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Molecular properties of a venom allergen-like protein suggest a parasitic function in the pinewood nematode *Bursaphelenchus xylophilus*

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ABSTRACT

The pinewood nematode, *Bursaphelenchus xylophilus*, is a destructive pest in several countries including Japan, China and Korea. Of three genes encoding the venom allergen-like protein in *B. xylophilus*, *Bxvap-1* showed the highest transcript levels at the pine-grown propagative stage. In addition, western blot and immunohistochemical analyses using anti-BxVap-1 polyclonal antibody verified a specific increase in BxVap-1 expression levels at the pine-grown propagative stage. Using immunohistochemistry, BxVap-1 was detected around the putative oesophageal glands and metacarpus, suggesting that BxVap-1 is secreted into the host pine tree and is involved in the parasitic mechanism. To explain the parasitic role of BxVap-1, we measured the migration rate inside pine seedlings of *B. xylophilus* either with or without *Bxvap-1* knockdown by RNA interference. *Bxvap-1* knockdown resulted in a significantly lower migration rate in the >6 cm region compared with the control *B. xylophilus*. These results suggest that BxVap-1 is involved in *B. xylophilus* migration, perhaps by suppressing the pine tree defence mechanism.
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1. Introduction

The pinewood nematode, *Bursaphelenchus xylophilus* (Bx), is a destructive phytophagous nematode that infects pine species in several countries including Japan (Mamiya, 1975), China (Yang and Wang, 1989), Korea (Yi et al., 1989) and Portugal (Mota et al., 1999). Despite the serious damage it causes, little is known about its pathogenic mechanisms, especially at the molecular level. To establish effective control measures against *B. xylophilus*, the pathogenic factors and molecular components of the parasitic interaction between pine trees and the pinewood nematode must be identified.

Many plant parasitic nematodes, including *B. xylophilus*, secrete proteins that facilitate their invasion or migration within the host plant (Baum et al., 2007; Davis et al., 2008; Jones et al., 2008). These secretory proteins are produced in the oesophageal glands or other secretory organs, including the epidermis and amphids, and are subsequently secreted into the host plant (Vanholme et al., 2004). Secretory proteins with putative parasitic functions have been reported in several plant parasitic nematodes (Curtis, 2007; Jones et al., 2008). The most widely known secretory proteins are cell wall modification enzymes such as endoglucanases, pectate lyase and expansins which soften cell walls, thereby allowing penetration of the parasitic nematodes into the plant epidermis or migration within plant tissue by directly degrading or modifying host plant cells (Baum et al., 2007). Several *B. xylophilus* endoglucanases and pectate lyases have been characterised, which are suggested to have putative parasitic secretory functions that facilitate the feeding and migration processes within the pine tree (Kikuchi et al., 2005, 2006).

Similar to cell wall modification enzymes, many putative parasitic secretory proteins are produced from the oesophageal glands, such as chorismate mutase (Jones et al., 2003), ubiquitin extension protein (Tytgat et al., 2004), chitinase (Gao et al., 2002), calreticulin (Jaubert et al., 2002) and venom allergen-like protein (Vap) (Ding et al., 2000). The Vap family protein is intriguing because its members are highly transcribed during plant nematode parasitism; however, their functions still remain to be elucidated (Ding et al., 2000; Gao et al., 2001; Wang et al., 2007).

Vap proteins belong to the class of sperm-coating protein (SCP)-like extracellular proteins that have a SCP/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS) domain, which is found in many eukaryotes (Chalmers et al., 2008). Many SCP/TAPS domain-containing proteins are secreted extracellularly. Their physiological functions remain unclear, although they have been proposed to function in the

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immune response (Alexander et al., 1993; Murphy et al., 1995), sperm development (Kovalick and Griffin, 2005) and host invasion in some parasitic nematodes (Hawdon et al., 1999; Murray et al., 2001). Nematode Vap was first reported in the animal parasitic nematode, *Ancylostoma caninum*. It represents a major protein of the excretory/secretory products collected from infective L3s (Hawdon et al., 1996). In plant parasitic nematodes, *vap* genes have been characterised in the soybean cyst nematode, *Heterodera glycines* (Gao et al., 2001) and the root-knot nematode, *Meloidogyne incognita* (Ding et al., 2000; Wang et al., 2007), and have been reported in the expressed sequence tag (EST) database of the peanut pod nematode, *Ditylenchus africanus* (Haegeman et al., 2009). The *vap* genes have also been reported and characterised in *B. xylophilus*. Two *Bxvap* genes (*Bxvap1* and *Bxvap2*) were found in the EST database of propagative-stage *B. xylophilus* (Kang et al., 2009) and three *Bxvap* genes (*Bx-vap-1*, *Bx-vap-2* and *Bx-vap-3*) were identified in a gland region-specific cDNA library of *B. xylophilus* (Lin et al., 2011). Amino acid sequence alignment showed that *Bxvap2* is identical to *Bx-vap-1* whereas *Bxvap1* differs from the other two *Bx-vaps*. The relative transcript levels of *Bxvap1* and *Bxvap2* were analysed in dispersal fourth-larva stage (D4S), pine-grown propagative mixed stage (PGPS) and media-grown propagative mixed stage (MGPS) organisms. The results suggested that *Bxvap2* has parasitic functions due to its significantly increased levels of transcription in PGPS nematodes (Kang et al., 2009). The genomic features and developmental expression patterns of the three *B. xylophilus vap* genes (*Bx-vap-1*, *Bx-vap-2* and *Bx-vap-3*) were examined and their transcript distribution patterns in oesophageal glands were analysed by in situ hybridisation (Lin et al., 2011). The study of the *B. xylophilus* genome has suggested that *vap* genes are important parasitic genes in *B. xylophilus* (Kikuchi et al., 2011).

In this study, we investigated the host-dependent expression and tissue distribution patterns of BxVap-1 protein (identical to BxVap2, Kang et al., 2009). In addition, the migration rate in host pine trees of the *Bxvap-1* knockdown *B. xylophilus* was measured and compared with control nematodes to determine the role of BxVap-1 in parasitism.

2. Materials and methods

2.1. Nematodes

Bursaphelenchus xylophilus was grown on a lawn of *Botrytis cinerea* cultured on potato dextrose agar (PDA) plates at 28 °C for 1 week. To obtain PGPS nematodes, approximately 10,000 *B. xylophilus* were injected into three 5- to 6-year-old pine trees. After 1 month, when the trees had withered, worms were separated using the Baermann method (Viglierchio and Schmitt, 1983). All nematodes were rinsed with M9 buffer to remove bacteria and fungi and subsequently stored in water at 4 °C.

2.2. Cloning of Bxvap-1 full-length cDNA

Total RNA was isolated using TRI reagent (MRC, Cincinnati, OH, USA), from nematodes ground by a Bullet Blender (Next Advance, Averill Park, NY, USA) using metal beads and then treated with DNaseI (Takara, Shiga, Japan). The partial sequence of *Bxvap-1* was obtained from the pinewood nematode EST database (http://pinewoodnematode.net) (Kang et al., 2009), and the full-length *Bxvap-1* sequence was obtained by 5′- and 3′-rapid amplification of cDNA ends (RACE) using a SMART RACE cDNA amplification kit (Clontech, Madison, WI, USA). The resulting sequence was identified by conducting a BLAST search (http://blast.ncbi.nlm.nih.gov) and compared with *vap* genes of other nematodes by using MegAlign (DNA Star Inc., Ver. 5.01).

2.3. Analysis of relative transcript levels by quantitative real-time PCR (qPCR)

The transcript levels of the three *Bxvap* genes (*Bxvap-1*, *Bxvap-2* and *Bxvap-3*) of the PGPS and MGPS nematodes were estimated by qPCR. Total RNA was extracted from the PGPS and MGPS nematodes, reared independently in three replicates as described in Section 2.2. First-strand cDNAs were synthesised from each total RNA sample using the SuperScript cDNA synthesis system (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA quantities were estimated with a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) to normalise the cDNA for qPCR, which was conducted with a 2 × DyNAmo™ HS SYBR® Green qPCR master mix (Finnzymes, Espoo, Finland). The cycling profile used was as follows: 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 20 s and 72 °C for 20 s. *β-Tubulin* was used as a reference gene. Relative transcript levels were calculated using the $2^{-\Delta ct}$ method (Pfaffl, 2001). Primers used are listed in Supplementary Table S1. The qPCRs were conducted for three biological replicates, which included three technical replicates for each reaction.

2.4. Expression of recombinant BxVap-1 using baculovirus

BxVap-1 was expressed using a bEASYBac baculovirus expression system (Choi et al., 2009). The full-length open reading frame (ORF) of BxVap-1 with His-tag sequences at the C-terminus was inserted into the pDualBac baculovirus vector and translocated into the *Autographa californica* nuclear polyhedrosis virus (AcNPV) chromosome by homologous recombination. Sf9 insect cells (5×10^4 cells) were transfected with recombinant AcNPV by incubating in TC-100 insect cell media (Invitrogen) containing 10% FBS at 27 °C for 1 week. Infected Sf9 cells were amplified three times before use as a seed stock for the transfection of 5×10^6 Sf9 cells. Infected cells were incubated at 27 °C for 3 days, harvested and separated by centrifugation at 1,500*g* for 5 min. Recombinant BxVap-1 was purified using a HisTrap affinity chromatography column (GE Healthcare, Uppsala, Sweden).

2.5. Generation of the BxVap-1 polyclonal antibody

Purified baculovirus-expressed recombinant BxVap-1 was used as an immunogen for antibody generation by injecting a rabbit at three independent time points. BxVap-1 polyclonal antibody (BxVap1Pab) serum was harvested and pre-absorbed with the lysate of Sf9 cells infected by wild-type AcNPV. A mixture of Sf9 cell lysate and BxVap1Pab serum (w/w, 2:1) was incubated with gentle shaking for 5 h at room temperature (RT) and centrifuged at 18,000*g* for 15 min at 4 °C. The supernatant was stored at −80 °C and used as the BxVap1Pab serum for western blots and immunohistochemistry.

2.6. SDS–PAGE and western blot

Both the PGPS and MGPS of *B. xylophilus* were homogenised with Tris buffer (0.1 M Tris–HCl, 0.02 M NaCl, pH 7.8) in a Bullet Blender (Next Advance) and centrifuged at 15,000*g* for 15 min at 4 °C. The supernatant was decanted and used for crude protein samples. Protein concentration was estimated using Bradford reagent (Sigma–Aldrich, St. Louis, MO, USA) and O.D. at 595 nm. After SDS–PAGE using 15 μg of protein samples, the gels were transferred to a nylon membrane using a western blotting system (Invitrogen), blocked with 5% skim milk in PBS with Tween-20 (PBST) for 3 h at RT and treated with BxVap1Pab serum and anti-rabbit secondary antibody in blocking solution

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(1:10,000). After incubating the membrane in Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA), the membrane was exposed to X-ray film for 5 min and the image was developed.

2.7. Immunohistochemistry

Both the PGPS and MGPS of *B. xylophilus* were used for whole-body immunohistochemical analysis according to the protocol in WormBook (http://www.wormbook.org). The nematodes were rinsed with M9 buffer at least three times to remove surface fungi and bacteria. The rinsed nematodes were fixed in 4% paraformaldehyde (PFA) for 2 h at 37 °C after freeze-cracking in liquid nitrogen. β-Mercaptoethanol solution (5% β-mercaptoethanol, 0.1% Triton X-100, 0.1 M Tris–HCl, pH 7.2) was added to the fixed nematodes for 2 h at 37 °C. After washing with PBST, collagenase (type VII, Sigma–Aldrich) was added for 8 h at 37 °C to increase permeability for the antibody. Collagenase-treated nematodes were blocked with blocking solution and antibody buffer (0.1 M PBS, 0.5% Triton X-100, 1 mM EDTA, 0.1% BSA and 0.05% sodium azide, pH 7.2) containing 10% goat serum albumin (Jackson ImmunoResearch, West Grove, PA, USA) was added for 1 h at RT. The primary antibody (BxVap1Pab) was diluted in blocking solution (1:50–200) and then added for an overnight incubation at RT. Anti-rabbit Alexa568 (Invitrogen) diluted in blocking solution (1:400) was then added for 4 h at RT. The nematodes treated with primary and secondary antibodies were mixed with Vectashield (Vector, Burlingame, CA, USA) and then mounted on glass slides. Whole-mount samples were photographed using a Zeiss Laser Scanning Microscope (LSM) 710 (Carl Zeiss, Oberkochen, Germany) and an Axio Image A1 fluorescence inverted optical microscope (Carl Zeiss). Digital images were processed using the LSM image browser (Carl Zeiss) and Adobe Photoshop (Adobe Inc., San Jose, CA, USA).

2.8. Bxvap-1 knockdown by RNA interference (RNAi) and movement assay

To amplify DNA templates used for double-strand RNA (dsRNA) synthesis, primers containing the T7 polymerase promoter (Supplementary Table S1) were designed using the BLOCK-iT™ RNAi designer program (Invitrogen, http://rnaidesigner.invitrogen.com). The *pQE30* plasmid sequence, which does not exist in *B. xylophilus*, was used as a negative control. *Bxvap-1* and *pQE30* DNA templates were amplified using *Ex Taq* polymerase (Takara) and used to synthesise dsRNA with the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA), which was followed by DNaseI digestion. Each *Bxvap-1* dsRNA (approximately 400 bp) and *pQE30* dsRNA (approximately 200 bp) was confirmed by gel electrophoresis.

For RNAi, mixed-stage nematodes were soaked in 20 µl of RNAi solution (0.25× Mg^{2+}-free M9 buffer) containing 3 mM spermidine (Sigma–Aldrich), 0.05% gelatin (Sigma–Aldrich), lipofectin (Invitrogen) and dsRNA (4 µg/µl) for 24 h at 28 °C. *Bursaphelenchus xylophilus* soaked in RNAi solution without dsRNA were used as the positive control.

After 24 h, approximately 200 nematodes were used to verify the efficiency of *Bxvap-1* knockdown by qPCR as described in Section 2.3. The qPCR was conducted for three biological replicates, each including three technical replicates. The remaining nematodes were used for migration assays which were modified from a previous method (Ichihara et al., 2000) with inoculation into 2 year old pine seedlings over 20 cm. To inject the nematodes, a 2 mm-deep hole was made in the middle of the tree and a small pipette tip containing approximately 300 nematodes was inserted (Supplementary Fig. S1A and B). The *pQE30* dsRNA-treated *B. xylophilus* and the untreated *B. xylophilus* (treated with RNAi solution lacking dsRNA) were used as the positive controls. Inoculated pine seedlings were incubated for 6 days at 25 °C in a 16 h-light:8 h-dark cycle. Pine seedlings were cut at 2 cm intervals and soaked in water for 1 day. The worms were then collected and counted (Supplementary Fig. S1C). Three or four biological replicates were conducted for all experiments. The mean comparison between the groups was calculated by Tukey's Honestly Significant Difference (HSD) with measured ANOVA.

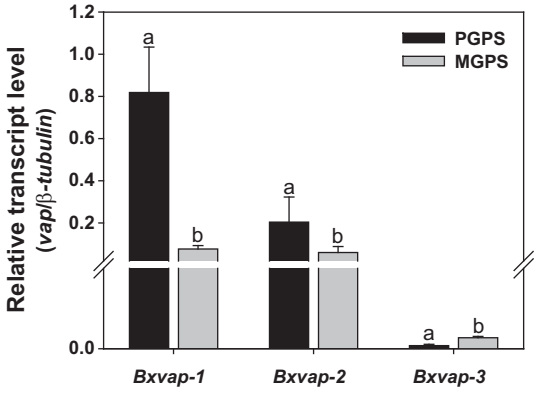


Fig. 1. Relative transcript levels of three *vap* genes of *Bursaphelenchus xylophilus* in the pine-grown propagative stage and the media-grown propagative stage. Bx venom allergen-like protein 1 displayed the highest transcript levels in pine-grown propagative stage (black bars). BxVap-1 showed the highest increase in transcript levels from media-grown propagative stage (gray bars) compared with pine-grown propagative stage. *β-Tubulin* was used as a reference gene. Three biological replicates were analysed, each with three technical measurements. Mean comparisons between groups were assessed by *t*-test and the different letters on top of the bars indicate statistically significant differences ($P < 0.05$).

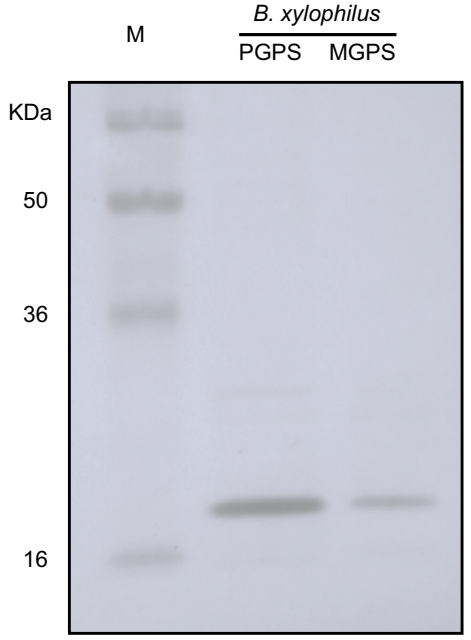


Fig. 2. Western blot with *Bursaphelenchus xylophilus* venom allergen-like protein 1 polyclonal antibody. Bands of 22 kDa were observed for both the pine-grown propagative stage (lane 2) and the media-grown propagative stage (lane 3) samples. The band intensity was stronger in pine-grown propagative stage than in media-grown propagative stage. M, protein marker.

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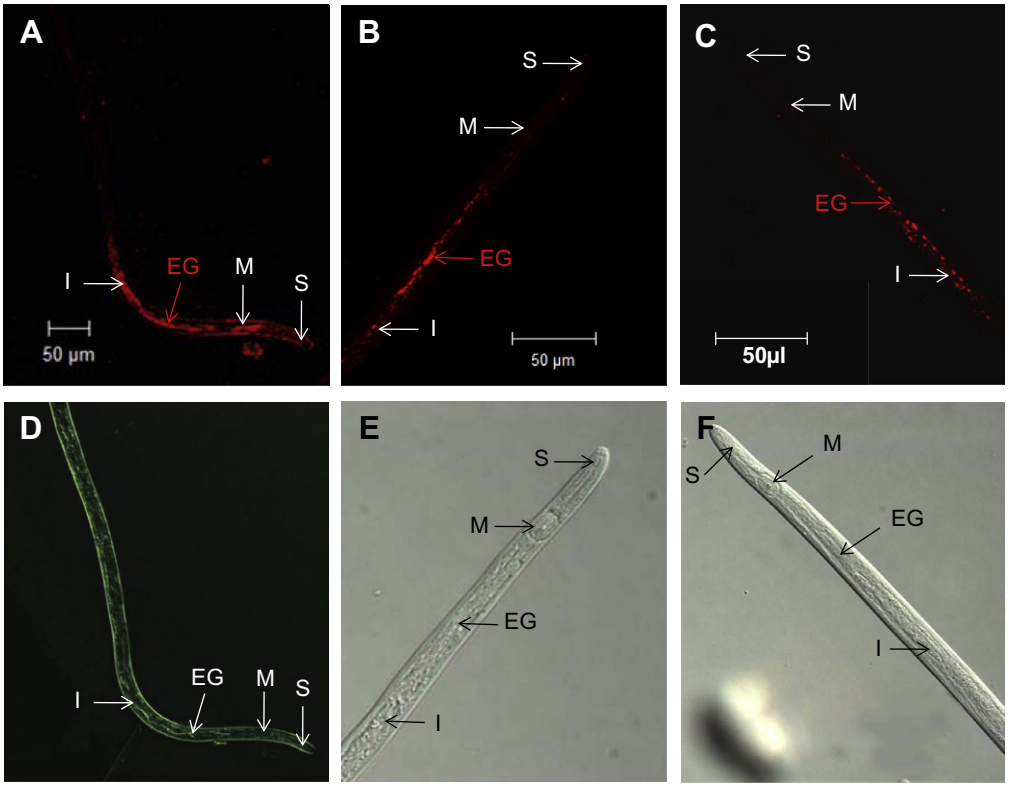


Fig. 3. Immunohistochemistry of *Bursaphelenchus xylophilus* venom allergen-like protein 1 in Bx-pine-grown propagative stage. (A–C) Signals were observed around putative oesophageal glands. (D–F) Optical microscopy photographs of A–C. Antibody dilutions: A, 1:100; B and C, 1: 200. Magnification: A and D, 100×; B, C, E and F, 200×. M, metacorpus; S, stylet; I, intestine.

3. Results

3.1. Sequence analysis of Bxvap-1

From the partial sequence of *Bxvap2* reported in our previous study, we sequenced the complete *Bxvap2* ORF, which yielded a predicted 204 amino acid peptide from 615 nucleotides. *Bxvap2* was determined to be identical to *Bx-vap-1* at the nucleotide level, which represents one of the three *B. xylophilus vap* genes reported previously (*Bx-vap-1*, *Bx-vap-2* and *Bx-vap-3*; GenBank Accession Nos. GQ272489, GQ272490 and GQ272491) (Lin et al., 2011) (Supplementary Fig. S2). We suggested a new nomenclature system for the *Bxvap* genes by slightly modifying that proposed by Lin et al. (2011). Based on the sequence identity, the *Bxvap2* gene was renamed as *Bxvap-1*, which was modified from *Bx-vap-1*. Other *Bxvap* genes, such as *Bx-vap-2* and *Bx-vap-3*, were also changed in this paper to *Bxvap-2* and *Bxvap-3*, respectively.

3.2. Relative transcript levels of the Bxvap genes

In a previous study, *Bxvap-1* displayed a remarkable pattern of elevated transcript levels at the propagative stage (Kang et al., 2009). Therefore, we analysed the relative transcript levels of *Bxvap-1*, *Bxvap-2* and *Bxvap-3* using qPCR. Among the three *B. xylophilus vap* genes, *Bxvap-1* displayed the highest transcript levels, whereas *Bxvap-3* showed the lowest transcript levels in both PGPS and MGPS nematodes (Fig. 1). Transcript levels were higher in PGPS than in MGPS for both *Bxvap-1* (10.6-fold) and *Bxvap-2* (3.4-fold). *Bxvap-3* transcript levels were 0.3-fold lower in PGPS than MGPS.

3.3. BxVap-1 protein levels

After determining that the transcript levels of *Bxvap-1* increased in PGPS, we measured the corresponding protein levels at various developmental stages by western blotting. Recombinant BxVap-1 expressed by a baculovirus expression system (Supplementary Fig. S3) was clearly detected by western blotting (Fig. 2). In a western blot of crude protein from the freshly harvested *B. xylophilus* of PGPS and MGPS, BxVap-1 was detected in both the PGPS and MGPS samples. The corresponding BxVap-1 band was more intense in PGPS than MGPS, indicating that BxVap-1 is expressed to a higher level in PGPS compared with MGPS (Fig. 2). The BxVap1Pab likely also recognises other BxVap proteins based on their high amino acid sequence similarities. Therefore, it appears that the BxVap-1 band pattern observed in the western blot is also contributed to by additional BxVap proteins. Nonetheless, because BxVap-1 is a predominant protein among the other BxVap proteins based on its high transcription levels (4- and 2,000-fold higher than BxVap-2 and BxVap-3, respectively), the increased band pattern observed in the PGPS immunoblot is predominately attributed to BxVap. The specific BxVap-1 expression pattern observed in the PGPS samples correlated to the *Bxvap-1* transcription pattern.

3.4. Histological distribution of BxVap-1

The histological features of *B. xylophilus* were examined based on the anatomy of the genus *Bursaphelenchus* (Choi, 2001; Zhao et al., 2008). Strong BxVap-1 expression signals were detected in PGPS nematodes in the regions of the putative oesophageal glands and metacarpus, which are involved in pumping proteins to external regions for secretion (Fig. 3). The oesophageal gland is adapted

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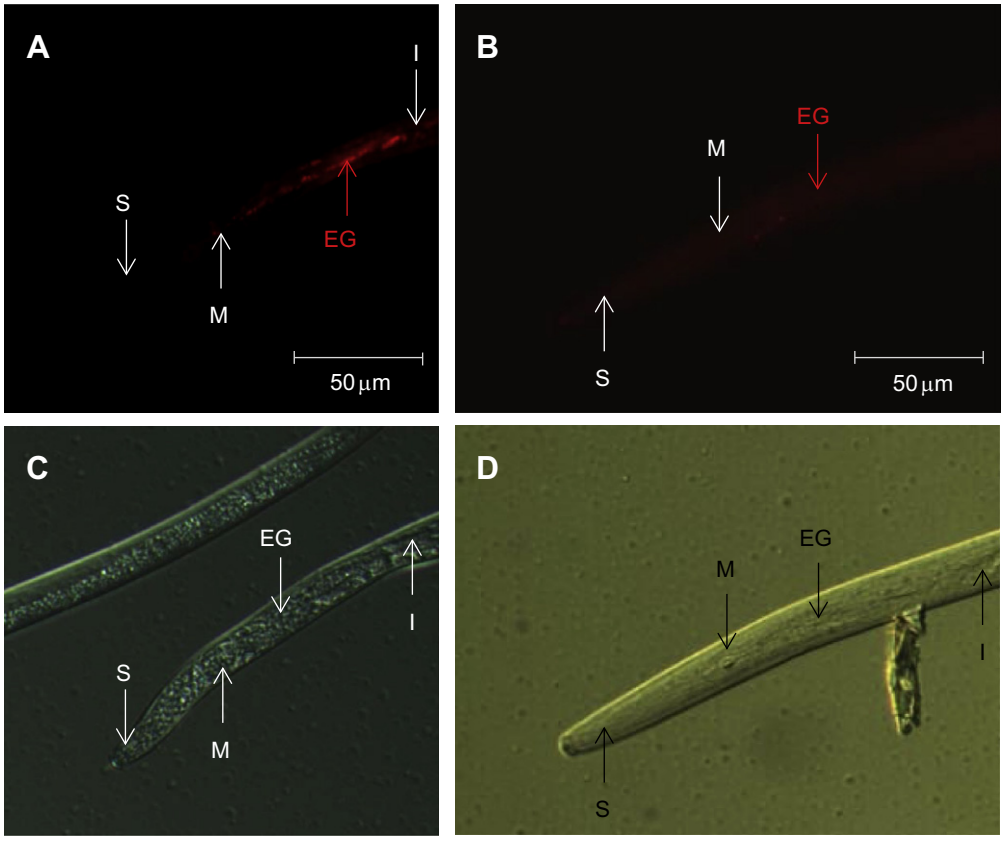


Fig. 4. Immunochemistry of *Bursaphelenchus xylophilus* venom allergen-like protein 1 in Bx-media-grown propagative stage. (A, B) Signals were detected around the putative oesophageal glands of Bx-media-grown propagative stage. (C, D) Optical microscopy photographs of A and B. Antibody dilutions: A, 1:50; B, 1:200. Magnification: A–D, 200×. M, metacarpus; S, stylet; I, intestine.

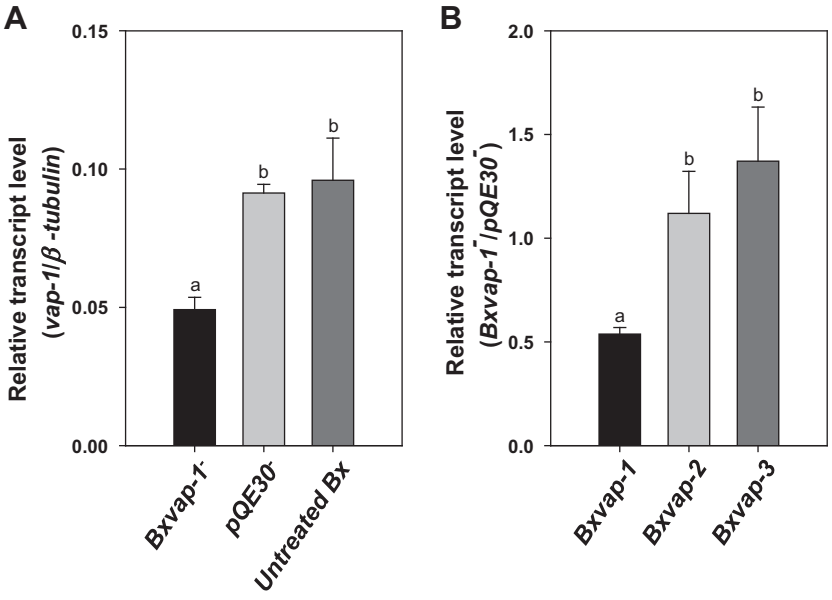


Fig. 5. Relative transcript levels of the gene for *Bursaphelenchus xylophilus* venom allergen-like protein after 24 h of RNA interference. (A) In *Bursaphelenchus xylophilus* treated with *Bxvap-1* double-stranded RNA (*Bxvap-1*$^{-}$), *Bxvap-1* transcript levels decreased to 48% compared with *B. xylophilus* treated with *pQE30* (plasmid, which does not exist in *B. xylophilus*) dsRNA (*pQE30*$^{-}$). The untreated *B. xylophilus* (untreated Bx) was used as a positive control. *β-Tubulin* was used as a reference gene. The mean comparison between groups was assessed by Tukey's Honestly Significant Difference with measured ANOVA ($P < 0.05$). (B) The transcript levels of *Bxvap-2* and *Bxvap-3* were analysed in *Bxvap-1*$^{-}$ and *pQE30*$^{-}$. The ratios of the relative transcript levels of these two *Bxvap* genes to the control were approximately 1.0, thereby indicating that the two *Bxvap* genes were not affected by *Bxvap-1* RNAi. The different letters on top of the bars indicate statistically significant differences ($P < 0.05$).

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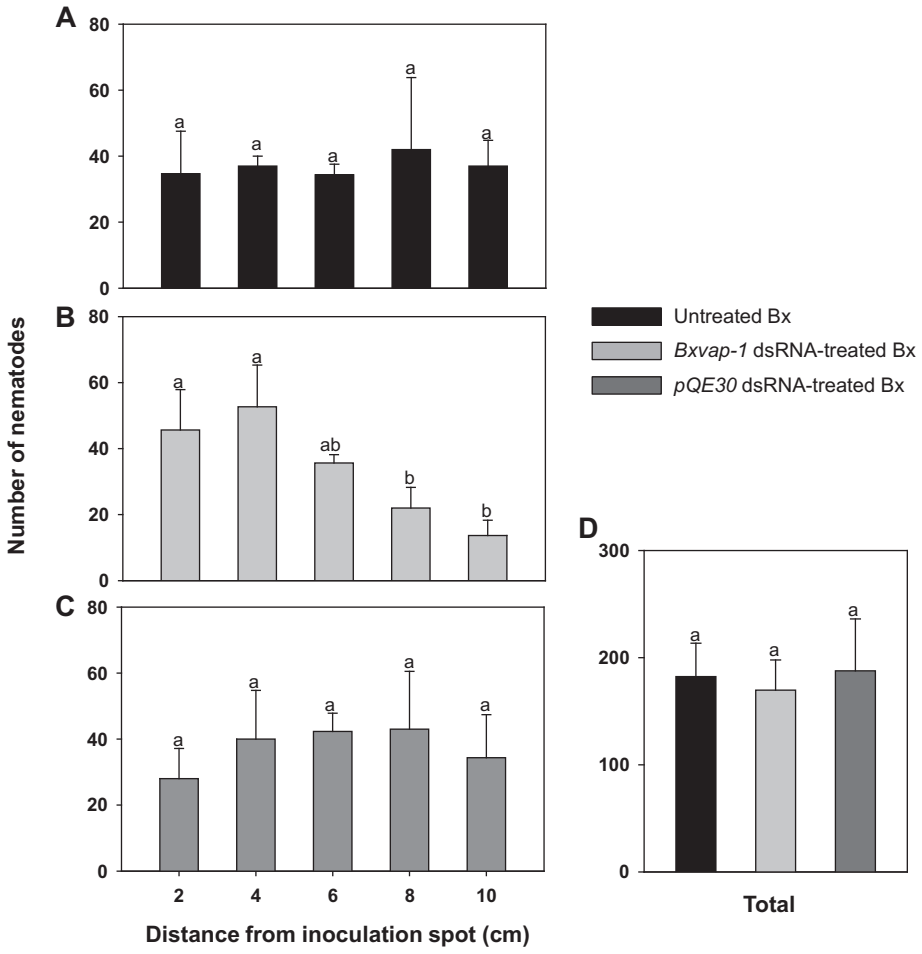


Fig. 6. Migration assay after RNA interference. (A–C) Untreated *Bursaphelenchus xylophilus* (A) and *pQE30* (plasmid sequence, which does not exist in *B. xylophilus*) double-stranded RNA-treated Bx (C) displayed even distribution in all ranges. *Bursaphelenchus xylophilus* venom allergen-like protein 1 dsRNA-treated Bx (B) showed a significantly reduced number of nematodes in >6 cm regions. (D) Numbers of collected *Bursaphelenchus xylophilus* nematodes under the three conditions were not significantly different. The mean comparisons between groups were assessed by Tukey's Honestly Significant Difference with measured ANOVA and the different letters on top of the bars indicate statistically significant differences ($P < 0.05$).

for high secretory activity and produces many secretory proteins. The metacorpus in the head has a triradiate pump chamber and is also involved in the secretion of proteins via pumping. Signals were also observed around the putative oesophageal glands in MGPS nematodes, but their intensity was significantly weaker in PGPS (Fig. 4A and B). Interestingly, some BxVap-1 signals were detected around the putative intestine immediately behind the oesophageal glands in both the PGPS and MGPS nematodes. The signals observed at the putative cuticle and hypodermis, shown in Fig. 3A, appeared to be non-specific as they were not detected at a lower BxVap1Pab concentration (1:200) (displayed in Fig. 3B and C). No signals were detected in the negative controls of the three nematodes, which lacked primary BxVap1Pab (Supplementary Fig. S4).

3.5. Migration rate of *Bxvap-1* knockdown nematodes in pine trees

The knockdown of *Bxvap-1* by RNAi resulted in a 48 ± 3% reduction in *Bxvap-1* transcript levels, as estimated by qPCR (Fig. 5A). In contrast, the transcript levels of *Bxvap-1* in the *pQE30* dsRNA-treated nematodes were comparable to those of the untreated *B. xylophilus* (Fig. 5A). In addition, the transcript levels of *Bxvap-2* and *Bxvap-3* in both dsRNA-treated nematodes (*Bxvap-1* and *pQE30* dsRNA) were analysed, from which two *Bxvap* genes were verified to be unaffected by *Bxvap-1* RNAi (Fig. 5B). The results of the migration assay are displayed in Fig. 6. The untreated and *pQE30* dsRNA-treated *B. xylophilus* were evenly distributed over all ranges (0–10 cm) (Fig. 6A and C). However, *Bxvap-1* dsRNA-treated *B. xylophilus* showed a significantly reduced number of nematodes in the >8 cm regions (Fig. 6B). For the total numbers of *B. xylophilus* nematodes collected from the infested pine trees, no significant difference was observed among the three treatments: *Bxvap-1* dsRNA-treated, *pQE30* dsRNA-treated, and untreated (Fig. 6D). Total collection rates from the infested pine tree were 45–75% for *B. xylophilus*.

4. Discussion

Vap proteins are thought to be associated with parasitism in parasitic nematodes; however, little is known of their physiological functions. Three *vap* genes have been discovered in *B. xylophilus*, whereas one has been identified in *B. mucronatus*. Of the three *B. xylophilus* Vap proteins, BxVap-1 appears to be closely associated with parasitism, as it shows higher expression levels than the other two BxVap proteins as well as a highly specific expression pattern in PGPS nematodes. We confirmed that the transcription profile of *Bxvap-1*, according to growth-environmental conditions, coincides precisely with its protein expression profile. Both western blot and immunohistochemical analyses demonstrated that BxVap-1 is

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selectively and predominantly expressed in PGPS and is, therefore, likely to be involved in parasitism. Protein expression was detected in the regions of the oesophageal glands and metacorpus, thereby further suggesting that BxVap-1 is secreted into host pine trees through the *B. xylophilus* stylet. Taken together, these results clearly indicate that BxVap-1 is functional during parasitism. Moreover, a migration assay using *Bxvap-1* knockdown *B. xylophilus* demonstrated that BxVap-1 enabled *B. xylophilus* to maintain normal migration activity against the pine tree defence mechanism. However, the small difference in the numbers between the untreated and *Bxvap1*-knockdown nematodes collected implied that BxVap-1 does not directly affect survival.

The migration activity of plant parasitic nematodes is an important factor for efficient parasitism. Many plant sedentary parasitic nematodes, such as root-knot nematodes and cyst nematodes, secrete cell wall degradation enzymes to enable their migration to an optimum feeding site, where they settle and take nutrients from the host. In contrast to sedentary plant parasitic nematodes, *B. xylophilus* moves through the pine tree resin canal during its entire life cycle. Vap expression in sedentary nematodes is primarily observed during an early parasitism stage but the expression of BxVap-1 in *B. xylophilus* is observed throughout the life cycle (Lin et al., 2011). This characteristic indicates that BxVap-1 is functional during the entire parasitic stage. Previous studies have suggested that the migration activity of *B. xylophilus* within pine tree closely correlates with its pathogenicity, as determined by movement assays (Ichihara et al., 2000; Odani et al., 1985). Odani et al. (1985) showed that the population size and the distance travelled by *B. xylophilus* increased at 6 days after inoculation, while this measurement decreased for *B. mucronatus* (Odani et al., 1985). These differences between *B. xylophilus* and *B. mucronatus* reached a peak at 9 days after inoculation. Besides, virulent *B. xylophilus* was shown to move a significantly farther distance through the resin canal from the inoculation spot than avirulent *B. xylophilus* under high temperature conditions (25–30 °C) (Ichihara et al., 2000). Because BxVap-1 is required for the normal migration activity of *B. xylophilus* inside pine tree, as determined from our migration assay (Fig. 6), BxVap-1 is most likely one of the determining factors of pathogenicity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2011.10.006.

References

Alexander, D., Goodman, R.M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E., 1993. Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. Proc Natl Acad Sci U S A 90, 7327–7331.

Baum, T.J., Hussey, R.S., Davis, E.L., 2007. Root-knot and cyst nematode parasitism genes: the molecular basis of plant parasitism. Genet Eng (NY) 28, 17–43.

Chalmers, I.W., McArdle, A.J., Coulson, R.M., Wagner, M.A., Schmid, R., Hirai, H., Hoffmann, K.F., 2008. Developmentally regulated expression, alternative splicing and distinct sub-groupings in members of the *Schistosoma mansoni* venom allergen-like (SmVAL) gene family. BMC Genomics 9, 89.

Choi, Y.E., 2001. Tylenchida and Aphelenchida. Economic insects of Korea 20. Ins. Koreana Suppl. 27, 171–186.

Choi, J.Y., Kim, Y.S., Wang, Y., Kang, J.N., Roh, J.Y., Shim, H.J., Woo, S.D., Jin, B.R., Je, Y.H., 2009. Improved baculovirus vectors expressing barnase using promoters from *Cotesia plutellae* bracovirus. Mol. Cells 28, 19–24.

Curtis, R.H.C., 2007. Plant parasitic nematode protein and the host–parasite interaction. Brief Funct. Genomic Proteomic, 1–9.

Davis, E.L., Hussey, R.S., Mitchum, M.G., Baum, T.J., 2008. Parasitism proteins in nematode-plant interactions. Curr. Opin. Plant Biol. 11, 360–366.

Ding, X., Shields, J., Allen, R., Hussey, R.S., 2000. Molecular cloning and characterisation of a venom allergen AG5-like cDNA from *Meloidogyne incognita*. Int. J. Parasitol. 30, 77–81.

Gao, B., Allen, R., Maier, T., Davis, E.L., Baum, T.J., Hussey, R.S., 2001. Molecular characterisation and expression of two venom allergen-like protein genes in *Heterodera glycines*. Int. J. Parasitol. 31, 1617–1625.

Gao, B., Allen, R., Maier, T., McDermott, J.P., Davis, E.L., Baum, T.J., Hussey, R.S., 2002. Characterisation and developmental expression of a chitinase gene in *Heterodera glycines*. Int. J. Parasitol. 32, 1293–1300.

Haegeman, A., Jacob, J., Vanholme, B., Kyndt, T., Mitreva, M., Gheysen, G., 2009. Expressed sequence tags of the peanut pod nematode *Ditylenchus africanus*: the first transcriptome analysis of an Anguinid nematode. Mol. Biochem. Parasitol. 167, 32–40.

Hawdon, J.M., Jones, B.F., Hoffman, D.R., Hotez, P.J., 1996. Cloning and characterization of *Ancylostoma*-secreted protein. A novel protein associated with the transition to parasitism by infective hookworm larvae. J. Biol. Chem. 271, 6672–6678.

Hawdon, J.M., Narasimhan, S., Hotez, P.J., 1999. *Ancylostoma* secreted protein 2: cloning and characterization of a second member of a family of nematode secreted proteins from *Ancylostoma caninum*. Mol. Biochem. Parasitol. 99, 149–165.

Ichihara, Y., Fukuda, K., Suzuki, K., 2000. Early symptom development and histological changes associated with migration of *Bursaphelenchus xylophilus* in seedling tissues of *Pinus thunbergii*. Plant Dis. 84, 675–680.

Jaubert, S., Ledger, T.N., Laffaire, J.B., Piotte, C., Abad, P., Rosso, M.N., 2002. Direct identification of stylet secreted proteins from root-knot nematodes by a proteomic approach. Mol. Biochem. Parasitol. 121, 205–211.

Jones, J.T., Furlanetto, C., Bakker, E., Banks, B., Blok, V., Chen, Q., Phillips, M., Prior, A., 2003. Characterization of a chorismate mutase from the potato cyst nematode *Globodera pallida*. Mol. Plant Pathol. 4, 43–50.

Jones, J.T., Moens, M., Mota, M., Li, H., Kikuchi, T., 2008. *Bursaphelenchus xylophilus*: opportunities in comparative genomics and molecular host–parasite interactions. Mol. Plant Pathol. 9, 357–368.

Kang, J.S., Lee, H., Moon, I.S., Lee, Y., Koh, Y.H., Je, Y.H., Lim, K.J., Lee, S.H., 2009. Construction and characterization of subtractive stage-specific expressed sequence tag (EST) libraries of the pinewood nematode *Bursaphelenchus xylophilus*. Genomics 94, 70–77.

Kikuchi, T., Shibuya, H., 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. Biochem. J. 389, 117–125.

Kikuchi, T., Shibuya, H., Aikawa, T., 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.

Kikuchi, T., Cotton, J.A., Dalzell, J.J., Hasegawa, K., Kanzaki, N., McVeigh, P., Takanashi, T., Tsai, I.J., Assefa, S.A., Cock, P.J., Otto, T.D., Hunt, M., Reid, A.J., Sanchez-Flores, A., Tsuchihara, K., Yokoi, T., Larsson, M.C., Miwa, J., Maule, A.G., Sahashi, N., Jones, J.T., Berriman, M., 2011. Genomic insights into the origin of parasitism in the emerging plant pathogen *Bursaphelenchus xylophilus*. PLoS Pathog. 7, e1002219.

Kovalick, G.E., Griffin, D.L., 2005. Characterization of the SCP/TAPS gene family in *Drosophila melanogaster*. Insect Biochem. Mol. Biol. 35, 825–835.

Lin, S., Jian, H., Zhao, H., Yang, D., Liu, Q., 2011. Cloning and characterization of a venom allergen-like protein gene cluster from the pinewood nematode *Bursaphelenchus xylophilus*. Exp. Parasitol. 127, 440–447.

Mamiya, Y., 1975. The life history of the pine wood nematode, *Bursaphelenchus lignicolus*. Jpn. J. Nematol. 5, 16–25.

Mota, M.M., Braasch, H., Bravo, M.A., Penas, A.C., Burgermeister, W., Metge, K., Sousa, E., 1999. First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. Nematology 1, 727–734.

Murphy, E.V., Zhang, Y., Zhu, W., Biggs, J., 1995. The human glioma pathogenesis-related protein is structurally related to plant pathogenesis-related proteins and its gene is expressed specifically in brain tumors. Gene 159, 131–135.

Murray, J., Gregory, W.F., Gomez-Escobar, N., Atmadja, A.K., Maizels, R.M., 2001. Expression and immune recognition of *Brugia malayi* VAL-1, a homologue of vespid venom allergens and *Ancylostoma* secreted proteins. Mol. Biochem. Parasitol. 118, 89–96.

Odani, K., Sasaki, N., Yamamoto, N., Nishiyama, Y., Tamura, H., 1985. Differences in dispersal and multiplication of two associated nematodes, *Bursaphelenchus xylophilus* and *Busaphelenchus mucronatus* in pine seedlings in relation to the pine wilt disease development. J. Jpn. Forest Soc. 67, 398–403.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Tytgat, T., Vanholme, B., De Meutter, J., Claeys, M., Couvreur, M., Vanhoutte, I., Gheysen, G., Van Criekinge, W., Borgonie, G., Coomans, A., 2004. A new class of ubiquitin extension proteins secreted by the dorsal pharyngeal

gland in plant parasitic cyst nematodes. Mol. Plant–Microbe Interact. 17, 846–852.

Vanholme, B., De Meutter, J., Tytgat, T., Van Montagu, M., Coomans, A., Gheysen, G., 2004. Secretions of plant–parasitic nematodes: a molecular update. Gene 332, 13–27.

Viglierchio, D.R., Schmitt, R.V., 1983. On the methodology of nematode extraction from field samples – Baermann funnel modifications. J. Nematol. 15, 438–444.

Wang, X., Li, H., Hu, Y., Fu, P., Xu, J., 2007. Molecular cloning and analysis of a new venom allergen-like protein gene from the root-knot nematode *Meloidogyne incognita*. Exp. Parasitol. 117, 133–140.

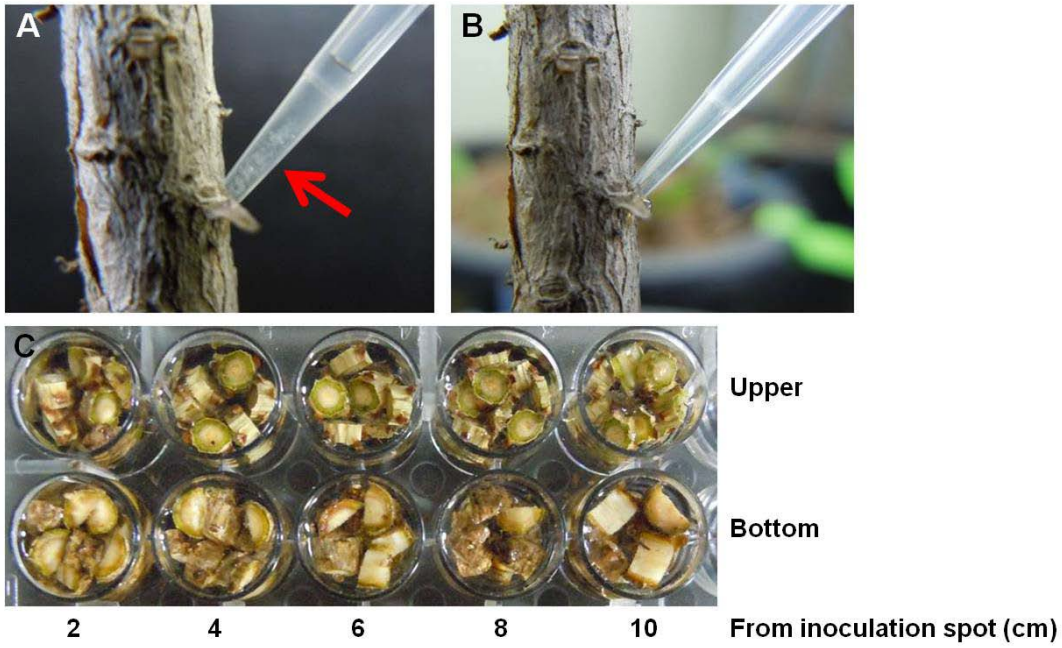
Yang, B., Wang, Q., 1989. Distribution of the pinewood nematode in China and susceptibility of some Chinese and exotic pines to the nematode. Can. J. Forest Res. 19, 1527–1530.

Yi, C.K., Byun, B.H., Park, J.D., Yang, S.I., Chang, K.H., 1989. First finding of the pine wood nematode, *Bursaphelenchus xylophilus* and its insect vector in Korea. Res. Rep. For. Res. Inst. 38, 141–149.

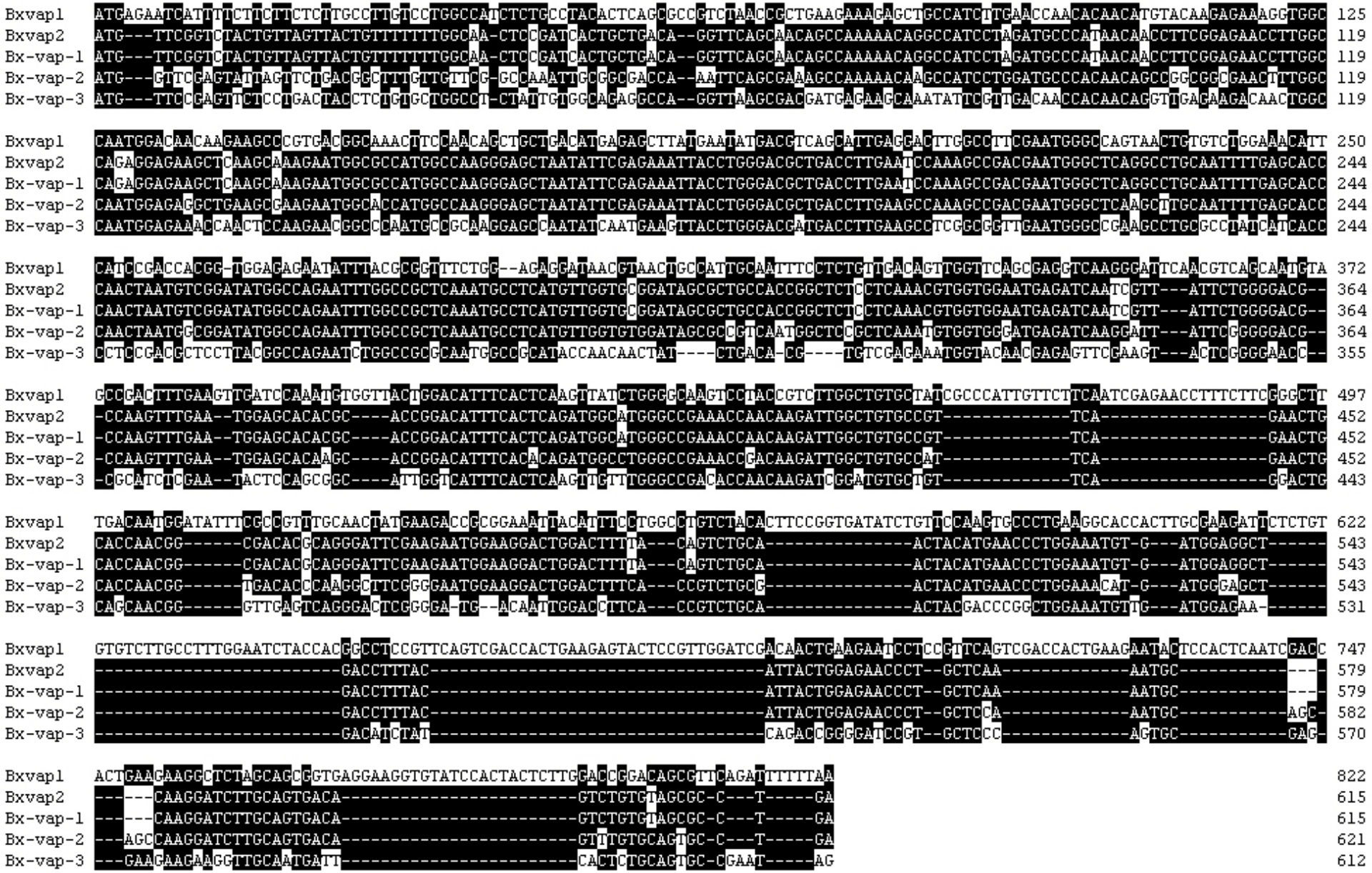
Zhao, B.G., Futai, K., Sutherland, J.R., Takeuchi, Y., 2008. Pine Wilt Disease. Springer, Tokyo.

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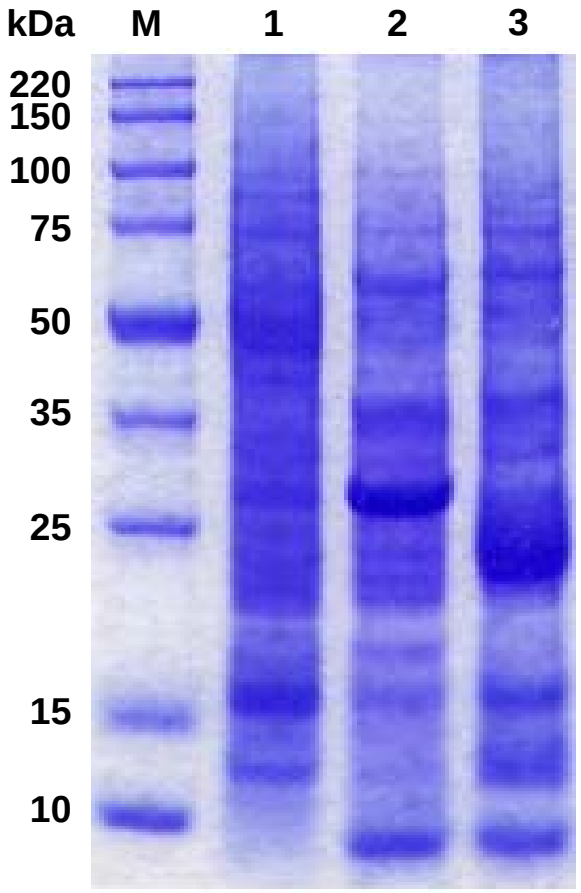
Supplementary Figure S1



Supplementary Figure S2

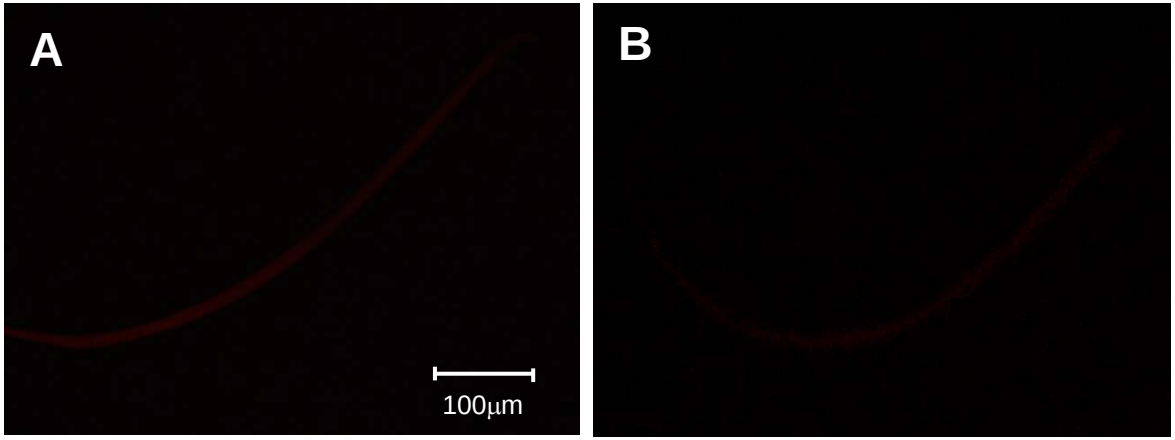


Supplementary Figure S3



M: Protein marker
1: Sf9 infected with AcNPV
2: Cell lysate
3: Sf9 infected with AcBxVap-1

Supplementary Figure S4



Supplementary Table S1. The primers used in quantitative real time PCR (qPCR) and double-stranded RNA (dsRNA) synthesis for RNA interference (RNAi)

Primer name	Sequence	Description
5rtBxvap-1	GGACTTTTACAGTCTGCAACTACATG	Used for qPCR
3rtBxvap-1	TCAGGCGCTACACAGACTGTCAC	
5rtBxvap-2	AGGCTGAAGCGAAGAATGGC	
3rtBxvap-2	ATCCGCCATTAGTTGGGTGC	
5rtBxvap-3	ACCTGGGACGATGACCTTGAAG	
3rtBxvap-3	CCATTTCTCGACACGTGTCAGAT	
5BxrtTub	TCCGTACCCTGAAGTTGGCTAACC	
3BxrtTub	AAGTGGAGACGAGGGAATGGAACC	
BxVap-1RNAi_F	TAATACGACTCACTATAGGGAGAGGTTCAGCAACAGCCAAAAACA	Used for synthesis of dsRNA for RNAi
BxVap-1RNAi_R	TAATACGACTCACTATAGGGAGAGTCCAGTCCTTCCATTCTTCGAA	

Supplementary Figure legends

Supplementary Fig. S1. The migration assay method used in this study. The *Bursaphelenchus xylophilus* venom allergen-like protein (*Bxvap-1*) knockdown worms (red arrow) were inoculated into pine seedling (A) and verified to have entered the pine seedling after 5-6 h (B). After 6 days, the pine seedling was chopped into 2 cm pieces and soaked in water for 1 day, and the worms were then collected from the chopped pine wood (C).

Supplementary Fig. S2. The alignment of venom allergen-like protein (*vap*) genes from *Bursaphelenchus xylophilus*. The *Bxvap2* was identical to the *Bx-vap-1* in nucleotide sequences. The black boxes represent identical sequences.

Supplementary Fig. S3. The SDS-PAGE results of the recombinant *Bursaphelenchus xylophilus* venom allergen-like protein 1 (BxVap-1) expressed by the baculovirus expression system. Over-expressed BxVap-1 was observed at approximately 22 kDa (black arrow, lane 3), which was not shown in the cell lysate (lane 2). M, Protein marker; 1, Sf9 insect cells infected with *Autographa californica* nuclear polyhedrosis virus (AcNPV); 2, Cell lysate; 3, Sf9 infected with AcBxVap-1.

Supplementary Fig. S4. The immunohistochemical results of the negative control nematodes. The negative controls were treated exclusively with secondary anti-rabbit antibody conjugated Alexa568 (primary antibody BxVap1Pab was omitted). (A) *Bursaphelenchus xylophilus*-pine-grown propagative stage (Bx-PGPS); (B) *Bursaphelenchus xylophilus*-media-grown propagative stage (Bx-PGPS).