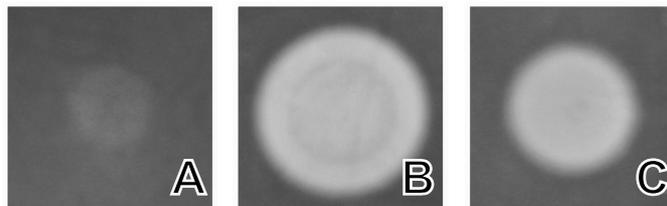


Fig. 2-1-3. Cellulase activity assay on carboxymethylcellulose (CMC) agar plate. Clear halos were detected in total proteins from *E. coli* expressing Bx-ENG-1 (B) and from secretions of *B. xylophilus* (C). No halo was found in the same quantity of total proteins from *E. coli* lacking the *Bx-eng-1* insert (A).

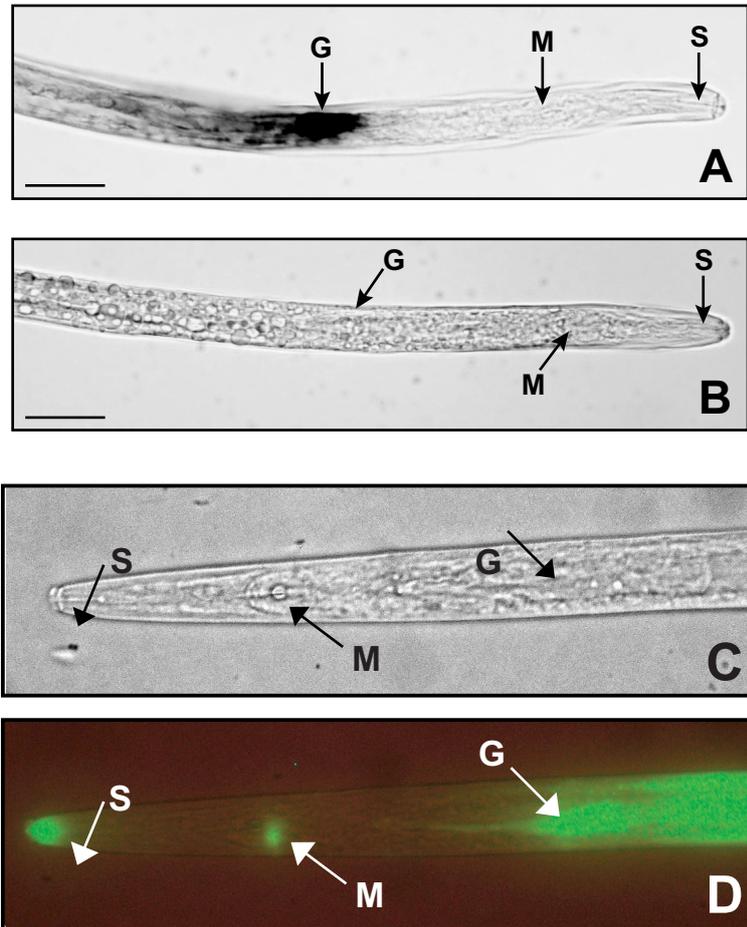


Fig. 2-1-4. Localization of the Bx-ENG-1 transcript and protein. A and B, Localization by in situ hybridization of *Bx-eng-1* transcripts in the oesophageal gland cells of *B. xylophilus* adult female with antisense (A) and sense (B) *Bxeng-1* digoxigenin-labelled cDNA probes. Expression is restricted to oesophageal gland cells. (Bar, 20 μ m.) C and D, Immunofluorescence localization with antiserum against recombinant Bx-ENG-1, showing that the protein is present in the oesophageal gland cells of the nematode and on the exterior of nematode's head. C, illustrates the bright-field image, whereas D, illustrates the same specimen viewed under fluorescence optics. G, oesophageal glands; S, stylet; M, metacarpus.

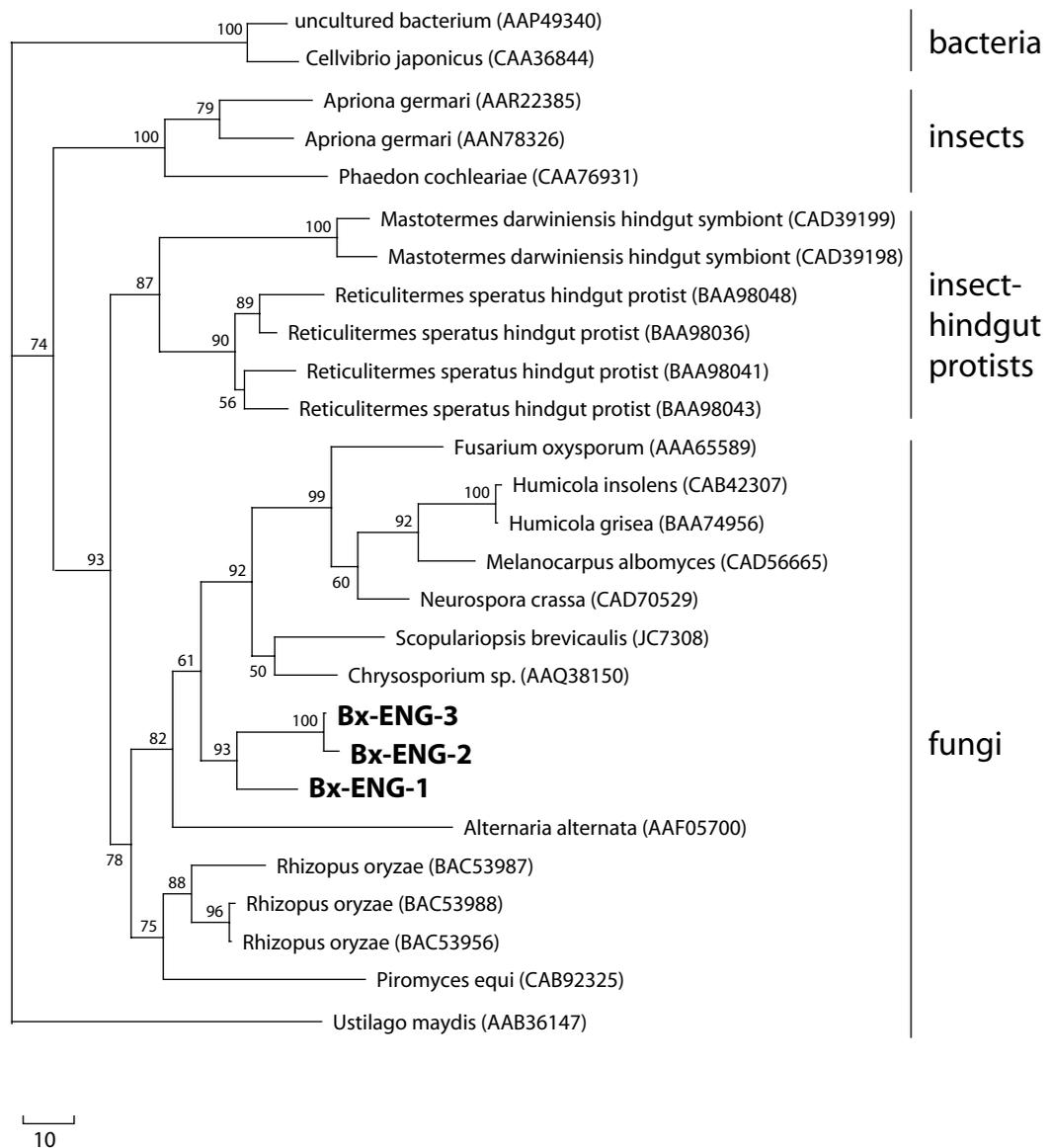


Fig. 2-1-5. Unrooted phylogenetic tree of GHF45 cellulases generated using ML analysis. Bootstrap probabilities for each node are estimated by the REL method. The scale bar represents 10 substitutions per 100 amino acid positions.

2-2. β -1,3-Glucanases

Objectives

β -1,3-Glucanases are widely distributed among bacteria, fungi and higher plants. They are classified into two groups: exo- β -1,3-glucanase (EC 3.2.1.58) and endo- β -1,3-glucanases (EC 3.2.1.6 and EC 3.2.1.39). β -1,3-Glucanases catalyze the hydrolysis of β -1,3-D-glucosidic linkages in β -1,3-D-glucan. This polymer is a major component of fungal cell walls and a major structural and storage polysaccharide (in the form of laminarin) in marine macro-algae (Hong et al., 2002). The physiological functions of β -1,3-glucanases are distinct and depend on their source. In plants, the enzymes are thought to be involved in cell differentiation and defense against pathogenic fungi (Castresana et al., 1990). In bacteria, the enzymes are released to break down fungal cell walls in order to allow them to be used as a food source (Watanabe et al., 1992). In fungi, β -1,3-glucanases may play roles in development and differentiation, β -glucan mobilization, and interactions of plant pathogenic fungi with their hosts (de la Cruz et al., 1995). Although they have the same hydrolytic activity, the bacterial enzymes are classified in glycosyl hydrolase family 16 (GHF16), whereas most plant and fungal enzymes are grouped in GHF17, on the basis of differences in their amino acid sequences (Henrissat and Bairoch, 1993).

In the animal kingdom, functionally characterised β -1,3-glucanases are restricted to marine invertebrates. Genes encoding β -1,3-glucanases have been cloned from the sea urchin *Strongylocentrotus purpuratus* (Bachman and McClay, 1996) and the bivalve mollusk *Spisula sachalinensis* (Kozhemyako et al., 2004). They are classified in GHF16 and thought to be involved in digestion of algal food. In addition, many β -1,3-glucanase-like proteins have been isolated, and the encoding genes cloned, from insects (Dimopoulos et al., 1997; Kim et al., 2000; Ma and Kanost, 2000; Ochiai and Ashida, 2000; Zhang et al., 2003) and other invertebrates (Beschlin *et al.*, 1998; Lee *et al.*, 2000; Seki *et al.*, 1994; Sritunyalucksana *et al.*, 2002). Although these sequences contain regions that are very similar to the activation region of GHF16 β -1,3-glucanases, they have not been shown to exhibit glucanase activity. These proteins bind specifically to β -1,3-glucan, which is found in the cell surface of microbes but is absent in the host, and they have been shown to play a role in the innate immune system by recognizing foreign material. For example, *Manduca Sexta* BGRP (β -1,3-glucan recognition protein) (Ma and Kanost, 2000), crayfish LGBP (lipopolysaccharide- and β -1,3-glucan binding protein) (Lee et al., 2000) and earthworm CCF-1 (coelomic

cytolytic factor-1) (Beschlin et al., 1998) have been shown to mediate activation of the prophenol oxidase activating system (proPO system), which is thought to be a recognition and defense system in many invertebrates (Soderhall and Cerenius, 1998).

Bursaphelenchus is a large group of nematodes that has a world-wide distribution. Most *Bursaphelenchus* species are solely fungal feeders and all species rely on fungi as a food source at some stage of their life cycle. Many *Bursaphelenchus* species feed on fungi colonizing dead trees. *B. xylophilus* and the few other pathogenic species described to date are unique in their capacity to feed on live trees as well as feeding on fungi. Many *Bursaphelenchus* species, including *B. xylophilus*, therefore have a close association with fungi. Since β -1,3-glucan is the main structural component of fungal cell walls, it seems likely that β -1,3-glucanases may play an important role in the life cycle of these nematodes.

In Chapter 2-1, we presented identification and functional characterization of a family of cellulase (endo- β -1,4-glucanase) genes from *B. xylophilus* and showed that these genes were likely to have been acquired by horizontal gene transfer not from bacteria but from fungi (Chapter 2-1) (Kikuchi et al., 2004). It was suggested that these genes were acquired from fungi on which ancestors of the current nematode species fed. These cellulases may have a role in the parasitic process. In this sub-chapter, we present the cloning and biochemical characterization of the nematode β -1,3-glucanase genes, first identified from *B. xylophilus* and *B. mucronatus* during an expressed sequence tag (EST) project.

Methods

Isolation of cDNA and gDNA clones

A cDNA library was constructed using mRNA derived from mixed-stage *B. xylophilus* that were vigorously growing on *B. cinerea* at 25°C. The β -1,3-glucanase gene was identified during an EST project carried out using this library (Chapter 1). One full-length cDNA (clone 03BK1-02-C05) encoding a β -1,3-glucanase, designated *Bx-lam16A* (see Results section below) was identified during BLAST analysis of sequences generated in this project. The plasmid clone from which the sequence was obtained was identified and re-sequenced in both directions in order to obtain full-length cDNA sequences.

The *Bx-lam16A* genomic coding region was obtained by polymerase chain reaction (PCR) amplification from *B. xylophilus* genomic DNA, using gene-specific primer

flanking each open reading frame (ORF). PCR products were cloned using the pGEM-T Easy vector (Promega) and sequenced.

Expression and purification of recombinant protein

The *Bx-lam16A* coding region without the putative signal sequence was amplified by PCR from the original plasmid using the primers bl3-1s (5' GGAGTCATTTGGCAAGAGGAC 3') and bl3-0a (5' CACCGAAAACACTACAACGT 3'). The resulting PCR product was ligated directly into the pQE-30UA vector (Qiagen) and then transformed into *Escherichia coli* M15[pREP4] (Qiagen). Plasmids with the insert in the correct reading frame and which showed no sequence changes during the PCR process were selected following sequencing of the plasmid clones. Recombinant *E. coli* containing these constructs were grown at 25°C in 500 ml LB medium containing 100 mg/ml ampicillin and 25 mg/ml kanamycin until an OD₆₀₀ of 0.5 was reached. Isopropyl-thio-β-D-galactoside (IPTG) was added to a final concentration of 0.1 mM and incubation was continued for a further 10 h. The cells were harvested by centrifugation at 9,000 g for 15 min and resuspended in 1 ml of lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0). The cells were sonicated twice for 1 min, and cell debris was removed by centrifugation. The enzymes were purified from the supernatant using HisTrap HP in accordance with the manufacturer's protocols (Amersham Biosciences).

SDS/PAGE and western blot analysis

An antiserum was raised against BxLAM16A by injecting recombinant protein purified from *E. coli* into rabbits. For SDS-PAGE analysis, protein samples were prepared by boiling for 5 min in an equal volume of 2x sample buffer (100 mM Tris, pH 6.8, 12% 2-mercaptoethanol, 4% SDS, 20% glycerol, and 0.01% bromophenol blue) and run on 12% poly acrylamide gels prepared using standard protocols. Proteins within the gels were either stained with Coomassie blue or transferred to poly-vinylidene-difluoride membrane for Western blot analysis. Membranes were blocked with 1% (w/v) blocking reagent (Roche) and 1% (v/v) normal goat serum in phosphate-buffered saline (PBS) with 0.2% Tween 20 for 16 h at 23°C. Blots were then immunolabeled for 3 h at 23°C with the antiserum against BxLAM16A diluted 1:1000 in PBS containing 0.2% Tween 20. Bound antiserum was detected with alkaline phosphatase conjugated secondary antibodies and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) color reaction.

Total homogenate and secretions from *B. xylophilus* were prepared for western blot

analysis as previously described (Chapter 2-1) (Kikuchi et al., 2004), except that 0.1% laminarin was added to the collection buffer.

Enzyme assays

Assays were performed in a reaction mixture containing 50 μ l of 0.5% (w/v) laminarin (Sigma) (or other substrates where appropriate), 40 μ l of citrate-phosphate buffer (0.1 M citric acid - 0.2 M Na₂HPO₄), pH 4.9, and 10 μ l of appropriately diluted enzyme solution. The pH value of the citrate-phosphate buffer was confirmed at room temperature. After incubation at 40°C for 10 min, the reducing power released was measured by the *p*-hydroxybenzoic acid hydrazide method (Lever, 1972), using D-glucose as the standard. One unit of β -glucanase activity was defined as the amount of the enzyme liberating 1 μ mol of reducing sugar per minute. Xyloglucan endotransferase activity was measured according to the procedure of Nishitani and Tominaga (Nishitani and Tominaga, 1992), using 2-aminobenzamide labeled xyloglucan oligomer (Ishii et al., 2002) as an acceptor and Tamarind xyloglucan as a donor molecule. A mixture of a fluorescence labeled acceptor molecule (11 nmol) and a nonlabeled high M_r polymer (250 μ g) was incubated with the enzyme preparation in 100 μ l of 0.4 x citrate-phosphate buffer (pH 4.9) at 40°C for 10 min. The reaction product was chromatographed on Superdex 75HR (Amersham Biosciences) and detected by refractometry and fluorometry. Protein concentrations were determined by the method of Lowry using the DC Protein Assay system (Bio-Rad), with bovine serum albumin as the standard.

The substrates laminarin (from *Laminaria digitata*), pustulan (from *Umbilicaria papullosa*) and carboxymethyl cellulose were obtained from Nacalai Tesque and glycol chitin from Seikagaku Corporation. Lichenan (from *Cetraria islandica*), β -D-glucan (from barley and bakers yeast) and κ -carrageenan (from *Eucheuma cottonii*) were obtained from Sigma while Xylan (from birch wood) was obtained from Fluka. Agarose was obtained from Wako and Tamarind xyloglucan (Glyloid 6c) was from Dainippon pharmaceutical. 2-Aminobenzamide labeled xyloglucan oligomer was kindly provided by Dr. Tadashi Ishii of Forestry and Forest Products Research Institute.

Detection of hydrolytic products

The hydrolytic products of laminarin were determined by high performance liquid chromatography. 0.25% (w/v) laminarin was digested in 0.4x citrate-phosphate buffer (pH 4.9) at 40°C for various time intervals. The reaction was stopped by incubating at 98°C for 5min. The hydrolysis products were separated at 60°C in H₂O using tandem

connected SUGAR KS-802 columns fitted with a guard column (SUGAR KS-G, Shodex) and detected by refractometry.

Temperature and pH optima, and thermal stability

The optimum temperature was determined by running the standard assay at temperatures from 30 to 80°C. The optimum pH of the enzyme was determined by running the standard assay at 40°C using citrate-phosphate buffer and potassium dihydrogenphosphate-sodium tetraborate buffer (0.1 M KH₂PO₄ – 0.05 M Na₂B₄O₇) for pH ranges 3.0 - 7.4 and 6.4 - 8.9, respectively. Thermal stability was determined using diluted enzyme (0.65 mg/ml of purified enzyme was diluted 100 times in 0.4x citrate-phosphate buffer, pH 4.9), incubated at various temperatures for 60 min, with the residual activity determined using the standard assay at 40°C.

Phylogenetic analysis

The deduced amino acid sequences of *B. xylophilus* and *B. mucronatus* β-1,3-glucanases were compared with sequences of GHF16 β-1,3-glucanase and β-1,3-glucanase-like proteins from bacteria, fungi and animals in a phylogenetic analysis. Protein sequences were aligned with ClustalX version 1.81 (Thompson et al., 1997), using Blosom 30 as the protein weight matrix. Several values for both gap opening and gap extension penalties were tested and a combination of 6 and 1 for these factors was finally adopted as this gave rise to an alignment in which the conserved domains were aligned most accurately with few gaps. Minor corrections were then performed manually on the alignment based on the crystal structure of the endo-β-1,3-1,4-glucanases from *Bacillus licheniformis* and *B. macerans* (Hahn *et al.*, 1995; Keitel *et al.*, 1993). Tentative phylogenetic trees for the alignment were calculated with neighbour joining (NJ) and maximum parsimony (MP) methods using PAUP* v. 4.0b10 (Swofford, 2000). The tree topologies thus obtained were subjected to maximum likelihood (ML) analysis using PROTML in the MOLPHY v.2.3b3 package (Adachi and Hasegawa, 1996) with the local rearrangement and the JTT-F options of amino acid substitution model. Local bootstrap probability was estimated using the resampling of estimated log likelihood (RELL) method with the best tree.

Results

Isolation of *B. xylophilus* β -1,3-glucanase gene

During an EST project performed on a *B. xylophilus* cDNA library, a clone with similarity to β -1,3-glucanases was identified. This cDNA was designated *Bx-lam16A* and the predicted protein BxLAM16A in keeping with the terminology suggested by (Henrissat et al., 1998). The putative full length *Bx-lam16A* cDNA comprised 803 bp containing an ORF of 753 bp that ended with a TAA stop codon. The cDNA contained a 46 bp 3' UTR, which contained a polyadenylation signal (AATAAA) 15nt upstream of the polyA tail. This spacing is similar to that seen for many *Caenorhabditis elegans* genes. The predicted amino acid sequence includes a hydrophobic signal peptide which is predicted by the SignalP program (Nielsen et al., 1997) to be cleaved between Ala-15 and Gly-16, giving rise to a mature peptide with a predicted molecular weight of 26441.73 Da and a predicted isoelectric point of 4.7.

A similarity search based on the BLASTP program showed that the deduced amino acid sequence BxLAM16A was most similar to GHF16 β -1,3-glucanases from bacteria. BxLAM16A shared 49 to 43% identity with β -1,3-glucanases from *Xanthomonas campestris*, *X. axonopodis* and *Pseudomonas sp.* Multiple alignments of the deduced amino acid sequence encoded by *Bx-lam16A* and other β -1,3-glucanases showed that conserved residues of the GHF16 glucanases were present in BxLAM16A (Fig. 2-2-1). A cDNA that could encode a similar protein was found amongst ESTs generated from a related, but solely fungal feeding species, *B. mucronatus*. This cDNA, designated *Bm-lam16A*, encodes a product of 217 amino acid residues exhibiting 73% identity with BxLAM16A (Fig. 2-2-1). The signal peptides of the *Bursaphelenchus* predicted proteins had features expected of eukaryotic signal peptides (a short hydrophobic region followed by a predicted cleavage site) rather than those of bacterial signals for secretion (Nielsen et al., 1997). The *Bursaphelenchus* signal peptides are short (16 amino acids for BxLAM16A) whereas prokaryotic sequences tend to be longer. In addition, the sequence immediately following the peptide cleavage site does not contain Alanine, Aspartic or Glutamic acid, Serine or Threonine residues. Such residues are usually present within 5 amino acids of the cleavage site of prokaryotic signal peptides. However, the BxLAM16A signal peptide does contain Alanine residues at positions -1 and -3 relative to the predicted cleavage site, a feature almost always seen in prokaryotic signal peptides, although this character cannot be considered to be diagnostic.

Endogenous origin of the *B. xylophilus* β -1,3-glucanase

Analysis of genomic DNA was performed to confirm the nematode origin of *Bx-lam16A*.

A Southern blot containing genomic DNA from *B. xylophilus*, and from the fungus on which the nematodes were reared, (*B. cinerea*), as a negative control, was made and hybridized with a DNA probe generated from the *Bx-lam16A* cDNA. The probe hybridized specifically to fragments in both the *Eco*RI and *Hind*III digests of *B. xylophilus* DNA (Fig. 2-2-2). No signal was obtained from *B. cinerea* genomic DNA. In addition, the entire coding region of the *Bx-lam16A* gene was amplified from *B. xylophilus* gDNA, and the resulting PCR product was cloned and sequenced. Analysis of this sequence showed that it was exactly the same as the cDNA sequence, except that one intron was present at the site indicated in Fig. 2-2-1. This intron is 401 bp in length, is bordered by canonical *cis*-splicing sequences, and is AT rich (59.7%). This sequence and the banding pattern observed on the Southern blot suggested that the β -1,3-glucanase is present as a small gene family in *B. xylophilus* as no *Eco*RI or *Hind*III recognition sites are present in the sequenced region of the gene recognized by the probe. Southern blotting of *B. mucronatus* genomic DNA suggested a gene family of similar size to that in *B. xylophilus* is present in *B. mucronatus* (not shown).

Expression and purification of recombinant BxLAM16A

The BxLAM16A coding region without the putative signal sequence was amplified by PCR from the original plasmid, cloned into expression vector, and expressed in *E. coli* as a polyhistidine-tagged fusion protein. The resulting recombinant protein had the expected molecular mass of 29 kDa, corresponding to 26.5 kDa from the *Bx-lam16A* open reading frame and 2.5 kDa encoded by the expression vector from the translational start codon to the cloning site, including a hexa-histidine tag. The recombinant protein was then purified using metal affinity column chromatography to electrophoretic homogeneity (Fig. 2-2-3A).

BxLAM16A was detected in nematode homogenate and secretions by Western blot analysis using antisera raised against recombinant BxLAM16A (Fig. 2-2-3B). The product of nematode appeared to be slightly smaller than that expressed in *E. coli*, which is in agreement with the expected sizes of these proteins. No signal was detected in crude cell extracts of *B. cinerea* on which the nematodes were reared or when pre-immune serum was used to probe Western blots (not shown).

Biochemical analysis of recombinant BxLAM16A

The activities of purified recombinant BxLAM16A with various substrates are summarized in Table 2-2-1. These results indicate that the enzyme had the highest specificity for the soluble β -1,3-glucan laminarin. Lower activity was found with

β -1,3-glucan from bakers yeast, probably because of its low solubility. Very low levels of activity were observed on β -1,3-1,4-linked glucans, such as barley β -D-glucan and lichenan. The β -1,3-1,6-linked glucan (pustulan) and the β -1,4-linked substrates (carboxymethyl cellulose, chitin, and xylan) were not hydrolyzed. Although some GHF16 proteins exhibit agarase, κ -carrageenase or xyloglucan endotransferase activity, BxLAM16A did not have these activities (Table 2-2-1). The mode of action of this enzyme was then examined by analyzing the hydrolysis products by high performance liquid chromatography (Fig. 2-2-4). Laminarin was immediately oligomerized and products larger than laminaribiose appeared. After prolonged incubation the oligosaccharides gradually degraded into laminaribiose and glucose. These results confirm that BxLAM16A is an endo- β -1,3-glucanase (EC 3.2.1.39).

The laminarinase enzyme activity was investigated at different pH values and temperatures (Fig. 2-2-5). The optimum pH was found to be 4.9 and the enzyme retained 90% of its activity between pH 4.5 and 5.8 (Fig. 2-2-5A). There was a sharp decrease in activity below pH 4 and a gradual decrease in activity at increasingly alkaline pH values. The temperature at which maximum activity was observed was 65°C (Fig. 2-2-5B). Thermal stability was investigated by incubating the purified BxLAM16A at various temperatures for 60 min (Fig. 2-2-6). Although the enzyme retained almost all of its activity at 10°C, the residual activities of the enzyme gradually decreased as the incubation temperature increased.

Expression site and protein localization

To determine which nematode tissues express *Bx-lam16A*, *in situ* mRNA hybridization was performed. Digoxigenin-labeled anti-sense probes generated from *Bx-lam16A* specifically hybridized with transcripts in the oesophageal gland cells of *B. xylophilus* (Fig. 2-2-7A). No hybridization was observed in *B. xylophilus* with the control sense cDNA probes (Fig. 2-2-7B). Hybridization signals were detected in female, male, and propagative larvae of *B. xylophilus*. We could not determine precisely which gland cell or cells were the sites of *Bx-lam16A* expression. This is because it is difficult to distinguish each gland cell as the three oesophageal gland cells of *B. xylophilus* are dorsally overlapping and all connect to similar positions in the large median oesophageal bulb (Nickle et al., 1981).

Immunolocalization studies with antibodies raised against the recombinant BxLAM16A showed that the protein was present in the oesophageal glands of the nematodes (Fig. 2-2-7C, D). No such binding was observed when using pre-immune serum in place of antiserum (not shown).

Phylogenetic analysis

A phylogenetic tree generated using maximum likelihood analysis from an alignment of the BxLAM16A and BmLAM16A deduced proteins with selected GHF 16 proteins from bacteria, fungi and animals is shown in Fig. 2-2-8. Analysis using neighbour joining generated trees with similar topology. This analysis showed that the BxLAM16A and BmLAM16A sequences are more closely related to bacterial sequences than animal sequences.

Discussion

In this sub-chapter, we describe the first β -1,3-glucanase gene from any nematode and confirmed its nematode origin.

The presence of a hydrophobic N-terminal sequence (signal peptide) in the deduced amino acid sequence of *Bx-lam16A* and its mRNA expression and protein localization in the oesophageal glands suggest that BxLAM16A is secreted from the stylet of the nematode. Western blot analysis using nematode secretions supports this. Although *B. xylophilus* is a pathogen of trees, it can complete its life cycle feeding solely on fungi, including *Botrytis*, *Alternaria* and blue stain fungi such as *Ophiostoma*. β -1,3-Glucan is the main structural component of the cell walls of these fungi. It is likely therefore that BxLAM16A is secreted from the nematode stylet and weakens fungal cell walls to allow nematode to feed more easily. Further evidence that the enzyme plays a role in fungal feeding rather than in parasitism of plants comes from the fact that a similar cDNA was identified in ESTs from a related nematode species, *B. mucronatus*, that feeds exclusively on fungi.

The biochemical properties of BxLAM16A were similar to those of bacterial β -1,3-glucanases from GHF16. BxLAM16A efficiently hydrolyzed β -1,3-glucans and to a lesser degree lichenan. Similar substrate preferences have been found in bacterial β -1,3-glucanases including those from *Streptomyces sioyaensis* (Hong et al., 2002), *Thermotoga neapolitana* (Zverlov et al., 1997) and *Cellvibrio mixtus* (Sakellaris et al., 1990). The optimum temperature of BxLAM16A is 65°C and its optimum activity was observed at pH 4.9. Many bacterial β -1,3-glucanases show optimal activities between pH 4.0 and 6.0. BxLAM16A seems to be particularly active at temperatures that are much higher than those found in the normal environmental conditions that the nematodes would encounter. This is also the case for several non-thermophilic

bacterial hydrolases including those from *B. circulans* (Asano et al., 2002) and *S. sioyaensis* (Hong et al., 2002). Although the optimum temperature of BxLAM16A was high (65°C), BxLAM16A was heat labile as the enzyme retained only 20% of its original activity after incubation for 1 hour at 60°C. This might indicate that laminarin in the reaction solution contributes to the stabilization of the enzyme.

Many β -1,3-glucanases and β -1,3-glucanase-like proteins from a variety of invertebrates are present in GHF16 (Henrissat and Bairoch, 1993) (<http://afmb.cnrs-mrs.fr/CAZY/>). Phylogenetic analysis showed that these animal proteins were clustered in one clade with the exception of one protein, coagulation factor G, from the horseshoe crab *Tachypleus tridentatus*. BxLAM16A was clustered together with glucanases from bacteria and this was supported by high bootstrap values, indicating that *B. xylophilus* β -1,3-glucanase is more closely related to those from bacteria than similar proteins from eukaryotes. In addition, no nematode sequences similar to the *Bx-lam16A* sequence described here are present in any publicly accessible database, despite the fact that genome sequences for *C. elegans* and *C. briggsae* and over 200,000 EST sequences from other nematodes from across the phylum are now available (Parkinson et al., 2003). It is therefore likely that the *B. xylophilus* endo- β -1,3-glucanase described here was acquired by horizontal gene transfer from bacteria.

In the previous sub-chapter, we suggested that cellulase (endo- β -1,4-glucanase) genes of *B. xylophilus* were acquired by horizontal gene transfer from fungi due to a close association of the ancestor of *B. xylophilus* with fungi rather than with bacteria (Chapter 2-1) (Kikuchi et al., 2004). The findings of this study support the independent horizontal gene transfer hypothesis which suggests that horizontal gene transfer has occurred on several independent occasions within the Nematoda and that it has been an important factor in the evolution of the ability to parasitise plants on each occasion. The data presented here suggest that similar horizontal gene transfer processes have enhanced the ability of a group of nematodes (*Bursaphelenchus* spp) to feed on fungi. It is possible that these nematodes acquired β -1,3-glucanase genes from bacteria to obtain a fungal feeding ability, and a sub group subsequently acquired cellulase genes from fungi which permitted them to parasitise plants.

There are now a number of documented horizontal gene transfer events from both prokaryotes and eukaryotes to nematodes. At least three horizontal gene transfer events have occurred within Tylenchid plant parasitic nematodes (Scholl et al., 2003) and the data we present here shows that at least two events have occurred within the *Bursaphelenchus* group. In addition, a large scale analysis of the complete *C. elegans*

genome sequence suggested that four alcohol dehydrogenase encoding genes were present in *C. elegans* and a closely related nematode that had been acquired by horizontal gene transfer from fungi (Parkinson and Blaxter, 2003). Fewer studies have been performed on horizontal gene transfer in other phylogenetic groups but there have been occasional reports of cell wall degrading enzymes in other invertebrates (e.g. Sugimura et al., 2003). As the number of genome sequencing and EST projects has increased it has become clear that horizontal gene transfer to eukaryotes is a more widespread phenomenon than previously thought. It is also clear that on a limited number of occasions it has allowed organisms to exploit niches that may have previously been unavailable to them.

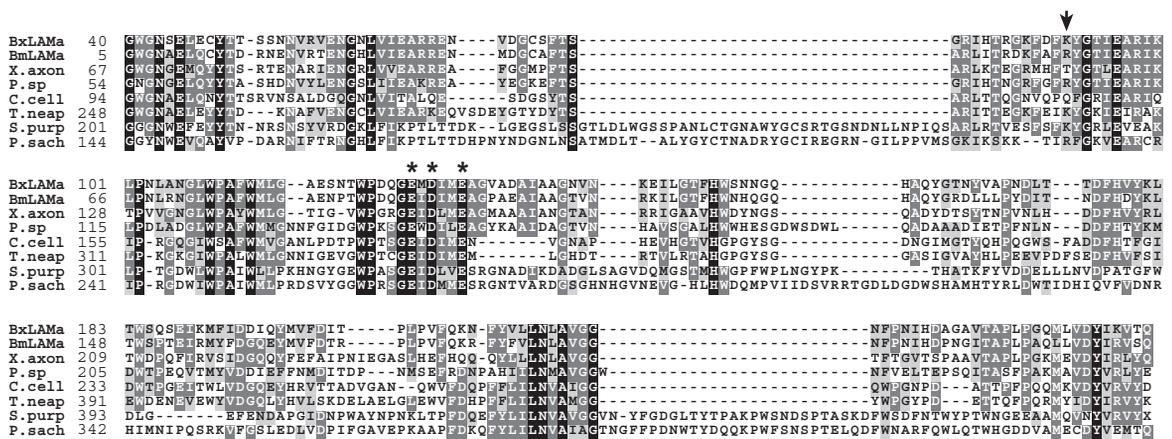


Fig. 2-2-1. Multiple alignment of the amino acid sequence of BxLAM16A and BmLAM16A with other GHF16 glucanases from bacteria and invertebrates. The black shading indicates identical amino acids, and the gray shading indicates conservative replacements. The numbers to the left indicate the amino acid position of the respective proteins. Residues identified as catalytic amino acids are labelled with asterisks. The position of the intron in the *B. xylophilus* sequence is indicated by an arrow above the alignment. BxLAMA, *B. xylophilus* sequence from the present study; BmLAMA, *B. mucronatus* sequence from the present study; X.axon, bacteria *Xanthomonas axonopodis* (AAM36156); P.sp, bacteria *Pseudomonas sp.* (BAC16331); C.cell, bacteria *Cellulosimicrobium cellulans* (AAC44371); T.neap, bacteria *Thermotoga neapolitana* (CAA88008); S.purp, sea urchin *Strongylocentrotus purpuratus* (AAC47235); P.sach, bivalve mollusk *Pseudocardium sachalinensis* (AAP74223).

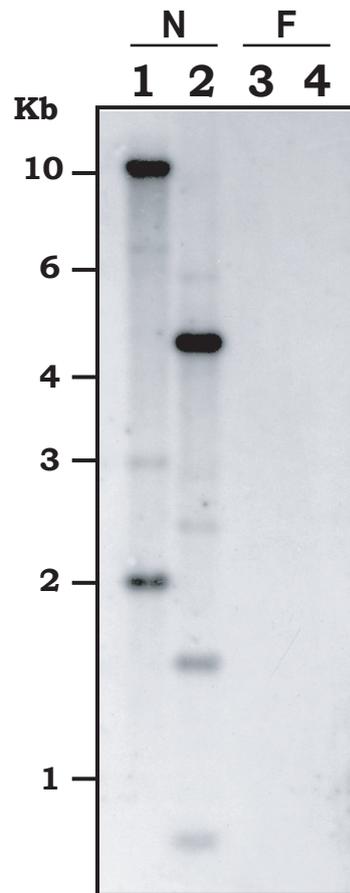


Fig. 2-2-2. Southern blot analysis of *Bx-lam16A*. Genomic DNA from *B. xylophilus* (N) and *B. cinerea* (F) were digested with *EcoRI* (1&3) or *HindIII* (2&4). The blot was hybridized with a probe generated from *Bx-lam16A* cDNA.

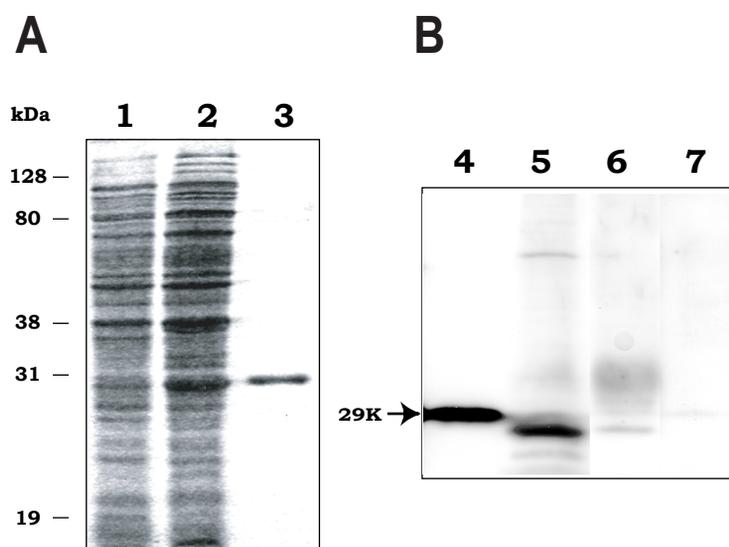


Fig. 2-2-3. Detection of native and recombinant BxLAM16A. A, Coomassie blue stained SDS/PAGE gel showing expression and purification of recombinant BxLAM16A. Lane 1 and 2, crude extract of *E. coli* containing expression plasmid, non-induced (1) or induced (2) with IPTG; Lane 3, purified recombinant BxLAM16A. B, Western blot analysis with antiserum raised against recombinant BxLAM16A. Lane 4, *E. coli* crude extract expressing BxLAM16A; Lane 5, *B. xylophilus* homogenate; Lane 6, *B. xylophilus* secretions; Lane 7, crude extracts of *B. cinerea* as a negative control.

Substrate	Specific activity (units/mg)
Laminarin	337 \pm 21
Yeastglucan	57 \pm 7
Barley β -D-glucan	10 \pm 4
Lichenan	7 \pm 3

Table 2-2-1. Substrate specificity of purified recombinant BxLAM16A.

The enzyme reaction was performed at 40°C for 10 min by incubating 0.065 mg of purified enzyme with 0.25% (w/v) substrate in a final 0.1 ml of buffer solution. The results are means for three independent experiments \pm S.D. No activity was detected in the case of pustulan, carboxymethylcellulose, glycol chitin, xylan, agarose, κ -carrageenan and xyloglucan. For xyloglucan, xyloglucan endotransferase activity was measured as described in the Materials&Methods section.

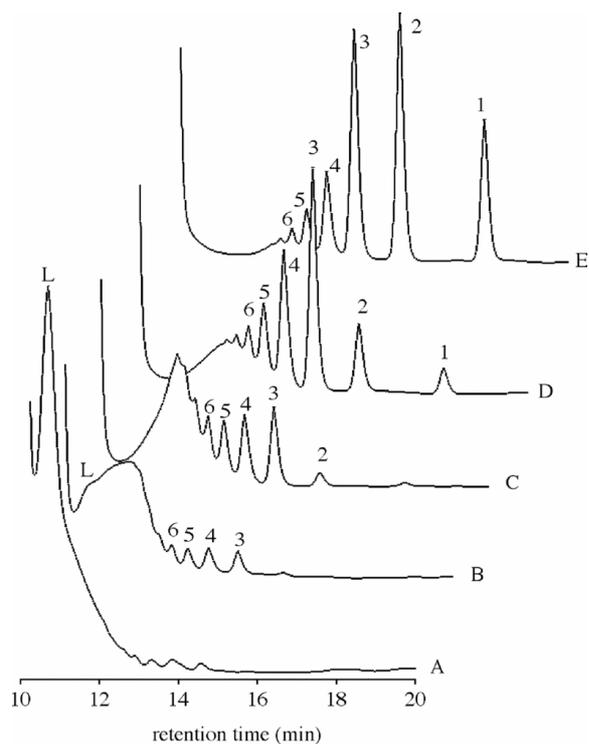


Fig. 2-2-4. HPLC analysis of BxLAM16A action on laminarin (0.25%, w/v). Traces A, B, C, D and E, the enzyme (0.65 mg/ml) was incubated at 40°C for 0, 3, 10, 210 min and overnight respectively. Samples were analysed on SUGAR KS-802 column \times 2 (Shodex). Identified products are glucose (1), laminaribiose (2), laminari-oligomers (3-6) and laminarin (L).

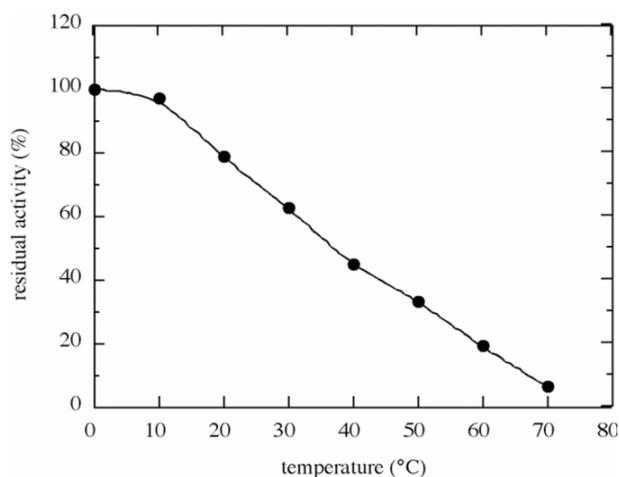


Fig. 2-2-6. Thermal stability of BxLAM16A. The enzyme (6.5 mg/ml) was incubated for 60 min at each temperature in citrate/phosphate buffer, pH 4.9. After the incubation, residual activity was measured at 40°C.

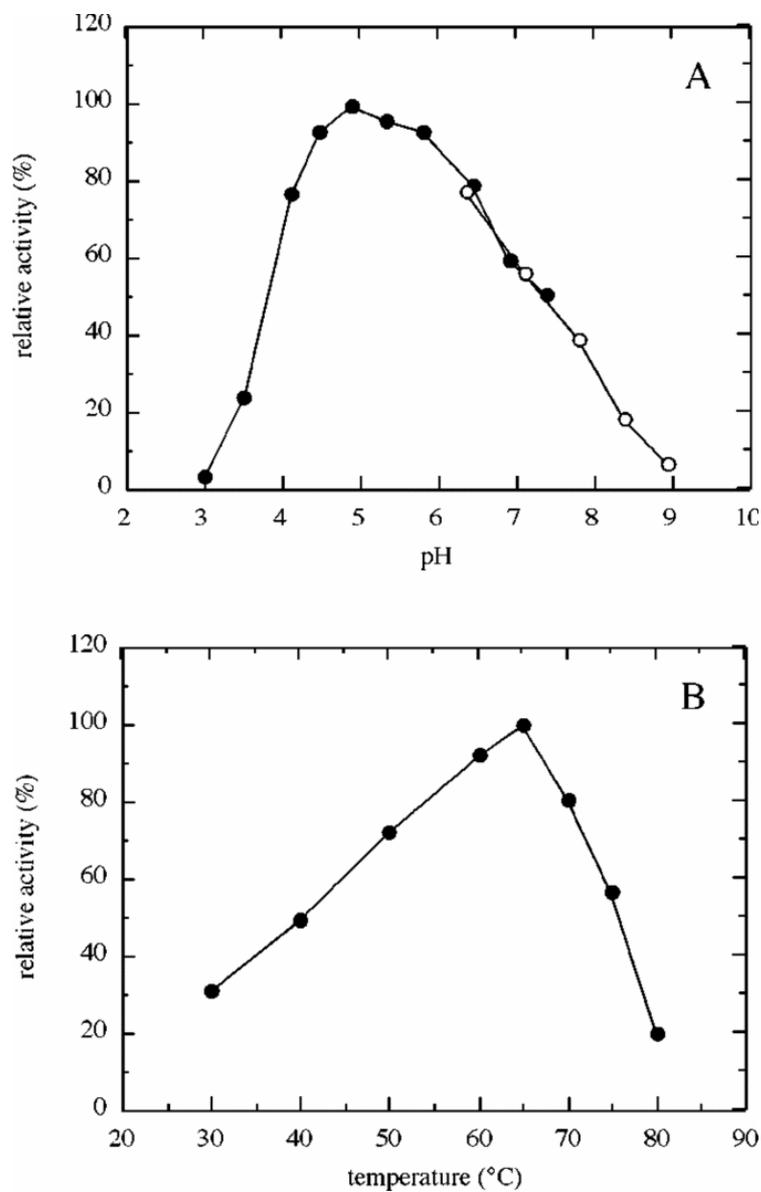


Fig. 2-2-5. Influence of pH and temperature on the activity of BxLAM16A. (A) Enzyme activity was measured at 40°C for 10 min, using laminarin as substrate, in citrate/phosphate buffer (●) at pH 3.0-7.4 and in KH₂PO₄/Na₂B₄O₇ buffer (○) at pH 6.4-8.9. (B) Enzyme activity was measured in citrate/phosphate buffer, pH 4.9, for 10 min, using laminarin as substrate. Each assay was performed with 0.65 mg of protein/ml.

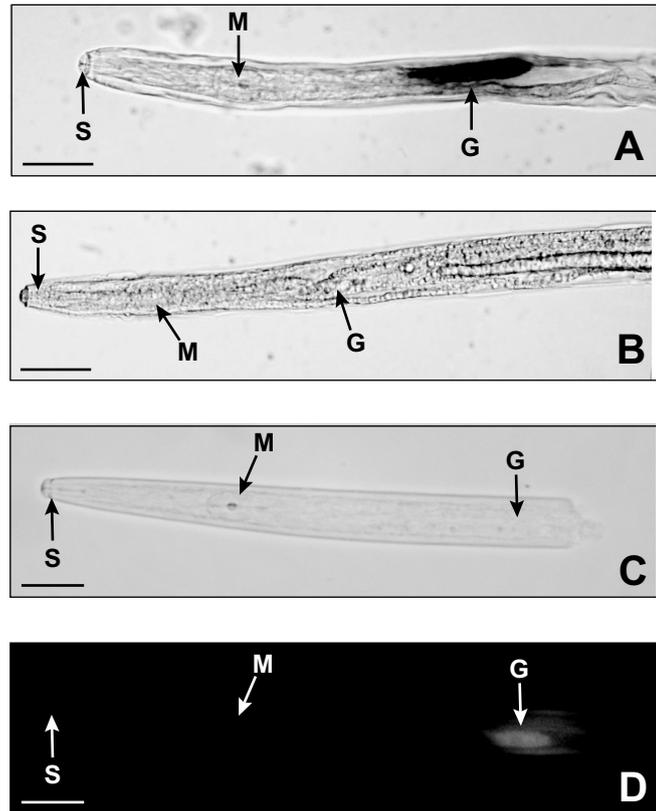


Fig. 2-2-7. Localization of the BxLAM16A transcript and protein. (A, B) Localization by in situ hybridization of *Bx-lam16A* transcripts in the oesophageal gland cells of *B. xylophilus* adult female with antisense (A) and sense (B) *Bx-lam16A* digoxigenin-labelled cDNA probes. Expression is restricted to oesophageal gland cells. (C, D) Immunofluorescence localization with antiserum against recombinant BxLAM16A, showing that the protein is present in the oesophageal gland cells of the nematode. (C) Illustrates the bright-field image, whereas (D) illustrates the same specimen viewed under fluorescence optics. G, oesophageal glands; S, stylet; M, metacarpus (scale bar, 20 μm).

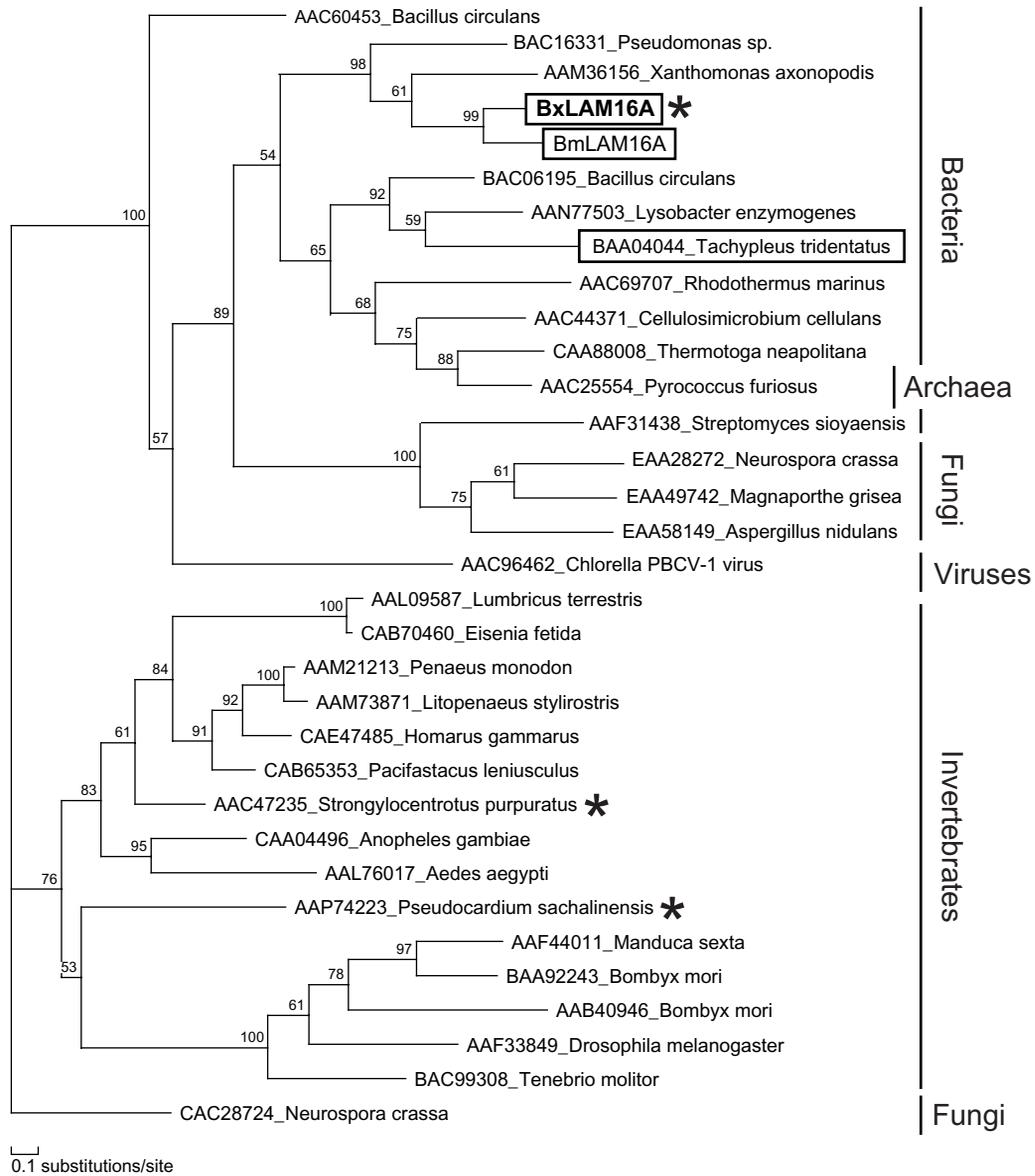


Fig. 2-2-8. Unrooted phylogenetic tree of selected GHF16 β -1,3-glucanases and β -1,3-glucanase-like proteins generated using maximum-likelihood analysis. Bootstrap probabilities are estimated for each node by the REML method. Functionally characterized β -1,3-glucanases from animals are labelled with asterisks. Animal sequences that cluster in the bacterial branch are boxed. Scale bar, 10 substitutions/100 amino acid positions.

2-3. Pectate lyases

Objectives

Pectin is a major structural component of the plant cell wall along with cellulose and hemicellulose. Pectin is located mainly in the middle lamella and primary cell wall and functions as a matrix anchoring the cellulose and hemicellulose fibres (Carpita and Gibeaut, 1993). The breakdown of pectin consequently leads to the maceration of plant tissues, the characteristic symptom of soft-rot diseases (Lietzke et al., 1994). Pectin degradation requires the combined action of several enzymes. These can be divided into two groups, namely pectin esterases, which remove the methoxyl groups from pectin and depolymerases (hydrolases and lyases) that cleave the backbone chain (Tamaru and Doi, 2001).

Pectate lyase (pectate transesterase, EC 4.2.2.2), which catalyzes cleavage of internal α -1,4-linkages of unesterified polygalacturonate (pectate) by β -elimination, is known to play a critical role in pectin degradation (Barras et al., 1994). Pectate lyases are classified as polysaccharide lyases and are present in 5 out of 15 families (1, 2, 3, 9 and 10) of this type of enzyme (Henrissat, 1991; Henrissat and Davies, 1997) (refer also to: <http://afmb.cnrs-mrs.fr/CAZY/index.html>).

Pectate lyases are widely distributed among bacterial and fungal plant pathogens and have been the focus of several studies that have aimed to ascertain their function as virulence factors (Barras et al., 1994). They are used by plant pathogens to degrade host cell walls in order to allow penetration and colonization. Plant parasitic cyst nematodes and root-knot nematodes are known to secrete pectate lyases. Genes encoding pectate lyases have been cloned from several species of plant parasitic nematodes including *Heterodera*, *Globodera* (cyst nematode) and *Meloidogyne* (root-knot nematode) species (de Boer et al., 2002; Doyle and Lambert, 2002; Huang et al., 2005; Popeijus et al., 2000). These pectate lyases are produced in the esophageal gland cells and are secreted from the stylet of the nematode. They are thought to play an important role in infection and parasitism of plant.

In Chapter 2-1, we presented the cloning and functional characterization of cellulase (β -1,4-endoglucanase) genes from this nematode (Kikuchi et al., 2004). It was shown that the *B. xylophilus* cellulases are secreted through the stylet of this nematode and, like the cellulases of other plant parasitic nematodes, may soften the plant cell wall to facilitate their feeding and migration. However, the *B. xylophilus* cellulases showed most similarity with fungal cellulases and were classified into glycosyl hydrolase family

(GHF) 45, while cellulases of cyst/root-knot nematodes belong to GHF5 and are most similar to bacterial cellulases. It was therefore proposed that cyst/root-knot nematodes and *Bursaphelenchus* spp might have evolved both the ability to digest cellulose and the ability to parasitise plants independently (Kikuchi et al., 2004). Here we report the cloning and characterization of genes encoding a second category of plant cell wall degrading enzyme, pectate lyase, from *B. xylophilus*.

Methods

Isolation of cDNA and gDNA Clone

The pectate lyase gene was cloned as a part of an EST project carried out using a cDNA library derived from mixed-stage *B. xylophilus* (Chapter 1). One full-length cDNA (clone 03EK1-03-E09) encoding a pectate lyase, designated *Bx-pel-1* was identified during BLAST analyses of sequences generated in this project. A further cDNA clone encoding a related but different sequence (*Bx-pel-2*) was also identified in the ESTs. The plasmid clones from which each of these sequences were obtained were identified and re-sequenced in both directions in order to obtain full-length cDNA sequences. Similar methods were used to identify pectate lyase encoding cDNAs from ESTs of a related species, *B. mucronatus*.

The genomic coding region of each cDNA clone was obtained by PCR amplification from *B. xylophilus* genomic DNA, using pairs of gene-specific primers flanking each open reading frame. PCR products were cloned using the pGEM-T Easy vector (Promega) and sequenced.

Expression and Purification of Recombinant Protein

The coding region of BxPEL1 without the putative signal sequence was amplified by PCR and then cloned into the bacterial expression vector pQE-30UA (Qiagen). The 6×His-tagged protein was expressed in *Escherichia coli* M15[pREP4] (Qiagen). After inducing protein expression for 6 h at 37°C with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), bacteria were harvested by centrifugation. The protein was purified from inclusion bodies using HisTrap HP Column (Amersham Biosciences) after being solubilized with 8 M urea. To recover the activity of the recombinant BxPEL1 (rBxPEL1), the protein was renatured on the column before elution with 0.5 M imidazole as described (Holzinger et al., 1996). The purity of the protein was assessed by SDS-polyacrylamide gel electrophoresis (SDS/PAGE).

SDS/PAGE and Zymography

Protein samples were boiled in an equal volume of 2x sample buffer (100 mM Tris, pH 6.8, 12% 2-mercaptoethanol, 4% SDS, 20% glycerol, and 0.01% bromphenol blue) for 3 min and analysed by SDS/PAGE in 12% polyacrylamide gel (Laemmli, 1970). After electrophoresis the gel was stained with Coomassie brilliant blue. For zymogram staining, samples were boiled for 3 min in 2x sample buffer without 2-mercaptoethanol before loading on a 12% SDS/PAGE gel containing 0.05% (w/v) PGA. Proteins were renatured by soaking the gel successively once in potassium phosphate buffer (50mM, pH 7.0) containing 2-propanol (25%, v/v) for 30 min, and twice in potassium phosphate buffer. After incubation at 37°C for 16 h, gels were stained with 0.05% (w/v) Ruthenium red (Nacalai Tesque) for 10 min and washed with water until pectate lyase bands became visible.

Nematode homogenate for zymography analysis was prepared as described previously (Chapter 2-1) (Kikuchi et al., 2004).

Enzyme Assays

Pectate lyase activity was assayed spectrophotometrically by measuring the formation of unsaturated products from PGA at 232nm (Collmer et al., 1988). The standard assay mixture contained 100 µl of 3% polygalacturonic acid (Sigma), 500 µl of 0.1 M Tris-HCl (pH 8.0), 100 µl of 10 mM CaCl₂ and 10 µl of appropriately diluted enzyme solution in a total volume of 1 ml. The subsequent increase in absorbance at 232 nm was monitored at 25°C over a period of 5 min. The pH values of these buffers were confirmed at room temperature.

The activity of recombinant protein on substrates with various degrees of methylation was determined by substituting 26%, 67%, and 89% esterified citrus fruit pectins (Sigma) or polygalacturonic acid (Sigma). The influence of Ca²⁺ was investigated by the addition of CaCl₂ to final concentrations ranging from 0 to 4 mM. The optimum pH was determined by using Tris-HCl buffer (pH 5.8-8.5) and glycine-NaOH buffer (pH 8.5-11), each at 50 mM. The pH values were determined in the final reaction mixture.

Phylogenetic analysis

Protein sequences which belong to polysaccharide lyase family 3 from bacteria, fungi and nematodes were obtained from the CAZY database (http://afmb.cnrs-mrs.fr/CAZY/PL_3.html) and aligned with the new sequences

(BxPEL1,2 and BmPEL1,2) using the ClustalX 1.8 program (Thompson et al., 1997) followed by manual refinement. Prior to phylogenetic analysis, signal peptide sequences and other N- and C- terminal extensions peculiar to individual taxa were excluded. In total, 129 characters were used for phylogenetic analysis. Neighbor-joining trees were constructed in MEGA v 3.1 (Kumar et al., 2004), using the JTT amino acid substitution matrix. Maximum likelihood analyses were carried out using Phym1 v 2.4 (Guindon and Gascuel, 2003), using the JTT amino acid substitution matrix. Support for the resulting Neighbor-joining and Maximum likelihood trees was assessed by bootstrap resampling.

Results

Identification of a pectate lyase gene from *B. xylophilus*

Expressed Sequence Tags (ESTs) obtained from the 5' end of genes present in a cDNA library constructed from mixed-stage *B. xylophilus* were searched using Blastn and Blastx programs for similar sequences in DNA and protein databases. A clone with significant similarity at the amino acid level with pectate lyases was identified and designated *Bx-pel-1*. The complete cDNA was 848 bp in length and contained an ORF of 252 amino acids with an ATG start codon at position 12 and a TGA stop codon at position 768. The cDNA possesses a polyadenylation signal located 9 bp upstream of the polyA tail. A signal peptide of 18 amino acids is predicted by the SignalP program (Nielsen et al., 1997) at the N-terminus of the deduced BxPEL1 polypeptide. The putative mature protein has a molecular mass of 25 kDa and theoretical pI of 8.45. The deduced amino acid sequence of BxPEL1 showed highest similarity with pectate lyases belonging to polysaccharide lyase family 3 from bacteria, fungi and nematodes. Subsequent EST analysis led to the identification of another cDNA from *B. xylophilus* that could also encode a protein (BxPEL2) similar to polysaccharide lyase family 3 pectate lyases and which also had a predicted signal peptide at the N-terminus. BxPEL1 and BxPEL2 share 64% identity in amino acid sequences (Fig. 2-3-1). In addition, two cDNAs (*Bm-pel-1* and *Bm-pel-2*) that could encode similar proteins with predicted signal peptides were found in ESTs from a related, but non-pathogenic nematode species, *B. mucronatus*.

Genomic analysis

Analysis of genomic DNA was performed to confirm the nematode origin of *Bx-pel-1*. A Southern blot containing genomic DNA from *B. xylophilus*, and from the fungus on

which the nematodes were reared, (*B. cinerea*), as a negative control, was made and hybridized with a DNA probe generated from the *Bx-pel-1* cDNA. The probe hybridized specifically with DNA in both the *Eco*RI and *Hind*III digests of *B. xylophilus* DNA (Fig. 2-3-2). No signal was obtained from *B. cinerea* genomic DNA. The *Bx-pel-1* cDNA hybridized to multiple fragments and since the genomic coding region does not contain *Eco*RI or *Hind*III sites *Bx-pel-1* could be a member of a small multigene family. In addition, the entire coding regions of the *Bx-pel-1* or *Bx-pel-2* gene were amplified from *B. xylophilus* gDNA, and the PCR products were cloned and sequenced. Analysis of these sequences showed that these were exactly the same as the cDNA sequences, except that one intron was present at the site indicated in Fig. 2-3-1. The introns are bordered by canonical *cis*-splicing sequences, and are AT rich (59.7%). The intron positions of these genes were identical to each other (Fig. 2-3-1). The genomic coding region of *Bm-pel-1* and *Bm-pel-2* were also amplified by PCR. Sequence analysis of the PCR products showed that each of these genes has one intron at the same position as *Bx-pel-1* and *Bx-pel-2* (Fig. 2-3-1).

The intron position in the *Bursaphelenchus* genes (*Bx-pel-1/2* and *Bm-pel-1/2*) were compared with those of other nematode pectate lyase genes. *Mi-pel-1* from *M. incognita* has three introns in the coding region, one of which is at the same position as that in the *Bursaphelenchus* genes (Fig. 2-3-1). *Gr-pel-1* from *G. rostochiensis* has six introns and *Mi-pel-2* from *M. incognita* has two introns. *Gr-pel-1* and *Mi-pel-2* share two intron positions but none of the introns of these genes have the same position as that in the *Bursaphelenchus* genes and *Mi-pel-1* (not shown).

Spatial localization of *Bx-Pel-1* mRNA

To determine which nematode cells express *Bx-pel-1*, *in situ* mRNA hybridization was performed. Digoxigenin-labeled anti-sense probes generated from *Bx-pel-1* specifically hybridized with transcripts in the esophageal gland cells of *B. xylophilus* (Fig. 2-3-3A). No hybridization was observed in *B. xylophilus* with the control sense cDNA probes of *Bx-pel-1* (Fig. 2-3-3B). Hybridization signals were detected in female, male, and propagative larvae of *B. xylophilus*. We could not determine precisely which of the three gland cells of the nematode expressed *Bx-pel-1*, because these cells overlap and are difficult to distinguish from each other (Nickle et al., 1981).

Over expression and purification of BxPEL1

The coding region of the *Bx-pel-1* gene without the signal peptide was amplified by PCR and cloned into an expression vector. Induction of exponentially growing cultures

of *Escherichia coli* transformants resulted in inclusion bodies containing most of the protein. However, solubilization in 8 M urea followed by on-column renaturation yielded milligram amounts of soluble recombinant BxPEL1 (rBxPEL1) protein in high purity as evidenced by electrophoretic homogeneity (Fig. 2-3-4).

Enzyme properties

Zymograms of rBxPEL1 displayed a prominent polygalacturonic acid (PGA) degrading activity band at approx. 27 kDa (Fig. 2-3-5). Similar analysis of nematode homogenate demonstrated an activity band at 25kDa (Fig. 2-3-5). These sizes are in agreement with the molecular mass predicted from the amino acid sequences of these proteins.

Of the pectic substrates tested, maximum activity was found on PGA (Fig. 2-3-6A). Moreover, as the percentage degree of methylation in pectin became higher, the activity of rBxPEL1 was reduced.

The effect of pH and temperature on the activity of purified rBxPEL1 on PGA were determined. The optimal temperature for rBxPEL1 activity was 55°C, and greater than 50% of maximum activity was found in the temperature range from 50°C to 65°C (Fig. 2-3-6B). rBxPEL1 was active on PGA in a broad alkaline pH range. The activity increased moderately with an increasing pH until 10.0, after which it decreased rapidly (Fig. 2-3-6C).

Since Ca^{2+} is known to be essential for the catalytic activity of pectate lyases, the influence of Ca^{2+} ions on enzymatic activity was examined using different CaCl_2 concentrations (Fig. 2-3-6D). Activity was undetectable without this ion. Maximum activity was observed at 0.25-0.5 mM CaCl_2 and activity decreased at higher concentrations. The addition of 1 mM EDTA to the standard mixture completely eliminated pectate lyase activity (data not shown). The effect of metal ions other than Ca^{2+} on pectate lyase activity was determined by performing enzyme assays on PGA in the presence of metal ions at 1 mM concentration. Ca^{2+} was not present in these assays. When rBxPEL1 was evaluated in the presence of Mg^{2+} , Mn^{2+} , Co^{2+} , Ni^{2+} , Zn^{2+} and Ba^{2+} , no pectate lyase activity was detected (data not shown), indicating that none of the ions tested could replace Ca^{2+} .

Phylogenetic analysis

A phylogenetic tree was generated from an alignment of *Bursaphelenchus* sequences with selected proteins belonging to polysaccharide lyase family 3 from bacteria, fungi and nematodes using the maximum likelihood method. Analysis using a

neighbor-joining method generated trees with similar topology. *Bx-pel-1* and 2 from *B. xylophilus* and *Bm-pel-1* and 2 from *B. mucronatus* were clustered together with strong bootstrap support (Fig. 2-3-7). However, other nematode sequences were not monophyletic but were clustered into three distinct clades (Fig. 2-3-7). In addition, no clear relationship between nematode sequences and either bacterial or fungal sequences emerged from the tree.

Discussion

In this sub-chapter, we describe pectate lyases from the pine wood nematode *B. xylophilus*. This is the first identification of a pectate lyase from a plant parasitic nematode belonging to the order Aphelenchida.

The observation that *Bx-pel-1* probe specifically hybridized to nematode DNA in Southern blot analysis, the presence of a poly-A tail at 3' end of the cDNA and the presence of introns within the coding region amplified from genomic DNA support a nematode origin for *Bx-pel-1*. In addition, in situ hybridization showed that the gene is expressed solely in the oesophageal gland cells of *B. xylophilus*, a tissue from which no endosymbiotic bacteria have ever been described. These lines of evidence demonstrate that the pectate lyase genes are endogenous nematode genes and were not derived from bacterial contaminants or symbionts or from the fungi on which the nematodes were reared.

The presence of a signal peptide in the deduced amino acid sequence of *Bx-pel-1* and its expression in the oesophageal glands suggest that BxPEL1 is secreted from the stylet of the nematode. *B. xylophilus* is required to migrate within plant tissue during its lifecycle. Once the nematode invades the pine tree, it migrates primarily through resin canals of the tree and feeds from parenchyma cells surrounding the canals (Mamiya, 1983). Although pectin substrates are scarce in the xylem of woody plants, they are present in the primary cell wall of the cambium and parenchyma cells of woody plants including pine trees (Hafren *et al.*, 2000; Westermarck *et al.*, 1986). It is likely therefore that BxPEL1 is secreted from the nematode stylet and helps the nematode migrate and feed within the tree. In a previous study we showed that the nematode secretes cellulase (Kikuchi *et al.*, 2004). In addition, we also showed that the nematode secretes endo- β -1,3-glucanase which degrade β -1,3-glucan, major component of fungal cell wall (Kikuchi *et al.*, 2005). *B. xylophilus* therefore secretes a mixture of enzymes to attack both the plant and fungal cell walls.

Recombinant BxPEL1 was produced as inclusion bodies in *E. coli* but could be recovered by a single-step purification and renaturation procedure. Renatured protein showed highest activity on PGA. The amino acid sequence of BxPEL1 shows high similarity with those of proteins belonging to polysaccharide lyase family 3 and the biochemical properties of rBxPEL1 were also similar to those of other polysaccharide lyase family 3 pectate lyases. For example, BxPEL1 has rather high optimal temperature (55°C), retains activity over a broad alkaline pH range and showed an absolute requirement for Ca²⁺ ion for activity. BxPEL1 seems to be particularly active at temperatures that are much higher than those found in the normal environmental conditions that the nematodes would encounter. This is also the case for several non-thermophilic bacterial pectate lyases (Soriano *et al.*, 2000; Tardy *et al.*, 1997). Furthermore, nematode homogenate showed highest pectate lyase activity on PGA at similar temperature to recombinant BxPEL1 (data not shown) indicating that this high optimum enzymatic temperature is not likely to be a result of bacterial expression or the subsequent denaturation and renaturation of the enzyme.

Polysaccharide lyase family 3 pectate lyases have been found from plant parasitic cyst and root-knot nematodes including *M. incognita*, *M. javanica*, *H. glycines* and *G. rostochiensis*, all belonging to the order Tylenchida (de Boer *et al.*, 2002; Doyle and Lambert, 2002; Huang *et al.*, 2005; Popeijus *et al.*, 2000). They are likely to play important roles in infection and parasitism. The pectate lyases described here come from nematodes belonging to the order Aphelenchida, indicating that pectate lyases are common in plant parasitic nematodes. Phylogenetic analysis of the pectate lyases, including those from bacteria, fungi and nematodes resulted in a tree in which the nematode sequences were not monophyletic. The sequences from *B. xylophilus* and *B. mucronatus* were clustered in the same clade but were not most closely related with sequences from cyst and root knot nematodes. Furthermore, sequences from cyst and root-knot nematodes were clustered in two separate groups. This is perhaps because multiple paralogous genes are present in the various nematode genomes. This analysis was also difficult due to the relatively high levels of similarity between the nematode genes and pectate lyases from both bacteria and fungi. Blast searching revealed that the cyst/root-knot nematode and *Bursaphelenchus* pectate lyase genes are most similar to genes from bacteria and the genes from all nematode species are most similar to pectate lyases from the same bacterial species. Although it is difficult to determine conclusively from this analysis whether the nematode pectate lyase genes have an ancient, common origin, it seems likely that this was the case and that the genes were acquired by a common ancestor in a single HGT event.

This work shows that pectate lyases are present in both *B. xylophilus* and *B. mucronatus*. *B. mucronatus* is very closely related to *B. xylophilus* and the two species share many characteristics in their life cycles. However, *B. mucronatus* is not pathogenic to plants and lives only in dead or dying pine trees where it feeds on colonizing fungi. This suggests that pectate lyases are used for migration through both living and dead plant tissues. It is therefore possible that cell wall degrading enzymes might be more widely used by nematodes in plant-nematode associations than previously thought.

Conclusion

Three distinct types of cell wall degrading enzyme genes were identified from the EST dataset; cellulase, β -1,3-glucanase and pectate lyase. Molecular characterization of these genes showed that these are endogenous nematode genes and that the nematode uses a mixture of enzymes to attack the plant or fungal cell wall.

It is likely that horizontal gene transfer has played an important role in the evolution of plant parasitism in at least two major groups of plant-parasitic nematodes. Studies on other nematodes, particularly the less intensively studied ectoparasites such as *Trichodorus* and the fungal feeding nematodes belonging to the groups different from *Bursaphelenchus* such as *Aphelenchus* and *Tylencholaimus*, would be useful in order to determine whether the presence of cellulases or other cell wall degrading enzymes is a requirement for nematode parasitism of plants and whether HGT has driven the evolution of plant parasitism in other nematode groups. As more genome sequences are obtained from a wider range of nematodes and as EST datasets are compared to one another and analysed in more detail it is possible that other horizontally acquired genes may be identified and the role that this process plays in the evolution of nematodes will be fully appreciated.

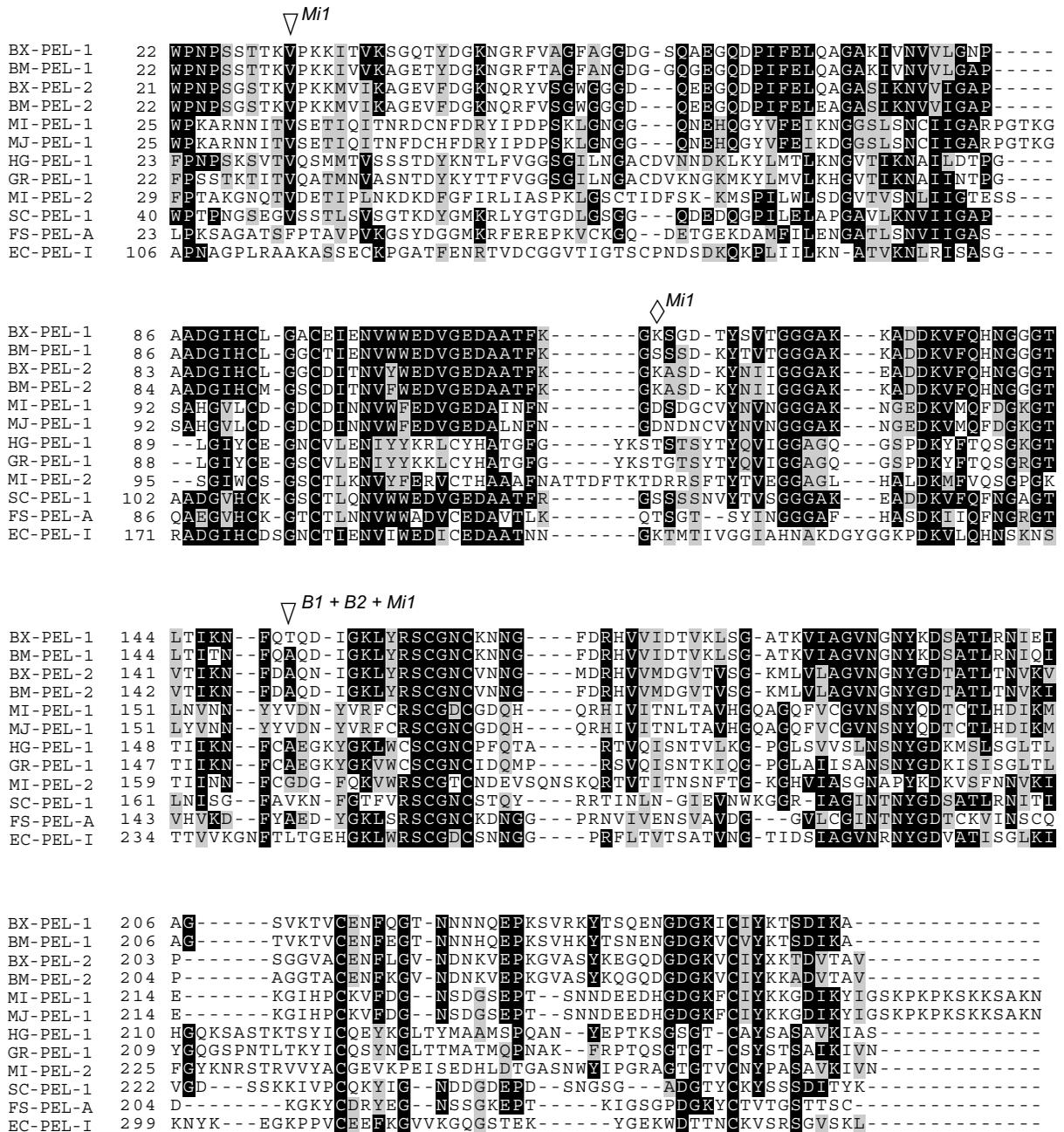


Fig. 2-3-1. Multiple sequence alignment of pectate lyases belonging to polysaccharide lyase family 3. BX-PEL-1, BX-PEL-2, BM-PEL-1, and BM-PEL-2 from this study; MI-PEL-1 (AAQ09004) and MI-PEL-2 (AAQ97032) from *Meloidogyne incognita*; MJ-PEL-1 (AAL66022) from *M. javanica*; HG-PEL-1 (AAK08974) from *Heterodera glycines*; GR-PEL-1 (AAF80747) from *Globodera rostochiensis*; SC-PEL-1 (CAC13062) from *Streptomyces coelicolor*; FS-PEL-A (AAA33338) from *Fusarium solani*; and EC-PEL-I (CAA73784) from *Erwinia chrysanthemi*. Identical residues are highlighted in black and functionally conserved are in gray. The positions of the intron in BxPEL1 and BmPEL1 is indicated by *B1*, that in BxPEL2 and BmPEL2 by *B2*, and those in MI-ENG-1 by *Mi1*. Triangles and diamonds represent phase 0 and 1 introns, respectively.

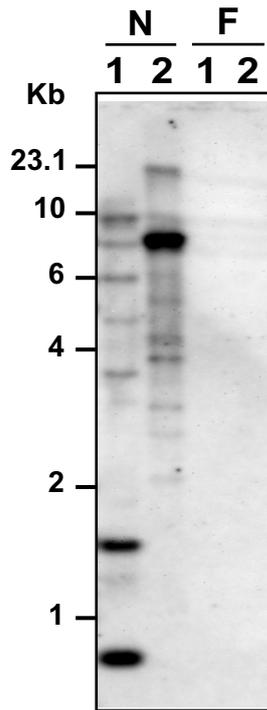


Fig. 2-3-2. Genomic Southern analysis of *Bx-pel-1*. A blot containing genomic DNA from nematode *Bursaphelenchus xylophilus* (N) and fungus *Botrytis cinerea* (F) digested with *EcoRI* (1) and *HindIII* (2) was hybridized with a *Bx-pel-1*-specific probe. The probe hybridizes to a series of bands in the nematode DNA indicating the presence of a multigene family.

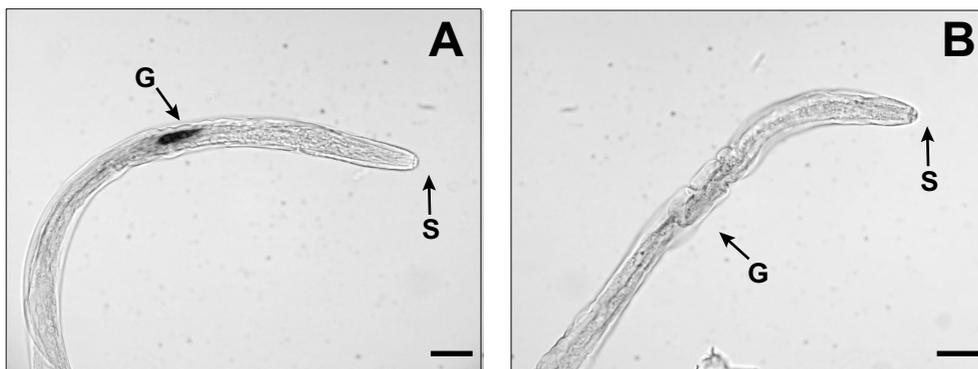


Fig. 2-3-3. Localization by in situ hybridization of the *Bx-pel-1* transcripts. Nematode sections were hybridized with A, antisense or B, sense *Bx-pel-1* digoxigeninlabeled cDNA probe. G: esophageal glands and S: stylet. Scale bar= 10 μ m.

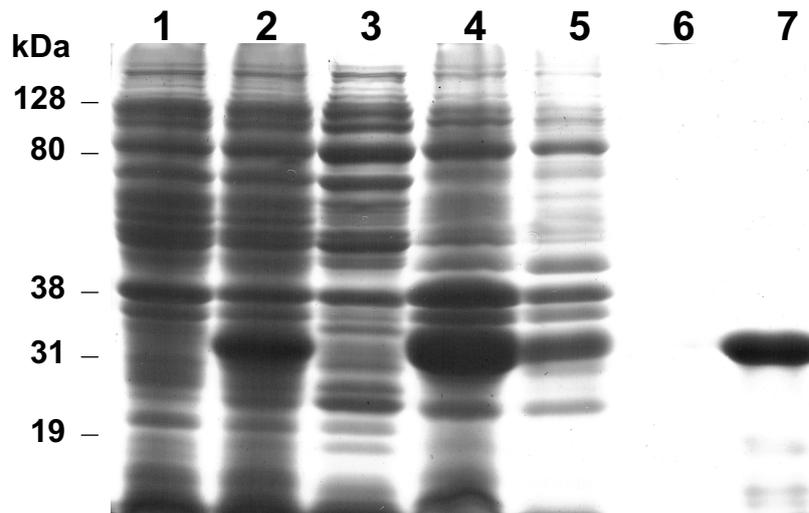


Fig. 2-3-4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of expression, purification, and refolding of recombinant BxPEL1. Lanes 1 and 2, crude extract from cells before (lane 1) and after (lane 2) induction with isopropyl- β -D-thiogalactopyranoside; lane 3 and 4, 10,000 x g supernatant (lane 3) and pellet (lane 4) fractions derived from cell lysate expressing BxPEL1; lane 5, flow-through from HisTrap HP column after solubilization with 8 M urea; lane 6, first wash-through; lane 7, proteins recovered after elution from the HisTrap HP column with imidazole buffer without urea.

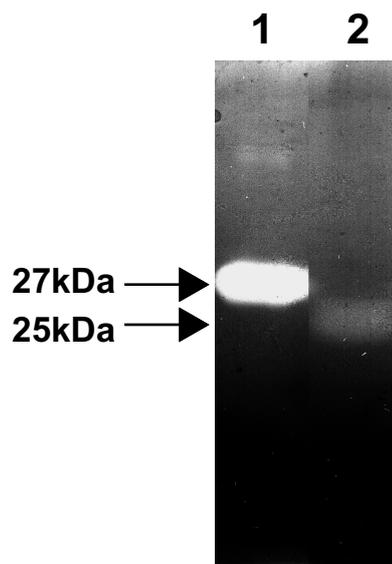


Fig. 2-3-5. Zymography showing the pectate lyase activity of the recombinant BxPEL1 (lane 1) and nematode homogenate (lane 2) in a sodium dodecyl sulfate-polyacrylamide gel containing polygalacturonic acid.

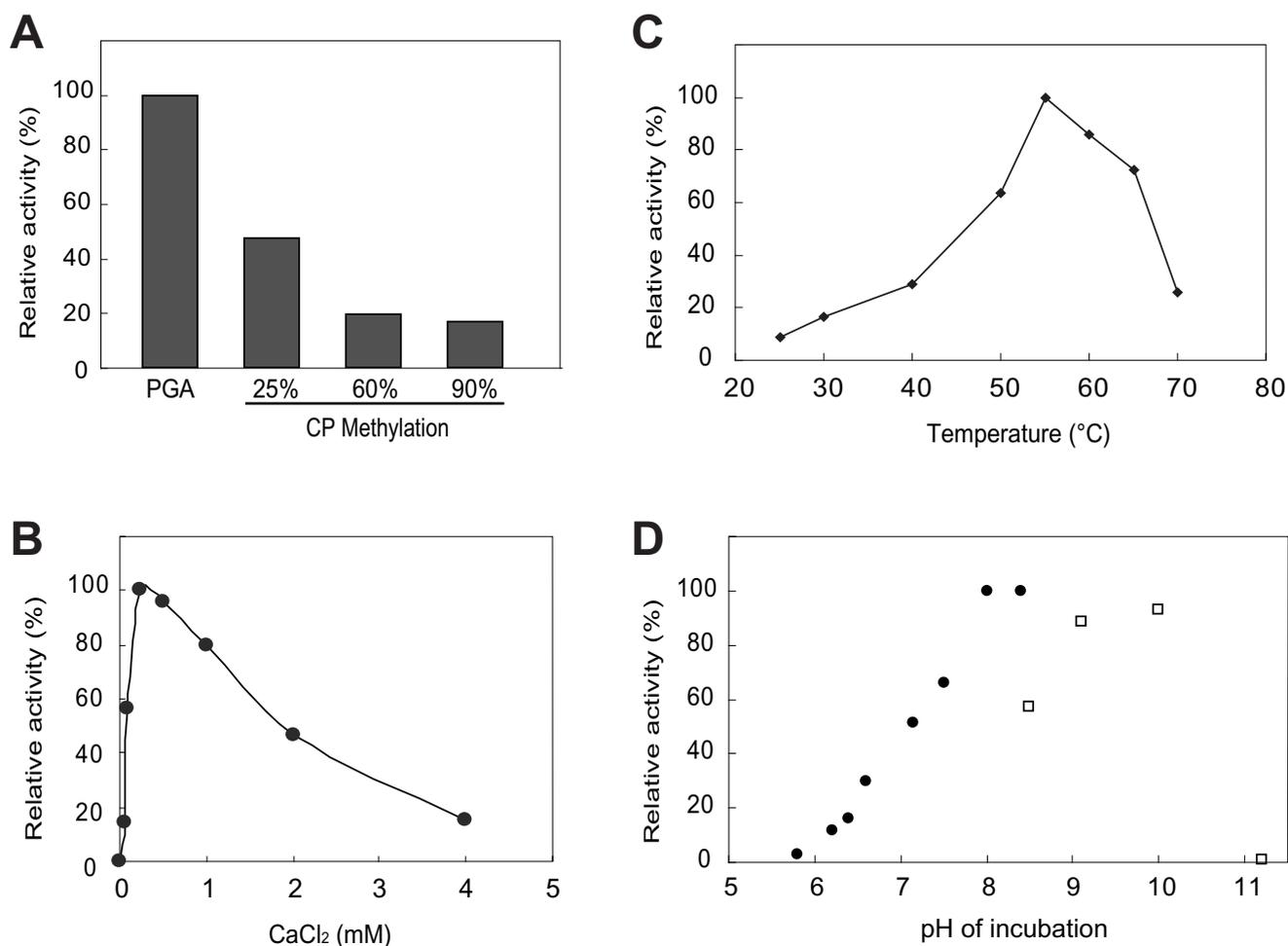


Fig. 2-3-6. Biochemical characterization of purified recombinant BxPEL1. A, Effect of the degree of pectin methylation on rBxPEL1 activity. Activity of rBxPEL1 on polygalacturonic acid (PGA) and citrus pectin (CP) with a degree of methyl esterification from 25 to 90% was determined in the standard assay mixture. B, Effect of Ca₂₊ concentration on rBxPEL1 activity on PGA. C, rBxPEL1 activity on PGA as a function of temperature. Activity was measured at the indicated temperatures in the standard assay mixture. D, rBxPEL1 activity on PGA as a function of pH. rBxPEL1 activity was measured in 50 mM Tris-HCl buffer (pH range, 5.8 to 8.3) (●) or 50 mM glycine-NaOH buffer (pH range, 8.5 to 11.3) (□). The pH values are those measured in the final reaction mixture.

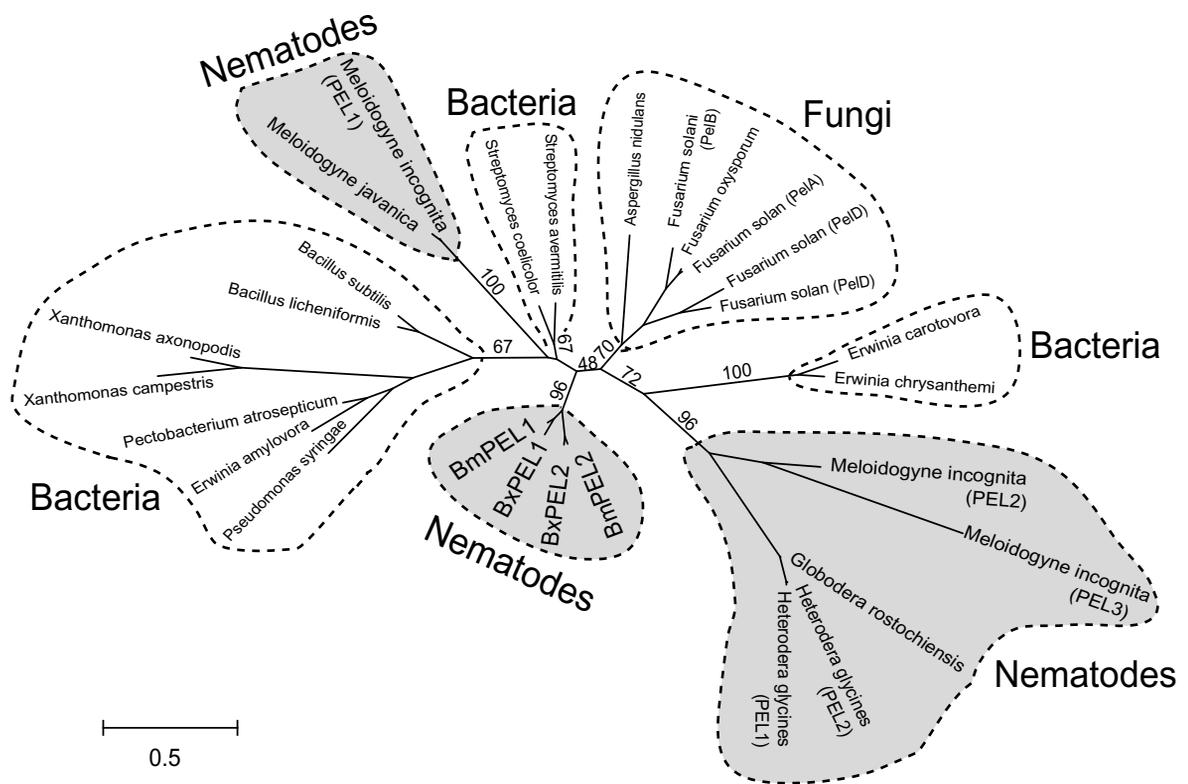


Fig. 2-3-7. Unrooted phylogenetic tree of selected polysaccharide lyase family 3 proteins generated using maximum likelihood analysis. The numbers on the node represent the bootstrap support percentages. The scale bar represents 50 substitutions per 100 amino acid positions.

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プロジェクトの概要

本研究は、交付金プロジェクト「マツノザイセンチュウの EST による遺伝子解析(H16 18)」によって行われた。

プロジェクトの目的

マツノザイセンチュウはマツ材線虫病の病原体である。わが国における本病による被害は甚大で、毎年日本各地で多くの松が枯死している。さらに近年、中国、韓国などの東アジア地域およびポルトガルにおいても本病の侵入が報告され、世界的なレベルで被害の拡大が危惧されている。市民の環境意識の高まりと被害地域の拡大から、これまでの農薬散布あるいは伐倒駆除に代わる新たな防御技術の開発が求められており、その開発には、まずマツノザイセンチュウの松寄生のメカニズムについての分子レベルでの深い理解が必要である。

これまで、マツノザイセンチュウについての生活史といくつかの生態的な側面についてはよく調べられてきた。一方で、本線虫の基礎的な生物学や宿主 - 線虫間の相互作用についての分子生物学的知見はほとんどなかった。本研究では、本線虫の分子生物学研究の基盤を整備することを目的として、マツノザイセンチュウのゲノム科学的な解析 (EST 解析: Expressed Sequence Tag Analysis) を行った。

第 1 章

マツノザイセンチュウの増殖型線虫、耐久型線虫それぞれから mRNA を抽出し、cDNA ライブラリーを作製した。同様にニセマツノザイセンチュウの増殖型線虫から cDNA ライブラリーを作製した。これらのライブラリーから、ランダムにクローンを取り出し、一方向から塩基配列を決定した。配列はアノテーションを付与した後、データベースに格納した。獲得した EST 配列数はマツノザイセンチュウで約 13,000、ニセマツノザイセンチュウで約 3,000 である。これらのデータは今後の遺伝子研究の基盤となりうるものである。

第 2 章

EST 配列の中から植物への寄生に関与する遺伝子として3種の細胞壁分解酵素遺伝子(セルラーゼ、 β -1,3 グルカナーゼ、ペクチン分解酵素)を単離し、それぞれを分子生物学・生化学的な手法を用いて解析した。その結果、これらの酵素はいずれも、ゲノム上に遺伝子ファミリーとして存在し、線虫の食道腺器官で生産され、口針から分泌されていることが明らかとなった。これらの酵素は細胞壁に作用することで線虫の寄生に積極的に関与していることが考えられた。また系統解析の結果はこれらの遺伝子が遺伝子水平転移によって獲得された可能性を示し、マツノザイセンチュウが他の植物寄生線虫とは異なるユニークな植物寄生能力の進化を経てきたことを示唆した。

「交付金プロジェクト」は、平成13年度に森林総合研究所が独立行政法人となるにあたり、これまで推進してきた農林水産技術会議によるプロジェクト研究（特別研究など）の一部、および森林総合研究所の経費による特別研究調査費（特定研究）を統合し、研究所の運営費交付金により運営する新たな行政ニーズへの対応、中期計画の推進、所の研究基盤高揚のためのプロジェクト研究として設立・運営するものである。

この冊子は、交付金プロジェクト研究の終了課題について、研究の成果を研究開発や、行政等の関係者に総合的且つ体系的に報告することにより、今後の研究と行政の連携協力に基づいた効率的施策推進等に資することを目的に、「森林総合研究所交付金プロジェクト研究成果集」として刊行するものである。

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