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Identification and Characterization of Expansins from *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae)

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We identified two novel expansin (EXP) genes in the expressed sequence tag database of *Bursaphelenchus xylophilus*, designated as Bx-EXPB2 and -EXPB3. Novel Bx-EXPBs encoded 150 amino acids and their similarities in coding sequence were 70.7–84.0% to the previously reported EXPB1 of *B. xylophilus*. Bx-EXPB2 and Bx-EXPB3 were clustered with Bx-EXPB1 and Bm-EXPB1, respectively, forming the independent phylogeny with other nematode EXPs. All identified Bx-EXPBs contained the signal peptide and were only expressed during the propagative stage, suggesting that they are secreted to facilitate nematode migration through hosts by loosening cell walls during infection. Quantitative real-time PCR analysis showed that the relative accumulation of Bx-EXPB3 mRNAs was the highest among the three Bx-EXPs examined and the order of mRNA accumulation was as follows: Bx-EXPB3 > Bx-EXPB2 >> Bx-EXPB1. Homology modeling of Bx-EXPBs showed that the structurally optimum template was EXLX1 protein of *Bacillus subtilis*, which shared residues essential for catalytic activity with Bx-EXPB1 and Bx-EXPB2 except for Bx-EXPB3. Taken together, Bx-EXPB1 and Bx-EXPB2 may be involved migration through plant tissues and play a role in pathogenesis.

Keywords : *Bursaphelenchus xylophilus*, expansin, homology protein modeling, pathogenicity, pine wilt disease

The pinewood nematode, *Bursaphelenchus xylophilus* is the known agent causative of pine wilt disease (Mamiya, 1975; Lee et al., 2011). *Bursaphelenchus xylophilus* are known to be transferred from infected pine trees to new ones by the pine sawyer beetle, *Monochamus alternates*

(Mamiya, 1975). To express their potent phytopathogenicity that is associated with infection and maintenance in the host trees, *B. xylophilus* must overcome the physical barriers such as plant cell walls, which are mainly composed of polysaccharides, lipids, and proteins. The architecture of plant cell wall is determined by cellulose, made of repeating glucose molecules joined by β -1,4 glycosidic bonds (McQueen-Mason and Cosgrove, 1994; van den Brink and de Vries, 2011). The cellulose framework of the wall is interpenetrated by a cross-linked matrix of non-cellulose molecules, hemicelluloses, or pectin. Even though pinewood nematode infected trees usually wither and die within a couple of months (Mamiya, 1975), we still do not understand the molecular and cellular pathophysiology underlying the abilities of pinewood nematodes to infection and propagate in the trees. However, the presence of diverse cell wall degrading enzymes such as endoglucanases (Kikuchi et al., 2004; Smant et al., 1998), pectate lyases (Kikuchi et al., 2006; Popeijus et al., 2000) and expansins (EXPs) (Kikuchi et al., 2009) that to play crucial roles in initiation and progression of the plant diseases are present in *B. xylophilus* genomes suggested that secreted cell wall degrading enzymes from *B. xylophilus* may be important factors involved with infections and propagation in host trees.

EXPs in plants play pivotal roles in a wide variety of developmental processes by loosening of their cell walls by weakening non-covalent interactions (McQueen-Mason and Cosgrove, 1994; McQueen-Mason and Cosgrove, 1995; Whitney et al., 2000), which makes cell walls susceptible to degradation and remodeling by other cell wall degrading enzymes (Cosgrove, 2000; van den Brink and de Vries, 2011). While most EXPs were identified from plants, recent findings that plant-parasitic nematodes express functional EXPs suggested that they could be one of the major factors regulating plant-parasite interactions (Qin et al., 2004; van den Brink and de Vries, 2011). However, compared with EXPs in plants, the understanding of *in vivo* functions of

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nematode EXPs are still rudimentary. To investigate the molecular and cellular mechanisms underlying infections and pathogenesis of *B. xylophilus*, we identified EXPs in *B. xylophilus* cDNA and EST library. The stage specific expression patterns of EXPs were further characterized by quantitative real-time Polymerase Chain Reaction (PCR) and their structures were predicted by performing three-dimensional homology modeling.

Materials and Methods

Nematode, media and culture conditions. *Bursaphelenchus xylophilus* and *B. mucronatus* were separated from chips of infected pine wood using Baermann methods (Viglierchio and Schmitt, 1983) and grown on a lawn of *Botrytis cinerea* cultured on potato-dextrose agar media at 25°C. The discrimination between two species was performed by microscopic observation and species-specific PCR (ssPCR) (Kang et al., 2004). The fourth stage dispersive juveniles were separated from newly emerged *M. alternatus* adults by dissection. The visual identification of nematodes isolated as *B. xylophilus* was confirmed by ssPCR (Kang et al., 2004).

Total RNA extraction and cDNA synthesis. Total RNAs were extracted using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) from the propagative, the dispersive, or *in vitro* propagative (media grown) stages of *B. xylophilus* (Chomczynski and Sacchi, 1987). cDNAs were synthesized with Superscript III™ reverse transcriptase according to manufacturer's instructions (Invitrogen). The reactions contained 2 µg of total RNA, 0.2 µM of Oligo-dT primer, 1 µl of reverse transcriptase, and a reaction mixture (0.4 mM of each dNTP and 2.4 mM MgSO₄), in a final volume of 20 µl. The reactions were incubated at 50°C for 50 min to synthesize cDNA and 37°C for 20 min to remove RNA by addition of RNase H (Invitrogen).

Cloning of EXPs from the pinewood nematode. To amplify partial fragment of *EXPB* gene, two degenerative primers (D-F1 and D-R1; Table 1) were used, based on the sequence similarity among nematode *EXPB* genes. All PCR reactions were conducted with *Pfx Taq* polymerase (Invitrogen). The following parameters were utilized to amplify *EXPB* gene from single-stranded cDNAs: 95°C for 1 min followed by 35 cycles at 95°C for 20 sec, 53°C for 20 sec, and 68°C for 40 sec, and one additional cycle at 68°C for 1 min. Five micro-liters of each PCR reaction were separated on 1% agarose gel containing GelStar® nucleic acid stain (BioWhittaker Molecular Applications, Rockland, ME, USA). The resultant PCR products were cloned into pGEMT-easy vector (Promega, Madison, WI, USA) and both strands were sequenced.

Quantitative real-time PCR of Bx-EXPBs. Total RNAs and cDNA synthesis were described above. For the qrt-PCR to determine relative expression level for each EXPB in *B. xylophilus*, total RNA (10 ng) was used as the template for the qrt-PCR. The qrt-PCR amplification was performed with specific primers for Bx-EXPB1 (Table 1). The tubulin gene from *B. xylophilus* was used as a reference gene for quantification (Kang et al., 2009). The amplification efficiency of each gene was estimated by using the equation, $E = 10^{-1/\text{slope}}$, where the slope was derived from the plot of amplification critical time (Ct value) versus serially diluted template cDNA concentration. Optimized PCR master mix (20 µl) contained the following components: 0.2 mM dNTPs, 5 reaction buffer, diluted SYBR Green I (1:40,000 final concentration; Invitrogen), 0.5 U AccuPrime™ *Pfx* DNA polymerase (Invitrogen), 2 µl cDNA (equivalent to 10 ng total RNA), and 2 µM sense and antisense primers for Bx-EXPBs. The qrt-PCR was performed using Chromo4i (Bio-Rad, Hercules, CA, USA). Optimized thermal program was 1 cycle of 95°C for 3 min, 40 cycles of 95°C for 20 sec, 60°C for 20 sec, and 68°C for 30 sec, and followed by a

Table 1. Primers and their sequences used in this study

Primer	Sequence	Experiment
D-F1	5'-ATYACKCCCMAGKYKAACAAGCC	Amplification of Bx-EXPBs
D-R1	5'-CABTYGTYKTCSRYKGGGAAGG	
RT-B1-F	5'-ATGAATCGCGTCTACTTGTGTCGC	qRT-PCR for Bx-EXPBs
RT-B1-R	5'-TTAACATCCGCTGGCAGGGCTAGC	
RT-B2-F	5'-ATGAATTCCTTGACTTGTTGG	
RT-B2-R	5'-TTAGCAGCTGCTGATGGTGGTCTGAT	
RT-B3-F	5'-ATGAATCCCTTGACTTGTTGGCAG	
RT-B3-R	5'-TCAACAGTTTGTGGTTGGGGTGGTGTCCG	
Tubulin-F	5'-TCCGTACCCTGAAGTTGGCTAACC	
Tubulin-R	5'-AAGTGGAGACGAGGGAATGGAACC	

final 1 cycle of 68°C for 3 min. Following qrt-PCR, homogeneity of PCR product was confirmed by the melting curve analysis. Quantification of the transcript level was conducted according to the $2^{-\Delta Ct}$ method (Pfaffl, 2001). The qrt-PCR was repeated two times with independently extracted total RNA (cDNA), and each reaction was conducted with 3 replicates to minimize intra-experiment variation. SigmaStat ver. 2.0 software (SPSS, USA) was used for *t*-test to detect statistical differences.

Multiple alignments and phylogenetic analysis of expansins. The deduced amino acid sequences of EXPB from *B. xylophilus* EST library and genomic DNA were compared with those of EXPs obtained from nematodes, plants and bacteria. The protein sequences of EXP domain were aligned with ClustalX ver. 1.83, using Gonnet as the protein weight matrix. Phylogenetic trees for the alignment were constructed with Neighbor-joining method (boot trap, $n = 1,000$). The tree was obtained by the protein sequence using Molecular Evolutionary Genetic Analysis ver. 4.1 software. EXPs used in this analysis were as follows: **Nematodes:** 3 EXPBs from EST library [*B. xylophilus* EXPB1 (AB374536; Bx-EXPB1), Bx-EXPB2 (T3-1245 clone), and Bx-EXPB3 (T3-2805 clone)] and 8 EXPs; *B. mucronatus* EXPB1 (AB374538; Bm-EXPB1); *Globodera rostochiensis* EXPB1 (AJ556781; Gr-EXPB1) and Gr-EXPB2 (AJ311902); *Ditylenchus destructor* EXPB1 (GU373911) and EXPB2 (*Heterodera glycines* EXPB1 (BF014507; Hg-EXPB1). **Plants:** *Arabidopsis thaliana* EXPB3 (NM_118965; At-EXPB3); *Nicotiana tabacum* EXPL protein (AF333386; Nt-EXPL); *Lycopersicon esculentum* EXPB2 (DQ205653; Le-EXPB2); *Solanum tuberosum* EXPB2 (DQ314759; St-EXPB2). **Bacteria:** *Bacillus subtilis* EXLX1 (GU327817; Bs-EXLX1), and *Clavibacter michiganensis* subsp. *michiganensis* NCPPB EXP-like protein (NC_009480; Cm-EXPL).

Prediction of protein structure via homology modeling. Template identifications of EXP domain in Bx-EXPBs were carried out using PHYRE (<http://www.sbg.bio.ic.ac.uk/servers/phyre/>) (Kelley and Sternberg, 2009) or the Swiss Model Workspace (<http://swissmodel.expasy.org/workspace>) (Arnold et al., 2006). Predicted 3-D structures of Bx-EXPBs were evaluated using the Qualitative Model Energy Analysis (QMEAN) server (<http://swissmodel.expasy.org/qmean>). The homology model that received the highest QMEAN score with the smallest estimated per-residue errors was used to represent the 3-D structure of Bx-EXPBs. Visualization and modification of models and structures were done with Swiss PDB viewer 4.0.1. (Swiss Institute of Bioinformatics) (Kaplan and Littlejohn, 2001).

Results

Three EXPBs are expressed in *B. xylophilus*. To identify expansin (*EXP*) genes from *B. xylophilus*, the degenerative primers (D-F1 and D-R1) were synthesized, based on the similarity of other nematode *EXPB* genes. The nucleotide sequences of resulting PCR products were compared with the previously reported expression sequence tag (EST) clones of *B. xylophilus* (Kang et al., 2004; Kang et al., 2009). Two *EXPB* genes were identical from the two EST clones from EST library and designated as Bx-EXPB2 and -EXPB3. All EXPBs shared the common structures of secreted proteins. For example, all Bx-EXPBs were predicted to contain signal peptides (16 amino acids in length). The full-length cDNA for each Bx-EXPB was 450 bp with a predicted molecular mass of 15.6 to 15.8 kDa. Identified Bx-EXPBs contained only an EXP domain and conserved cysteine residues, which are also found in EXPA and EXPB family. Bx-EXPB2 and Bx-EXPB3 exhibited 84% and 86% sequence similarity to Bx-EXPB1 (AB374536) and Bm-EXPB1 (AB374538), respectively. However they showed relatively low sequence similarity (54.4 to 60.4%) to Bx-EXP (EU143763). To examine the origin of Bx-EXPBs, genomic sequences were analyzed. An intron was found in the genomic DNA sequence in all Bx-EXPBs (Fig. 1A and Supplementary Figure 1).

EXPB genes from nematodes independently clustered. On the basis of the alignment of EXP domains deduced protein sequences from nematodes, bacteria, and plants, a phylogenetic tree was generated via neighbor-joining analysis (Fig. 1B). EXPs from nematodes, *B. xylophilus*, *B. mucronatus*, *G. rostochiensis* and *Heterodera glycines*, formed in an independent cluster which is distant from bacteria and plant EXPs. Expansins from nematodes were closely related with those of plants compared to those from bacteria.

Expression of Bx-EXPBs in propagative stage. The expression of EXPBs between the propagative stage and the dispersive stage *B. xylophilus* were compared. When degenerative primers (DF1 and DR1) were used, strong single band were detected from the cDNAs of propagative stage, but not from that of the dispersive stage (Fig. 2A). However, the primer sets designed from first reported Bx-EXP (EU143763; C-F1: 5'-GCGTTGTAATAGGTGAACCTCC; C-R1: 5'-AGGCCAAGGATGCTACTATCACC; Fig. 2A) did not produce any band. This result suggested that EXPBs were not expressed in the dispersion stage *B. xylophilus*. Since they were stayed in the pine sawyer beetles, there were no cell walls or barriers which were degraded by EXPBs. To further characterize expression patterns of the

Fig. 1. Multiple alignment and phylogenetic analysis of expansin domains from nematodes, plants and bacteria. (A), Amino acid sequences were aligned using CLUSTALW. The consensus residues and the putative polysaccharide binding site in EXLX1 were indicated with black boxes and asterisks, respectively. The location of modified HFD motif in nematode EXPs were indicated with the red box. The inverted triangle indicates the location of intron among Bx-EXPBs. (B), The phylogenetic tree was constructed by the Molecular Evolutionary Genetic Analysis ver. 4.0 software. Branch lengths are proportional to the numbers of amino acid substitutions, which are indicated by a scale bar below the tree. The bootstrap values are calculated from 1,000 replications. **Nematodes:** *Bursaphelenchus xylophilus* EXPBs (Bx-EXPB1, Bx-EXPB2 and Bx-EXPB3); *B. mucronatus* EXPB1 (Bm-EXPB1); *Globodera rostochiensis* EXPB1 (Gr-EXPB1) and Gr-EXPB2. **Plants:** *Arabidopsis thaliana* EXPB3 (At-EXPB3); *Nicotiana tabacum* EXP-like protein (Nt-EXPL); *Lycopersicon esculentum* EXPB2 (Le-EXPB2); *Solanum lycopersicum* EXPB (Si-EXPB); *S. tuberosum* EXPB2 (St-EXPB2). **Bacteria:** *Bacillus subtilis* EXLX (Bs-EXLX); *Clavibacter michiganensis* subsp. *michiganensis* NCPPB EXP-like protein (Cm-EXPL).

three EXPBs, qrt-PCR analysis were performed using specific primer sets for each EXPB. We found that mRNA for Bx-EXPB2 and Bx-EXPB3 accumulated at much

higher levels than that of Bx-EXPB1 in the *in vivo* (Fig. 2B) and *in vitro* propagation stages (Fig. 2C). The level of relative expression is as follows: Bx-EXPB3 > Bx-EXPB2

Fig. 2. Detection of Bx-EXP mRNAs from cDNAs of the propagative stage, the dispersive stage and the media in *Bursaphelenchus xylophilus*. (A), P and D represent the propagative stage and the dispersive stage, respectively. The arrow indicates the Bx-EXPB-specific DNA fragment. M: 100 bp DNA ladder, 1 and 3: PCR products amplified with D-F1 and D-R1. 2 and 4: PCR products amplified with C-F1 and C-R1. (B and C), detection of Bx-EXPB mRNA accumulation in the propagative stage (B) and in vitro propagation stage (media-grown; C) via qrt-PCR. Bars indicate the average of relative ratio + S.E.M. Different letters above each bar indicate statistical difference by *t*-test ($P < 0.001$).

>> Bx-EXPB1.

Putative polysaccharide binding sites and catalytic residues are conserved in Bx-EXPBs. In order to address the function of Bx-EXPs, protein homology modeling was

performed. The templates were identified via Swiss-Model Workspace and Phyre. The quality of templates was analyzed with QMEAN scores, representing a global score of the whole model, reflecting predicted model reliability ranging from 0 to 1 (Benkert et al., 2009). EXLX1 from *B. subtilis*,

Fig. 3. Three-dimensional homology models of EXP domain in Bx-EXPBs. (A) 3D30: EXLX protein from *Bacillus subtilis*, the most similar template of Bx-EXPB2, (B-D), predicted model of Bx-EXPB1, 2 and 3. The α -helix and β -strand were colored with magenta and light green, respectively. The putative polysaccharide binding site and the catalytic residue essential for activity were indicated with blue and red, respectively.

3D30, showed the highest QMEAN score among identified templates of Bx-EXPBs (Supplementary Table 1)(Kerff et al., 2008). The estimated per-residue errors of identified were also considered (Supplementary Figure 2). The EXP domain of each Bx-EXPB exhibited 16.5 to 25.2% sequence identity to D1 of EXLX1 from *B. subtilis*, homologous to EXP domain. However the overall structures of EXPBs were similar to that of D1 domain of EXLX1, there was structural difference found in the composition of EXP domain. The D1 domain of EXLX1 was composed of three α -helices and six β -strands flanked by short loops (Fig. 3A). Two α -helix (Bx-EXPB1; Fig. 3B), one α -helix and one β -strand (Bx-EXPB2; Fig. 3C), and two α -helix deletions and three β -strand additions (Bx-EXPB3; Fig. 3D) were predicted in the 3-D structures of the EXP domains in Bx-EXPBs. The catalytic D⁸² residue in EXLX1 corresponded to D⁸⁸ residue in Bx-EXPB1 and Bx-EXPB2 (Fig. 3B and 3C) whereas is replaced with N residue in Bx-EXPB3 (Fig. 3D). The putative polysaccharide binding sites (PPBS) in D1 domain 1 of EXLX1 were lined near catalytic D⁸² residue (Fig. 3A). The residues corresponding to PPBS based on multiple alignments were shown in the predicted 3-D structure (Fig. 3). Residues corresponding to PPBS in Bx-EXPB2 showed the most similar distribution pattern to that in EXLX1 (Fig. 3C) among Bx-EXPBs.

Discussion

EXPs are plant cell wall loosening enzymes and play various roles such as cell enlargement and the rearrangement of plant cell wall polysaccharides (Cosgrove, 2000). Since the first nematode EXP was discovered in the potato cyst nematode *G. rostochiensis* (Qin et al., 2004), a number of EXPs have been identified from other nematodes: Twenty putative EXPs were identified through the analysis of the genome of *Meloidogyne incognita* (Abad et al., 2008) and EXP-like proteins were also identified from *B. xylophilus* as well as *B. mucronatus* (Kikuchi et al., 2007; Kikuchi et al., 2009). In this study we identified two novel EXPB genes from *B. xylophilus*. Both EXPBs exhibiting high similarity to previously reported EXPB proteins of *Bursaphelenchus* spp. contained a signal peptide, indicating that they are secreted, consistent with their involvement in cell wall loosening during pathogenicity as shown for EXP of the potato cyst nematode (Gr-EXP) (Qin et al., 2004). Bx-EXPBs exhibited relatively low sequence similarity with plant EXPs (Fig. 1A). Plant EXPs are composed of two structural domains (Cosgrove, 1997; Sampedro and Cosgrove, 2005): The N-terminal domain (D1 in EXPB) has low similarity to the catalytic domain of endoglucanase 45 family (EG45) and the C-terminal domain (D2 in EXPB) has putative carbohydrate binding domain (CBD).

The EG45-like domain found in diverse EXP-related proteins (Kudla et al., 2005; Li et al., 2002; Xu et al., 2001) has multiple cysteine/tryptophan residues and conserved HFD amino acid sequence motif. Non-plant EXPs including nematode EXPs may have evolved independently of plant EXPs leading to the different gene structure. Bx-EXPBs were also distant from plant EXPs (less than 20% similarity; Fig. 1B) even though some characteristics were shared with plant EXPs such as conserved cysteine residues and modified HFD motifs (HVD or HVN; Fig. 1A).

There are four gene structures in nematode EXPs (Haegeman et al., 2010; Haegeman et al., 2011): (1) signal peptide-EXP domain structure found in *B. xylophilus* and *B. mucronatus*, *G. rostochiensis*, *M. arenaria*, *M. chitwood*, *M. hapla*, *M. incognita*, *M. javanica*, *M. paranaensis*, *Heterodera glycines*, and *Xiphinema index*; (2) signal peptide-EXP domain-CBM domain structure found in *Ditylenchus africanus*; (3) signal peptide-CBM domain-EXP domain structure found in *G. rostochiensis*, *M. hapla* and *M. incognita*; (4) signal peptide-avirulence domain structure found in *M. incognita* and *M. javanica*. Except for the signal peptide-avirulence domain structure, other nematode EXPs are expected to have been generated via domain shuffling (Haegeman et al., 2010). Even though nematodes EXPs shared the characteristics with plant EXPs, nematode EXP domains are much closer to bacterial proteins than plant EXPs via BLAST search. In addition, genomic DNA sequences of Bx-EXPBs showed that Bx-EXPBs likely did not originate from endogenous or symbiotic microorganisms. BLAST search and genomic DNA sequence of Bx-EXPBs led to suggestion that Bx-EXPBs could have originated from bacteria via horizontal gene transfer as shown in other cell wall degrading enzymes and biologically functional (Jones et al., 2005; Kudla et al., 2005; Scholl et al., 2003).

The predicted optimum template of Bx-EXPBs by homology modeling is EXP of *B. subtilis* (EXLX1) (Fig. 3 and Supplementary Table 1) (Kerff et al., 2008). EXLX1 is composed of two domains, D1 and D2 as shown in plant EXPs. The D1 domain forms a six-stranded double- ψ β -barrel with high structural similarity to D1 of plant EXPs. The nematode D2 domains are structurally related to those of plant EXPs and are proposed to act as carbohydrate binding modules (CBM), whose function is mediating EXLX1 binding to whole cell walls and cellulose (Georgelis et al., 2011). In contrast to EXLX1 structure, Bx-EXPBs comprised of an EXP domain that lacks a CBM. Recently, the structure-function relationship of EXLX1 were analyzed via site-directed mutagenesis (Georgelis et al., 2011): the residue (D⁸²) essential for catalytic activity in D1 domain and residues (W¹²⁵, W¹²⁶ and Y¹⁵⁷) important for cellulose binding and wall loosening activity in D2 domain were identified. Only the D⁸² residue in EXLX1 which corre-

sponded to D residues in the catalytic sites of EG45 and EG102 enzymes was conserved and located in the HFD motif (Fig. 1) (Kerff et al., 2008). D82A or D82N mutation in EXLX1 completely eliminated wall loosening activity, demonstrating that D⁸² residue is essential for catalytic activity (Georgelis et al., 2011). The catalytic D residue was also found in Bx-EXPB1 and Bx-EXPB2 whereas the N residue in Bx-EXPB3 and Bm-EXPB1, indicating that catalytic Bx-EXPB1 and -EXPB2 may be functional in *B. xylophilus*. Other residues as PPBS (T¹⁴, S¹⁶, D⁷¹, Y⁷³, E⁷⁵, K⁹⁵, and K⁹⁸) were identified in the D1 domain of EXLX1, which are near catalytic D⁸² residue in 3-D structure (Fig. 3A). Residues in the PPBS are not conserved in EXPs of nematode and plants. The D⁷¹ residues that are conserved in plant and bacteria are replaced with an N residue in nematode. Y⁷³ residue was only found in EXLX whereas E⁷⁵ residue except for Bx-EXPB3 was identical with EXPBs of nematode. Among the point mutations, D71N, Y73A, T14A, S16A, E75A and E75Q, only Y73A revealed severely reduced activity and the other mutations exhibited moderate reduction. K95A and K98A did not show any effect on enzyme activity (Georgelis et al., 2011), which were distant from the catalytic residue (Fig. 3).

Restriction in the distribution of nonpathogenic *B. mucronatus* in host trees was found whereas pathogenic *B. xylophilus* moved freely and caused severe symptoms (Futai, 1980; Kosaka et al., 2001; Odani et al., 1985). Plants in which parasitic nematodes have invaded aerial parts of the host normally lose water regulation, exceed the permanent wilting point and die (Chen et al., 2006). Plant parasitic nematodes encode at least one *EXP* in the pharyngeal gland, which is thought to be secreted into the host due to the presence of the signal peptide (Kikuchi et al., 2004). Therefore, it appears that EXPs enhance migration in the host in combination with other cell wall degrading enzymes. Based on similarities to EXLX1, EXPBs present in pathogenic *B. xylophilus* are catalytic whereas inactive EXPBs in nonpathogenic *B. mucronatus* are inactive due to the D82N mutation, indicating that EXPBs in pathogenic *B. xylophilus* are involved in migration through plant tissues as well as play a role in pathogenesis.

The qRT-PCR analysis and stage-specific expression of Bx-EXPBs suggests that Bx-EXPBs may be involved in migration within host and play a role as a pathogenic factor during the propagative stage of infections in susceptible hosts (Fig. 2). The life cycle of *B. xylophilus* is divided into the propagative stage for reproduction and parasitization, and the dispersive stage for dauer formation and migration, and the media (Ishibashi et al., 1978; Mamiya, 1975). The propagative stage including eggs, larvae and adults is the mixed developmental stage, in which most host-nematode

interactions such as parasitization, infection, proliferation and defense by the host occur. Therefore, the propagative stage would be closely related to the pathogenicity or proliferation of *B. xylophilus* rather than the dispersive stage at which the gonad formation is suppressed (Kondo and Ishibashi, 1978). Although we did not analyze the biochemical activity for Bx-EXPBs identified in this study (there was an experimental problem in functional expression; personal communication with Dr. Y. Lee, Chungbuk National Univ., Korea), the other evidences shown in this study strongly support that secreted Bx-EXPB1 and Bx-EXPB2 could loosen cell walls of host trees and allow *B. xylophilus* to migrate and propagate.

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