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A family of glycosyl hydrolase family 45 cellulases from the pine wood nematode *Bursaphelenchus xylophilus* ☆

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Abstract **We have characterized a family of GHF45 cellulases from the pine wood nematode *Bursaphelenchus xylophilus*. The absence of such genes from other nematodes and their similarity to fungal genes suggests that they may have been acquired by horizontal gene transfer (HGT) from fungi. The cell wall degrading enzymes of other plant parasitic nematodes may have been acquired by HGT from bacteria. *B. xylophilus* is not directly related to other plant parasites and our data therefore suggest that horizontal transfer of cell wall degrading enzymes has played a key role in evolution of plant parasitism by nematodes on more than one occasion.**

Keywords: Endoglucanase; Plant parasitic nematodes; Secretions; Horizontal gene transfer

1. Introduction

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. As cellulose is a major component of plant cell walls, cellulases (β-1,4-endoglucanases) are produced by many plant pathogens including bacteria and fungi [1]. Endogenous cellulase genes have also been identified from plant parasitic nematodes, including *Heterodera*, *Globodera* (cyst nematode) and *Meloidogyne* (root-knot nematode) species [2–4]. These cellulases are produced within the esophageal gland cells of these nematodes and secreted through the nematode stylet into plant tissues. They are therefore likely to facilitate penetration and migration of nematodes in root tissues [5]. 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 (HGT) from bacteria [e.g. 6]. Other genes thought to be involved in host-parasite interactions in plant parasitic nematodes are also thought to have been acquired by HGT from bacteria [7–9]. GHF5 cellulase genes have also been found in one migratory endoparasitic nematode *Pratylenchus penetrans* [10] 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.

The pine wood nematode, *Bursaphelenchus xylophilus*, is the causal agent of pine wilt disease. Most *Bursaphelenchus* species are solely fungal feeders and are transmitted by vector insects only to dead or dying trees during oviposition. *B. xylophilus* and the few other pathogenic species described to date are unique in their capacity to feed on live trees as well as fungi. Molecular phylogenetic analysis using small subunit ribosomal DNA sequences has shown that *B. xylophilus* is part of the same clade (IVb) as cyst/root-knot nematodes and *Pratylenchus* spp. [11]. However, *Bursaphelenchus* spp. are not directly related to these nematodes but form a distinct grouping with other fungal feeding nematodes including *Aphelenchoides* spp. [12]. In order to investigate molecular mechanisms underlying parasitism in *B. xylophilus*, we are currently undertaking an expressed sequence tag (EST) project on this nematode (T. Kikuchi, unpublished results). Here, we report the cloning and characterization of a family of GHF45 cellulase genes from *B. xylophilus* identified during this EST project.

2. Materials and methods

2.1. Biological material

Bursaphelenchus xylophilus used in this study was the Ka-4 isolate, from Ibaraki Prefecture, Japan and was cultured on *Botrytis cinerea* grown on autoclaved barley grains. Nematodes for experiments were separated from *B. cinerea* hyphae on a Baermann funnel [13] for 2 h at 25 °C. The nematodes were then washed 5 times in M9 buffer (42.3 mM Na_2HPO_4, 22 mM KH_2PO_4, 85.6 mM NaCl, and 1 mM $MgSO_4$, pH 7.0) to remove any remaining *B. cinerea* mycelium.

2.2. 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 genes, *Bx-eng-1*, *2* and *3*, were identified during an EST project carried out using this library (T. Kikuchi, unpublished results).

☆ Data deposition: The sequences reported in this paper have been deposited in the DDBJ database [Accession nos. AB179541 (*Bx-eng-1*), AB179542 (*Bx-eng-2*), AB179543 (*Bx-eng-3*)].

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Abbreviations: GHF, glycosyl hydrolase family; CMC, carboxymethyl cellulose; EST, expressed sequence tag

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The plasmid clones from which each of these sequences was obtained were 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 using standard protocols, using pairs of gene-specific primers flanking each open reading frame (ORF). PCR products were cloned using the pGEM-T Easy vector (Promega) and sequenced.

2.3. Genomic southern hybridization

5 μg of nematode genomic DNA or 7 μg of *B. cinerea* genomic DNA was digested with *Eco*RI or *Hin*dIII. The digestion products were separated on agarose gels and blotted onto Hybond-N+ Nylon membranes (Amersham bioscience) using standard protocols [14]. Hybridization and detection were performed with a digoxigenin-labeled DNA probe from *Bx-eng-1* made by PCR amplification of the full-length cDNA from the original plasmid with primers ENG00s (5′ TCTAAAATGAAGTCTCTTGTG 3′) and ENG00a (5′ AGTCCT-CTAAGCATCGTC 3′).

2.4. Expression of recombinant protein

The Bx-ENG-1 coding region without the putative signal sequence was amplified from the original plasmid using primers ENG10s (5′ CAGGATACCGGCAAAACCACG 3′) and ENG00a. The resulting PCR product was ligated into the pQE-30UA vector (Qiagen) and transformed into *Escherichia coli* M15[pREP4] (Qiagen). Plasmids with the insert in the correct reading frame and which showed no sequence changes were selected following sequencing of the plasmid clones. The *E. coli* cells containing these constructs were grown to an A_{600} of 0.5 and induced with 0.1 mM IPTG at 25 °C. Protein was harvested using standard protocols [14].

2.5. CMC plate tests

Cellulase activity assays were performed on carboxymethyl cellulose (CMC) agarose plates as described by Mateos et al. [15].

Total homogenate and secretions from *B. xylophilus* were also prepared for cellulase activity assays. Approximately 10^5 nematodes were suspended in 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. Half of the nematodes were homogenized and aliquots of the soluble fraction of this homogenate were used in CMC assays. The remaining nematodes were resuspended in 1 ml 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 to test for endoglucanase activity in nematode secretions. Buffer without nematodes put through the same procedures was used for negative controls.

2.6. In situ hybridization

In situ hybridizations were performed as described by de Boer et al. [16]. The nematode sections were hybridized with digoxigenin labeled sense or antisense DNA probes generated using the ENG00s and ENG00a primers shown above. Specimens were examined with differential interference contrast microscopy.

2.7. Phylogenetic analysis

The deduced protein sequences of *B. xylophilus* cellulases were compared with protein sequences of GHF45 cellulases from bacteria, fungi, protists and animals. All signal peptides and the N-terminal extensions of Bx-ENG-2 and Bx-ENG-3 were removed for generating the alignment shown in Fig. 1 and for phylogenetic analysis. This removal had no effect on the overall alignment or phylogenetic tree (not shown). Sequence alignments were constructed using CLUSTAL W [17] and SOAP V1.1 software [18]. 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. Aligned dataset was analyzed with maximum likelihood (ML) analysis using PROTML in the MOLPHY v.2.3b3 package [19] with 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. 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.

3. Results

3.1. 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 [20]. 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 Bx-ENG-1deduced protein sequence using the Signal P program [21]. Subsequent EST analysis led to the identification of two other cDNAs that could also encode proteins (Bx-ENG-2 and Bx-ENG-3) similar to GHF45 cellulases (Fig. 1) and which also had predicted signal peptides at their N-termini. Bx-ENG-2

Fig. 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 are marked in shades of gray, with greater levels of conservation indicated by darker shades of gray. 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.

and Bx-ENG-3 were slightly longer at their N-termini when compared to Bx-ENG-1. These additional sequences of 56 and 128 amino acids, respectively, were not similar to other sequences in the database and did not affect the sequence alignment as shown in Fig. 1.

Analysis using BLASTX software 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 No. 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. 1).

3.2. Endogenous origin of the B. xylophilus endoglucanases

A Southern blot containing genomic DNA from *B. xylophilus* and from the fungus (*B. cinerea*) on which the nematodes were reared was hybridized with a probe generated from the *Bx-eng-1* cDNA. The probe hybridized strongly to digests of DNA of *B. xylophilus* (Fig. 2). No signal was obtained from *B. cinerea* genomic DNA.

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. 1. These introns are bordered by canonical *cis*-splicing sequences, are small (98 and 132 bp, respectively) and AT rich (61.2% and 65.9%, respectively), all features commonly found in nematode introns [20]. The intron positions of *Bx-eng-2* and *3* were identical to each other (Fig. 1), but were different to those of a GHF45 cellulase gene from an insect, *Apriona germari* (AY451326) (Fig. 1).

3.3. Protein expression and cellulase activity assays

Recombinant Bx-ENG-1 protein showed cellulase activity in CMC plate assays (Fig. 3B). No CMC hydrolysis was observed with *E. coli* containing the expression plasmid without *Bx-eng-1* (Fig. 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. 3C) of *B. xylophilus* in the same assay. No hydrolysis was detected with buffer controls (data not shown).

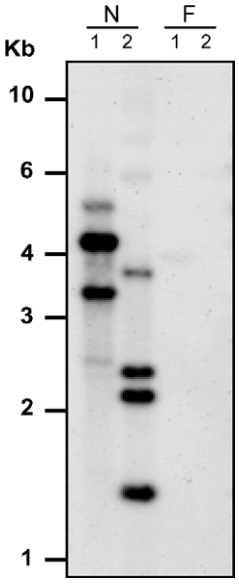

Fig. 2. Southern blot analysis of *Bx-eng-1*. Genomic DNA from nematode, *B. xylophilus* (N) and fungus, *B. cinerea* (F) were digested with *Eco*RI (1) or *Hin*dIII (2). The blot was hybridized with a probe generated from *Bx-eng-1* cDNA.

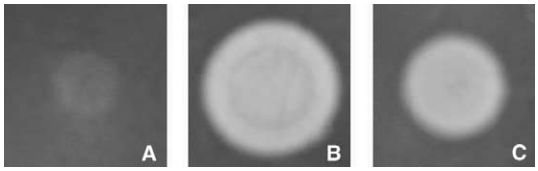

Fig. 3. Cellulase activity assay on CMC agar plate. Clear halos were detected in total proteins from *E. coli* expressing Bx-ENG-1 (B) and from secretions of *B. xylophilus* (C). No halo was found in the same quantity of total proteins from *E. coli* lacking the *Bx-eng-1* insert (A).

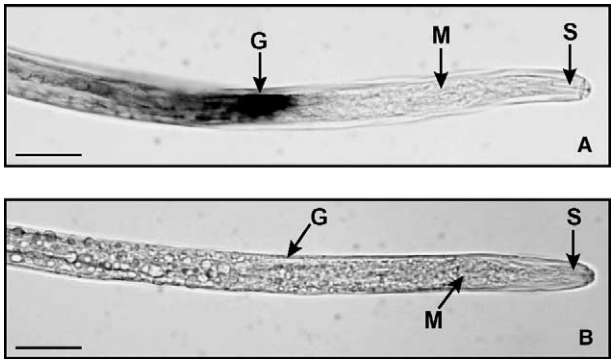

Fig. 4. Localization of *Bx-eng-1* transcripts in the esophageal gland cells of *B. xylophilus* adult female by in situ hybridization. Nematode sections were hybridized with antisense (A) or sense (B) *Bx-eng-1* digoxigenin-labeled cDNA probes. G, esophageal glands; S, stylet; M, metacarpus. (Bar = 20 μm.)

3.4. Spatial localization of Bx-eng-1 mRNA

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. 4A). No hybridization was observed in *B. xylophilus* with the control sense cDNA probes of *Bx-eng-1* (Fig. 4B). Hybridization signals were detected in female, male, and propagative larvae of *B. xylophilus*.

3.5. Phylogenetic analysis

A phylogenetic tree generated using maximum likelihood analysis from an alignment of the Bx-ENG-1, 2 and 3 deduced proteins with other GHF45 cellulases is shown in Fig. 5. Similar analysis using neighbor joining method 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.

4. Discussion

In this article, 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

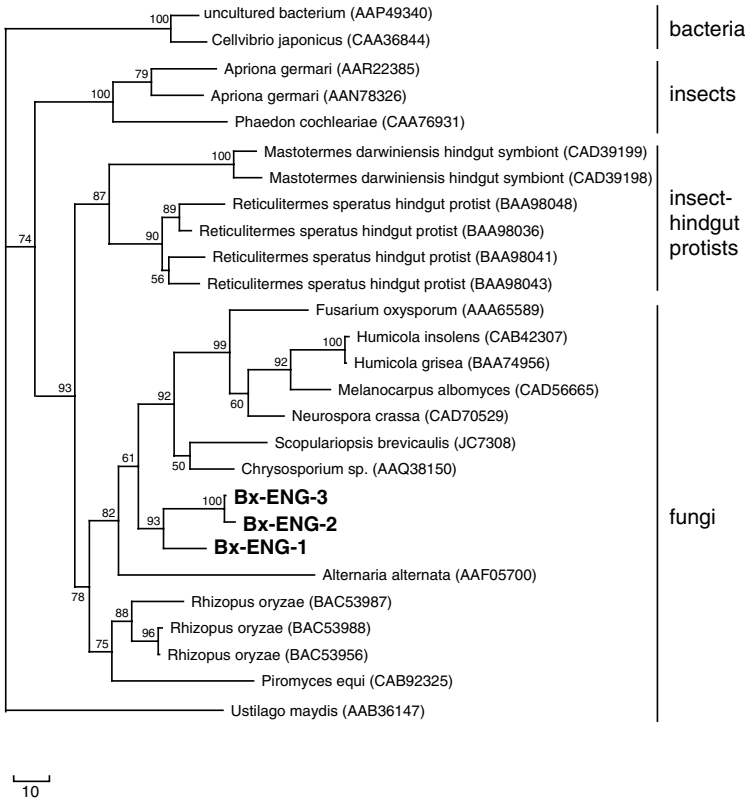

Fig. 5. Unrooted phylogenetic tree of GHF45 cellulases generated using ML analysis. Bootstrap probabilities for each node are estimated by the RELL method. The scale bar represents 10 substitutions per 100 amino acid positions.

of these genes, *Bx-eng-1*. This is the first example of a functional GHF45 cellulase from any nematode. Cellulase activity has been reported in homogenates and secretions of *B. xylophilus* [22] and was confirmed in this study. The presence of a predicted signal peptide at the N-terminus of each of the *B. xylophilus* ENG sequences, the localization of the *Bx-eng-1* in the esophageal glands and the presence of enzyme activity in secretions of *B. xylophilus* suggest that Bx-ENG-1 is secreted from the stylet of the nematode. 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 [23,24]. Bx-ENG-1, 2 and 3 could therefore be secreted through the nematode stylet into plant tissues and 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 used by the nematode to degrade the cell walls of fungi on which it feeds as cellulose, as well as chitin and other polysaccharides, have been shown to be present in the walls of some of the fungi on which *B. xylophilus* feed [25].

Several lines of evidence demonstrate the endogenous origin of the three cellulase genes, *Bx-eng-1*, *2* and *3*. 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 situ hybridization localized the transcripts of *Bx-eng-1* specifically to the esophageal glands of *B. xylophilus*, a region that has never been shown to contain symbiotic bacteria or fungi. In addition, 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 the genes were not a contaminant from the fungus on which the nematode was raised. These data strongly support a nematode origin for the *Bx-eng-1*, *2* and *3* genes.

GHF45 cellulases have been found from fungi, bacteria, protists, and a very small number of animals [26] (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. This 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 HGT from fungi.

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 nematodes [27,28]. The ancestor of *B. xylophilus* was probably a fungal feeder [29] 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 [3,10] and GHF5 cellulases are not present in the 5000 ESTs we have analyzed from *B. xylophilus* to date. 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 [11], 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 HGT seems to have played a key role in this process on both occasions. The role of HGT in the acquisition of GHF5 cellulases by cyst and root-knot nematodes has recently been questioned [30]. However, the presence of different cellulases in different nematode groups, as described here, strengthens the argument that HGT has played a key role in evolution of plant parasitism by nematodes and suggests that this has been the case on more than one occasion.

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