Thaumatocin-Like Protein-1 Gene (*Bx-tlp-1*) Is Associated with the Pathogenicity of *Bursaphelenchus xylophilus*

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ABSTRACT

The pine wood nematode *Bursaphelenchus xylophilus* is a destructive species affecting pine trees worldwide; however, the underlying mechanism leading to pathogenesis remains unclear. In this study, a *B. xylophilus* gene encoding thaumatocin-like protein-1 (*Bx-tlp-1*) was silenced by RNA interference to clarify the relationship between the *Bx-tlp-1* gene and pathogenicity. The in vitro knockdown of *Bx-tlp-1* with double-stranded RNA (dsRNA) decreased *B. xylophilus* reproduction and pathogenicity. Treatments with dsRNA targeting *Bx-tlp-1* decreased expression by 90%, with the silencing effect maintained even in the F3 offspring. Pine trees inoculated with *B. xylophilus* treated with *Bx-tlp-1* dsRNA decreased the symptom of wilting, and the disease severity index was

56.7 at 30 days after inoculation. Additionally, analyses of the cavitation of intact pine stem samples by X-ray microtomography revealed that the xylem cavitation area of pine trees inoculated with *B. xylophilus* treated with *Bx-tlp-1* dsRNA was 0.46 mm² at 30 days after inoculation. Results from this study indicated that the silencing of *Bx-tlp-1* has effects on *B. xylophilus* fitness. The data presented here provide the foundation for future analyses of *Bx-tlp-1* functions related to *B. xylophilus* pathogenicity.

Keywords: *Bursaphelenchus xylophilus*, thaumatocin-like protein, X-ray microtomography

The pine wood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner & Buhrer) (Nickle et al. 1981) is a critical plant-parasitic nematode that is harmful to conifers, particularly pine trees. Pine wilt disease (PWD) caused by *B. xylophilus* has caused considerable economic losses in Asia (Mamiya 1988; Togashi and Shigesada 2006; Yoshimura et al. 1999) and Europe (Abelleira et al. 2011; Branco et al. 2014; Mota et al. 2009; Vicente et al. 2012), especially in Japan, China, South Korea, Portugal, and Spain. This disease was first detected in 1982 in Nanjing, Jiangsu province, China (Sun 1982). During the past 30 years, PWD has spread to 588 counties in 18 provinces in China. *Monochamus* spp. beetles (Morimoto and Iwasaki 1972; Ryss et al. 2005) are the major vectors for *B. xylophilus* because they bore through and feed on more than one pine tree during the course of their lives, thereby introducing nematodes into multiple trees. Infected pine trees usually die rapidly, and there is currently no effective treatment to control the spread of the disease. Despite many studies concerning PWD and the PWN, the pathogenic mechanism of *B. xylophilus* has not been completely elucidated.

Plant-parasitic nematodes exhibit diverse interactions with their host plants to obtain nutrients and supplements important for their development and reproduction (Goellner et al. 2000). Additionally, some pathogenesis-related (PR) proteins that are crucial for plant defense systems are generally produced in higher plants and are involved in biotic stress responses (Hoffmann-Sommergruber 2002). The production of these proteins may be stimulated by pathogens or other external factors. Thaumatocin-like proteins (TLPs)

belong to the PR-5 family and are widely distributed in most plants and some lower animals. For example, they were first identified in *Caenorhabditis elegans* (Kitajima and Sato 1999); after which, they were detected in angiosperms, gymnosperms, bryophytes, arthropods (Brandazza et al. 2004; Shatters et al. 2006), and fungi (Grenier et al. 2000; Sakamoto et al. 2006). In a previous study, two *B. xylophilus* TLPs and one cysteine proteinase inhibitor with sequences that are highly similar to those of plant proteins were identified based on a proteomic analysis (Shinya et al. 2013). Additionally, the similarity between the TLPs from *B. xylophilus* (Bx-TLP-1) and *Pinus monticola* (TLP-S3; ADB97933.1) was confirmed by BLAST and structural analyses of the proteins. Moreover, the sequence of a *B. xylophilus* TLP was reportedly similar to that of a *P. massoniana* protein (Wang et al. 2014). Furthermore, Meng et al. (2017) cloned the gene encoding the *P. massoniana* TLP (*Pm-tlp*) and revealed that its expression is associated with the expression of *Bx-tlp-1* from *B. xylophilus*. The sequence homology between Bx-TLP-1 and Pm-TLP has been confirmed (Meng et al. 2017).

Methods involving RNA interference (RNAi) technology are useful for functionally characterizing genes. A previous study confirmed that dsRNA can induce RNAi in *C. elegans* (Fire et al. 1998). In nematodes, the uptake of exogenous dsRNA is enhanced by neurostimulants such as octopamine (Bakhiet et al. 2005; Dubreuil 2007; Rosso et al. 2005). Moreover, the effects of immersing nematodes in a solution containing dsRNA were investigated with fluorescein isothiocyanate (FITC), which is a fluorescent dye, as a marker. The dye was observed in the stylet, pharynx, and intestinal tract of nematodes. Because RNAi silencing of a gene that is critical for nematode development can decrease pathogenicity, the biotechnological application of RNAi technology may lead to more effective methods of controlling plant-parasitic nematodes (Gheysen and Vanholme 2007; Sindhu et al. 2009).

Cavitation is a typical pathological feature observed during the development of PWD. As PWD progresses, the pits of tracheids and the ray parenchyma become aspirated, and the epithelial cells surrounding the resin canals are denatured. Therefore, the anatomical deterioration of pine trees infected by PWD can more

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easily permit xylem cavitation (Ikeda and Ohtsu 1992; Utsuzawa et al. 2005). Additionally, X-ray microtomography (micro-CT) has been applied to examine xylem cavitation in intact samples (Cochard and Delzon 2013). Micro-CT provides a spatial resolution of approximately 1 µm, which is sufficient for analyzing the structure of xylem tracheids and resin canals. Furthermore, micro-CT enables scientists to conduct in-depth studies of plant physiological and pathological phenomena via direct observations (Cochard et al. 2015).

In this study, a *B. xylophilus* TLP gene (*Bx-tlp-1*) was targeted by dsRNA to evaluate the influence of silencing the TLP gene on the reproduction and pathogenicity of *B. xylophilus*. Additionally, the cavitation of intact pine samples was examined by micro-CT, thus representing a radiological method of studying PWN pathogenicity. The resulting data may provide the foundation for verifying the interaction between *B. xylophilus* and host pine trees.

MATERIALS AND METHODS

Materials. The *B. xylophilus* NXY61 strain analyzed in this study was isolated from the wood chips of infested *P. massoniana* trees growing in Zhejiang province in China and stored in the Forest Pathogen Integrated Biology Laboratory of the Chinese Academy of Forestry in Beijing, China. Three-year-old *P. massoniana* trees were grown in a greenhouse at 25°C with 80% RH.

Preparation of dsRNA for in vitro RNAi. Double-stranded RNA targeting *Bx-tlp-1* was used to evaluate the efficiency of RNAi in *B. xylophilus* (Table 1). A 467-nucleotide dsRNA corresponding to the *gfp* gene (Luo et al. 2017) was synthesized as the negative control. The dsRNA sequences were synthesized using the T7 RiboMAX Express RNAi System Kit (Promega, Madison, WI, U.S.A.) with PCR products as templates. The PCR program was as follows: 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; and 72°C for 10 min. Two ssRNAs were mixed together and incubated at 70°C for 10 min and 25°C for 30 min to synthesize the dsRNAs. The generated dsRNAs (2-µl aliquots) were evaluated by 1% agarose gel electrophoresis.

In vitro RNAi targeting *Bx-tlp-1*. We used FITC to assess the efficiency of the dsRNA uptake by nematodes. The PWNs (≈10,000 individuals comprising a mixture of juveniles and adults) were soaked in a buffer solution consisting of *Bx-tlp-1* dsRNA (2 mg/ml), FITC (1 mg/ml), 50 mM of octopamine, and M9 salts (43.6 M of Na₂HPO₄, 22 mM of KH₂PO₄, 2.1 mM of NaCl, and 4.7 mM of NH₄Cl) (Urwin et al. 2002). Samples were incubated on a rotator for 36 h at 25°C in darkness. To confirm the target-specific silencing, control samples were prepared with PWNs incubated in the buffer alone and in the buffer supplemented with *gfp* dsRNA. The PWNs were rinsed five times with ddH₂O and centrifuged. The nematodes were observed with a Zeiss Axio Imager A1 fluorescence microscope to determine the dsRNA uptake efficiency. The number of surviving nematodes was determined by microscopy following the 36-h treatment. Nematodes were considered dead if their bodies were straight and they did not move, even after mechanical prodding.

To investigate the effects of the silencing of *Bx-tlp-1* on nematode offspring (F1, F2, F3, F4, and F5 generations), we selected 500 fourth-stage propagative juveniles (J4s) to analyze the *Bx-tlp-1* expression level after the *Bx-tlp-1* dsRNA treatment (F0). A total of 500 J4s were inoculated with *Botrytis cinerea* in Petri dishes and

incubated at 25°C for 96 h to generate the J4s of the next generation (F1). We selected 500 J4s to analyze *Bx-tlp-1* expression and another 500 J4s to produce the J4s of the next generation (F2). We repeated this method to generate the J4s of the third, fourth, and fifth generations (F3, F4, and F5).

To analyze *Bx-tlp-1* expression in the dsRNA-treated nematodes, total RNA was isolated from the nematodes treated with *Bx-tlp-1* dsRNA (dsTLP1), *gfp* dsRNA (dsGFP), and buffer alone (CK). A quantitative real-time PCR (qRT-PCR) assay was performed using the LightCycler 480 II system (Roche Diagnostics Ltd., Switzerland) with TB Green Premix Ex Taq (TaKaRa, Japan). The PCR program was as follows: 95°C for 30 s; 40 cycles of 95°C for 5 s; and 60°C for 35 s. All samples were analyzed in triplicate, and melting curves were prepared for each data point to assess the specificity of the PCR. The *B. xylophilus* *β-actin* gene was used as an internal control for normalizing the expression data. Details regarding the qRT-PCR primers are provided in Table 1. Relative gene expression levels were calculated based on the cycle threshold changes (ΔΔCt) and were normalized against the *β-actin* expression levels. All experiments were performed in triplicate with three biological replicates.

Effects of silencing *Bx-tlp-1* on *B. xylophilus* reproduction. To investigate the effects of silencing *Bx-tlp-1* on *B. xylophilus* reproduction, 500 fourth-stage propagative juveniles (J4s) treated with dsRNA were inoculated with *B. cinerea* in Petri dishes and incubated at 25°C. We collected samples at 4, 8, 12, 16, and 20 days. Additionally, 500 RNAi-treated *B. xylophilus* J4s were cultivated in Petri dishes containing *B. cinerea*, after which the PWN populations in each dish were isolated and counted. The population structure was used to evaluate the effects of RNAi-mediated silencing of *Bx-tlp-1* on *B. xylophilus* reproduction.

***Pinus massoniana* symptoms after various PWN treatments.** Three-year-old *P. massoniana* trees grown in a greenhouse were disinfected with 75% ethyl alcohol and then inoculated with a 300-µl suspension comprising *B. xylophilus* (≈3000 nematods, including juveniles and adults) that had been treated with CK, dsGFP, or dsTLP1. Specifically, 0.5-cm-long wounds on the trees at ≈40 cm above the soil level were inoculated and then sealed with Parafilm. Sterile water was used as a control. All treatments were analyzed with three replicates. The inoculated *P. massoniana* trees were incubated in the greenhouse for 30 days. PWD symptoms and the disease severity index (DSI) were recorded as previously described (Yu et al. 2012). Disease symptoms were assessed based on a scale of 0 to 4 as follows: 0, all needles were green; 1, 0 to 25% of the needles were discolored and turned yellow; 2, 25 to 50% of the needles turned yellow; 3, 50 to 75% of the needles turned yellow; and 4, 75 to 100% of the needles turned yellow. The infection rates and DSI were calculated using the following formulae (Yu et al. 2012):

$$\text{Infection rate} = \frac{\sum \text{Number of infected trees}}{\text{Total number of trees}} \times 100\%$$

$$\text{DSI} = \frac{\sum \text{Number of diseased trees} \times \text{symptom stage}}{\text{Total number of trees} \times \text{highest symptom stage}} \times 100$$

Cavitation analysis by X-ray micro-CT. To detect cavitation in samples, cross-sections of the stems at 2 to 3 cm below the inoculation sites were examined by X-ray micro-CT (SkyScan 1172; Belgium) at 30 days after inoculations. The X-ray parameter settings

TABLE 1. List of primers used for RNAi experiments

Gene	Primer 5'-3'	
	Sense	Antisense
<i>dsTLP1</i>	T7 + CGGTATCGGATGTGCTATT	T7 + CTGGTACAAGTCAAGTTCTTC
<i>dsGFP</i>	T7 + TGGTCCCAATTCTCGTGGAAC	T7 + CTTGAAGTTGACCTTGATGCC
<i>Bx-tlp-1</i>	TGTGGCTGACACTTATGG	AGTCGCTGTTGTAGTTGATA
<i>β-actin</i>	TCCGTACCCTGAAGTTGGCTAAC	AAGTGGAGACGAGGGAATGGAAC

were 70 kV and 141 μ A, and 1000 images were acquired during the 360° sample rotation. After three-dimensional reconstruction, the spatial resolution of the image was $2.5 \times 2.5 \times 2.5$ μ m per voxel according to the CTvox and DataViewer programs. The cavitation area was measured using Image J (National Institutes of Health, U.S.A.) as described on the National Institutes of Health website

(<https://imagej.nih.gov/ij/>). The measurements were completed for three replicates.

Data analysis. All experiments involving PWNs included three replicates, with each treatment replicated five times. The qRT-PCR analysis of each sample also included three replicates. Excel 2013 was used to analyze the *Bx-tlp-1* expression and *B. xylophilus*

Fig. 1. Effects of RNA interference (RNAi) on *Bx-tlp-1* gene expression of *Bursaphelenchus xylophilus*. The relative expression of the *Bx-tlp-1* gene from pine wood nematodes (PWNs) after RNAi for **A**, different times; **B**, different treatments; and **C**, different generations. CK, PWNs treated with the buffer alone; dsGFP, PWNs treated with *gfp* dsRNA; dsTLP1, PWNs treated with *Bx-tlp-1* dsRNA; 12, 24, 36, 48, and 60 h, PWNs treated with *Bx-tlp-1* dsRNA for 12, 24, 36, 48, and 60 h; and F0–F5, F0–F5 generations. Each experiment was repeated three times. The error line means standard deviation of the mean. Bars with different letters indicate significant differences among treatments, as defined by Duncan's test ($P < 0.05$). ** Significant differences among treatments, as defined by t test ($P < 0.05$).

generations. All data are presented as the mean $\pm$ standard deviation. A single factor analysis of variance (ANOVA) and *t* test were completed using the SPSS 13.0 program (IBM China Company Ltd., Beijing, China). Differences between samples were considered significant at $P < 0.05$.

RESULTS

In vitro RNAi targeting *Bx-tlp-1* in *B. xylophilus*. To detect the silencing of *Bx-tlp-1* in vitro, PWNs ($\approx 10,000$ individuals comprising a mixture of juveniles and adults) were treated with a buffer containing dsRNA targeting *Bx-tlp-1*. A qRT-PCR analysis

Fig. 2. Fluorescence microscopy showing FITC uptake by *Bursaphelenchus xylophilus*. Fluorescence was apparent in the pharyngeal lumen and the median bulb. s = stylet; pl = pharyngeal lumen; m = median bulb.

indicated that the 36-h treatment period was most effective at silencing *Bx-tlp-1* (Fig. 1A). Accordingly, the subsequent RNAi experiments involved a 36-h incubation on a rotator at 25°C in darkness. The *Bx-tlp-1* transcript level was $\approx 90\%$ lower in the dsTLP1-treated PWNs than in the CK-treated or dsGFP-treated PWNs. There was no significant difference in the *Bx-tlp-1* expression level between the PWNs treated with CK or dsGFP ($P < 0.05$) (Fig. 1B). Additionally, most of the PWNs survived the RNAi treatment, regardless of the buffer solution (Supplementary Fig. S1). Moreover, the qRT-PCR results indicated that the effects of the dsTLP1 treatment were still observable in the F3 offspring (Fig. 1C).

Evaluation of dsRNA uptake with FITC as a marker. The uptake of dsRNA by the PWNs was assessed by fluorescence microscopy, with FITC as a marker. Nematodes treated with dsTLP1 were curled and formed aggregates (Supplementary Fig. S2). Obvious FITC signals were detected in the stylet, pharyngeal lumen, and median bulb of nematodes treated with dsTLP1 (Fig. 2). There was no difference between the PWNs treated with solutions with or without neurostimulants (e.g., octopamine), implying that neurochemicals are not required for the PWNs to take-up FITC. The treatment time was the decisive factor influencing FITC uptake, with fluorescence detected in most nematodes treated for 36 h. These results were consistent with those of a previous study (Cheng et al. 2010).

Influence of silencing *Bx-tlp-1* on *B. xylophilus* reproduction. After culturing *B. cinerea*, the PWN populations at each timepoint (4, 8, 12, 16, and 20 days) were counted. The dsTLP1 treatment resulted in fewer nematodes than the control treatment, especially at 20 days (Fig. 3A). The population structure for each timepoint revealed that most of the PWNs were at the larval stage (Fig. 3B). These results implied that the silencing of *Bx-tlp-1* markedly affects *B. xylophilus* reproduction.

Symptoms of *P. massoniana* inoculated with *B. xylophilus*. Diverse symptoms were observed on inoculated *P. massoniana* trees, depending on the solution used to treat *B. xylophilus*. At 27 days after inoculation, the trees inoculated with PWNs treated with the CK or dsGFP solutions had an infection rate of 100% and a DSI of 100 or 97.5, whereas the trees inoculated with dsTLP1-treated PWNs had an infection rate of 83.3% and a DSI of 43.3 (Table 2). At 30 days after inoculations, all of the *P. massoniana* trees were infected, but the trees inoculated with dsTLP1-treated PWNs had the lowest DSI (56.7). Additionally, almost all of the trees

Fig. 3. Effects of RNA interference (RNAi) on the population structure of *Bursaphelenchus xylophilus*. Five hundred RNAi-treated J4s of *B. xylophilus* after cultivation on a fungal plate of *Botrytis cinerea* with different treatments. **A**, The number of population of *B. xylophilus*. **B**, The population structure of *B. xylophilus*; CK, pine wood nematodes (PWNs) treated with the buffer alone; dsGFP, PWNs treated with *gfp* dsRNA; dsTLP1, PWNs treated with *Bx-tlp-1* dsRNA; T0, T4, T8, T12, T16, and T20, the populations of PWNs were counted at 0, 4, 8, 12, 16, and 20 days. There were five biological replications for each experiment. The error line indicates the standard deviation of the mean.

inoculated with CK-treated or dsGFP-treated PWNs were completely wilted. In contrast, the trees inoculated with sterile water remained healthy (Fig. 4). These results suggested that silencing *Bx-tlp-1* expression decreases the pathogenicity of PWNs.

Cavitation analysis. At 30 days after inoculation, changes to the longitudinal sections of the phloem and xylem were observable by the eye (Fig. 5). The stem segments of trees treated with sterile water were healthy, but half of the pith tissue was necrotic and had turned black in trees inoculated with dsTLP1-treated PWNs. Moreover, all of the phloem and pith tissue of the xylem core were completely necrotic and black in trees inoculated with CK-treated or dsGFP-treated PWNs (Fig. 5).

Pine stem samples collected from 2 to 3 cm below the inoculation sites were analyzed by micro-CT. The two-dimensional cross-sections were reconstructed to reveal functional (gray) and air-filled (black) resin and tracheids. The cross-sections of the stem samples from trees treated with sterile water were gray, indicating that resin and tracheids were filled with liquid and functional. However, the cross-sections of samples from trees inoculated with CK-treated or dsGFP-treated PWNs appeared black, implying resin and tracheids were filled with air and that the pith cells had ruptured to form a large cavity. In contrast, the cross-sections of stem samples collected from trees inoculated with dsTLP1-treated PWNs were half gray and half black, reflecting the partial cavitation of the resin and tracheids (Fig. 5). Additionally, the xylem cavitation area of pine trees inoculated with *B. xylophilus* treated with *Bx-tlp-1* dsRNA was 0.46 mm² at 30 days after inoculation (Fig. 5).

TABLE 2. Symptoms of *Pinus massoniana* caused by different treatments^a

Time of inoculation (days)	CK		dsGFP		dsTLP1	
	Infection rate (%)	DSI	Infection rate (%)	DSI	Infection rate (%)	DSI
15	50.0	33.3	46.7	32.5	6.7	1.7
21	66.7	61.7	66.7	62.5	56.7	25.8
27	100	100	100	97.5	83.3	43.3
30	100	100	100	99.2	100	56.7

^a Pines inoculated with CK = pine wood nematodes (PWNs) treated with the buffer alone; dsGFP = PWNs treated with *gfp* dsRNA; dsTLP1 = PWNs treated with *Bx-tlp-1* dsRNA. DSI = disease severity index.

Fig. 4. Symptoms of *Pinus massoniana* inoculated with different treatments after 30 days of inoculation. Pines inoculated with: CK, pine wood nematodes (PWNs) treated with the buffer alone; dsGFP, PWNs treated with *gfp* dsRNA; dsTLP1, PWNs treated with *Bx-tlp-1* dsRNA; and water, sterile water.

Regarding the three-dimensional reconstruction, the X-Z and Z-Y sections of the sterile water-treated stem samples revealed intact xylem and phloem tissues with cells arranged in rows and no projective image noise. However, projective image noise was detected in the X-Z and Z-Y sections of the stem samples from trees inoculated with CK-treated or dsGFP-treated PWNs, and the cells were somewhat disordered. The appearance of the corresponding sections of the stem samples from trees inoculated with dsTLP1-treated PWNs was between that of the sterile water-treated control and the sample from trees inoculated with dsGFP-treated PWNs (Fig. 6). Similar results were obtained for the three-dimensional reconstructions and the two-dimensional cross-sections.

DISCUSSION

In this study, silencing *Bx-tlp-1* expression adversely affected *B. xylophilus* reproduction and pathogenicity. We assessed the effects of soaking PWNs in a dsRNA-containing solution. The dsRNA was taken-up by the PWNs, even in the absence of a neurochemical, which was similar to the results of a previous investigation (Cheng et al. 2010) in which the target gene expression level decreased by 70% following RNAi induced by soaking samples in buffers supplemented with dsRNA (1.5 mg/ml). In our study, treatments with dsRNA-containing buffers (2 mg/ml) silenced the target gene (*Bx-tlp-1*) by more than 90%. Additionally, a qRT-PCR assay indicated that the effects of dsTLP1 treatment were maintained in the F3 offspring. RNAi treatment of *C. elegans* via the injection of dsRNA affects nontreated progeny. When genes were targeted by RNAi, the silencing responses were observed for at least three generations (until the F3 offspring). Therefore, these effects may qualify as transgenerational heritable responses (Rechavi and Lev 2017).

Variations in the PWN populations indicated there were fewer dsTLP1-treated nematodes than control-treated nematodes, especially at 20 days. Additionally, there was a measurable decrease in reproduction after the RNAi treatment, suggesting a decrease in nematode fitness. Thaumatin-like proteins exhibit (1, 3)- β -glucanase activity and may bind to (1, 3)- β -glucans in plants. Moreover, TLPs may be capable of binding the cell wall (1, 3)- β -glucans of fungi (Osmond et al. 2001). We speculated that the (1, 3)- β -glucanase activity decreased in dsTLP1-treated PWNs, and that these nematodes could only weakly feed on fungi after RNAi treatment, which may have contributed to the decrease in PWN

Fig. 5. Internal change of *Pinus massoniana* after inoculation and direct visualization of xylem cavitation by X-ray microtomography (micro-CT) technology. The scale bar of two-dimensional (2D) cross-sections was 1 mm. The xylem cavitation areas (mm²) of *P. massoniana* were measured by Image J. Pines inoculated with: CK, pine wood nematodes (PWNs) treated with buffer alone; dsGFP, PWNs treated with *gfp* dsRNA; dsTLP1, PWNs treated with *Bx-tlp-1* dsRNA; and water, sterile water. Data in the table are presented as mean ± SD.

reproduction. These results indicated that silencing *Bx-tlp-1* expression adversely affected PWN reproduction.

Three-year-old *P. massoniana* trees were inoculated with PWNs treated with CK, dsGFP, or dsTLP1. At 30 days after inoculation, 100% infection rates were recorded for all inoculated *P. massoniana* trees, but the lowest DSI (56.7) was calculated for the trees inoculated with dsTLP1-treated PWNs. Moreover, almost all of the trees inoculated with CK-treated or dsGFP-treated PWNs were completely wilted. Therefore, *B. xylophilus* pathogenicity appears to be influenced by *Bx-tlp-1*.

A previous study confirmed that the browning of the pith tissue of *Pinus thunbergii* infected with *B. xylophilus* for 5 days was observable with the eye (Sheng-Rong and Jian-Ren 2007). Cavitation is a characteristic symptom of PWD that may occur in infected trees in diverse ways (Ikeda and Ohtsu 1992; Utsuzawa et al. 2005). In our

study, we measured the cavitation of intact pine stems using micro-CT. Analyses of the stem cross-sections of trees inoculated with CK-treated or dsGFP-treated PWNs revealed extensive air-filled areas, with ruptured pith tissue cells forming relatively large cavities. However, incomplete cavitation was observed in the stem cross-sections of trees inoculated with dsTLP1-treated PWNs. Therefore, the silencing of *Bx-tlp-1* via dsTLP1 treatment inhibited the ability of *B. xylophilus* to induce cavitation in pine trees at 30 days after inoculation. The xylem cavitation area of pine trees inoculated with *B. xylophilus* treated with *Bx-tlp-1* dsRNA was 0.46 mm² at 30 days after inoculation. These results implied that *Bx-tlp-1* is crucial for *B. xylophilus* pathogenicity, and RNAi may be used to decrease *Bx-tlp-1* expression during infections by PWNs. A previous study confirmed the utility of micro-CT for examining xylem cavitation in

Fig. 6. Three-dimensional (3D) reconstruction (X-Y, X-Z, and Z-Y) of X-ray microtomography (micro-CT). The spatial resolution of the image was 2.5×2.5×2.5 μm per voxel. Pines inoculated with: CK, pine wood nematodes (PWNs) treated with the buffer alone; dsGFP, PWNs treated with *gfp* dsRNA; dsTLP1, PWNs treated with *Bx-tlp-1* dsRNA; and water, sterile water.

intact samples (Cochard and Delzon 2013). Consequently, micro-CT may be an ideal method for studying cavitation during infections by PWNs. In future studies, we will use micro-CT to further analyze the cavitation of pine trees inoculated with PWNs to clarify the pathogenicity of PWNs.

Previous studies suggested that the protein encoded by *Bx-tlp-1* is similar to the TLPs of *P. massoniana* regarding sequence and structure (Meng et al. 2017; Shinya et al. 2013; Wang et al. 2014). Molecular mimicry is an effective means for plant-parasitic nematodes to evade plant host defenses (Maizels and Yazdanbakhsh 2003). Additionally, *Brugia malayi* can suppress human macrophages by releasing a secreted homolog of the human cytokine macrophage migration inhibitory factor (Falcone et al. 2001). Moreover, *Globodera rostochiensis* secretes a CLAVATA3/ESR peptide that affects the host root meristem cells (Mitchum et al. 2012; Wang et al. 2011), which benefits the nematode in plant-parasite interactions. Accordingly, the similarity of the protein encoded by *Bx-tlp-1* to plant proteins suggests it may mimic a component of host plant defense systems. *B. xylophilus* may trigger defense responses that lead to xylem cavitation. Additionally, a *B. xylophilus* TLP may function as an effector molecule that manipulates plant hosts. Furthermore, the protein may have resulted from the co-evolution of *B. xylophilus* and pine trees or the co-evolution of a close ancestor of *B. xylophilus* and its hosts.

In conclusion, *Bx-tlp-1* is crucial for *B. xylophilus*, not only because it influences nematode reproduction but also because it influences the extent of PWD symptoms. The results of this study provide new insight relevant for future characterizations of *Bx-tlp-1* functions related to the interaction between *B. xylophilus* and host pine trees.

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