Specifically Expressed Genes of the Nematode
*Bursaphelenchus Xylophilus* Involved with Early Interactions with Pine Trees

Xiwen Qiu¹², Xiaoqin Wu¹², Lin Huang¹², Minqi Tian¹², Jianren Ye¹²* 

¹ Institute of Forest Protection, College of Forest Resources and Environment, Nanjing Forestry University, Nanjing, Jiangsu, China, ² Jiangsu Key Laboratory for Prevention and Management of Invasive Species, Nanjing, Jiangsu, China

**Abstract**

As the causal agent of pine wilt disease (PWD), the pine wood nematode (PWN), *Bursaphelenchus xylophilus*, causes huge economic losses by devastating pine forests worldwide. However, the pathogenesis-related genes of *B. xylophilus* are not well characterized. Thus, DNA microarrays were used to investigate differential gene expression in PWN where *Pinus thunbergii* was inoculated with nematodes, compared with those cultured on *Botrytis cinerea*. The microarrays comprised 31121 probes, 1310 (4.2%) of which were differentially regulated (changes of >2-fold, $P < 0.01$) in the two growth conditions. Of these 1310 genes, 633 genes were upregulated, whereas 677 genes were downregulated. Gene Ontology (GO) categories were assigned to the classes Cellular Component, Molecular Function, and Biological Process. The comparative gene expression analysis showed that a large number of the pathogenesis-related genes of *B. xylophilus*, such as pectate lyase genes, cytochrome P450s, UGTs, and ABC transporter genes, were highly expressed when *B. xylophilus* infected *P. thunbergii*. Annotation analysis indicated that these genes contributed to cell wall degradation, detoxification, and the reproduction process. The microarray results were validated using quantitative RT-PCR (qRT-PCR). The microarray data confirmed the specific expression of *B. xylophilus* genes during infection of *P. thunbergii*, which provides basic information that facilitates a better understanding of the molecular mechanism of PWD.

**Introduction**

Pine wilt disease (PWD) is one of the most serious diseases [1,2] that affects coniferous forests around the world and it is considered to be caused by pine wood nematodes (PWNs), *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle [3,4]. In Asia, PWD was introduced into Japan during the early 20th century [5] and it spread subsequently to other countries, including China [6] and Korea [7], where pine trees have no natural resistance to *B. xylophilus*. Later, PWD spread to European countries, including Finland [8], Portugal [9], and Russia [10]. *B. xylophilus* is considered to be a native of North America [11], where the local forests are generally resistant or tolerant [12]. *B. xylophilus* causes severe economic, environmental, and social impacts [13,14] in non-indigenous areas and it has attracted attention because of its high pathogenicity in host trees. As the main pathogenic agent of PWD [15], PWNs lead to the death of pine trees within several months, especially between May and September. *B. xylophilus* is vectored by longhorn beetles in the genus *Monochamus* [16–19] and is transmitted via an unusual tree to tree infection route [20]. It is widely known that the introduction and expansion of *B. xylophilus* into non-native areas was mediated mainly by the international wood trade via the short- or long-distance transportation of pine wood, as well as packing materials and wood products infected by PWN [21–23]. Therefore, there is an urgent need to develop effective management strategies to control PWD [24].

PWN is a serious threat to forests in Asia and Europe, and the risk of this problem is likely to increase due to climate change. However, the pathogenic mechanism of PWD is not clear at present [25]. Little was known about the molecular pathogenicity of *B. xylophilus* until ten years ago, but much progress has been made recently since the development of biotechnology techniques [21]. High-throughput sequencing, which is a powerful method for gene research, has been used
to screen resistant genes in trees infected by *B. xylophilus* [26]. Microsatellite markers have also been used to study the genetic diversity of PWN to understand its invasion route and the host colonization process [24].

Information about the pathogenesis-related genes of *B. xylophilus* is essential for understanding the pathogenic mechanism of PWD, but there has been little research in this area. Thus, we compared the differential gene expression of *B. xylophilus* in two growth conditions: growth on *Botrytis cinerea* and after inoculating *Pinus thunbergii* with PWNs. The goal of this study was to analyze the specifically expressed PWN genes involved with the early interactions between *B. xylophilus* and *P. thunbergii*, and to screen the pathogenesis-related genes of *B. xylophilus* using DNA microarrays. Quantitative RT-PCR (qRT-PCR) was used to validate the results obtained using the microarray assays.

Materials and Methods

*B. thunbergii* growth conditions and sampling

*P. thunbergii* seedlings (2 years old) obtained from the greenhouse at Nanjing Forest University were transplanted into pots (30 cm in diameter, 25 cm in height) and maintained with a relative humidity of 70%. The seedlings (height c. 80 cm) were watered every other day and maintained with a photoperiod of 14 h day (25°C) and 10 h night (20°C). The highly virulent AMA3cl strain of PWN was used in the experiment, which was maintained by Lihua Zhu in our laboratory [27]. Two treatments were applied: (i) a suspension of 5000 nematodes (a mixture of adults and juveniles) was used to inoculate the fungus *B. cinerea* (the fungus had been incubated at 25°C for 6 days) on potato dextrose agar medium and grown at 25°C for a further 7 days; (ii) the same amount of nematodes was pipetted into wounds (2 cm in length) in *P. thunbergii* seedlings at about 50 cm above the soil level. The inoculated seedlings were cultivated in the greenhouse for 7 days at 25°C during the daytime and 20°C at night, with 70% humidity. The nematodes were separated from *B. cinerea* and *P. thunbergii* seedlings using a Baermann funnel. Next, *B. xylophilus* was collected by centrifugation at 3000 rpm for 1 min and frozen in liquid nitrogen, before further RNA isolation.

RNA isolation

The RNA was extracted from frozen nematodes using an RNAprep Kit (Tiangen, China) and purified further with an RNAclean Kit (Tiangen, China), according to the manufacturer’s protocol. The RNA was quantified at 260 nm using a spectrophotometer and examined by electrophoresis on a 1.5% agarose gel.

Microarray construction, hybridization, and data analysis

The microarray experiments were performed with the help of Shanghai Biotechnology Corporation. In total, 31121 oligonucleotides were synthesized based on the whole genome sequences (obtained from http://www.ncbi.nlm.nih.gov/ genome) of *B. xylophilus*. A silane mixture was used to prepare hydrophobic glass slides, which contained exposed hydroxyl groups to facilitate nucleotide combining. The synthesized oligonucleotides were spotted robotically onto the hydrophobic surfaces of the glass slides. The first-strand cDNA was synthesized using a T7-oligo (dT) promoter primer. Subsequently, double-stranded cDNA was produced using a combination of Affinity Script RNase Block Mix, dNTP mix, DTT, and First Strand Buffer. The cRNA was generated from cDNAs using a Genechip IVT Labeling Kit (Affymetrix, USA) and purified with an RNAeasy Mini Kit (Qiagen, Germany). The cRNAs were then hybridized at 65°C for 17 h with a microarray that contained 31121 probe sets. After hybridization, the slides were washed with GE wash buffer, according to the manufacturer’s instructions, and scanned using an Agilent microarray scanner (Cat#G2565CA, Agilent Technologies, Santa Clara, CA, USA). The gene expression data were analyzed using a t-test (*P < 0.01*). Only genes with significantly different expression levels were screened for further analysis. The gene data analysis and functional annotation were performed using SBC Analysis System (http://www.sas.ebioservice.com). The results of microarray analysis were deposited in the NCBI database (Gene Expression Omnibus) and the accession number is GSE50481.

Quantitative real-time RT-PCR

Real-time quantitative RT-PCR (qRT-PCR) was used to verify the microarray results. Gene-specific primers (Table S1) were designed using Primer Premier 5 software. The actin gene of *B. xylophilus* was selected as the internal control. The first-strand cDNA was synthesized using a Prime Script 1st strand cDNA synthesis Kit (TaKaRa, Japan). The cDNA samples were diluted to 20 ng/µl. Real-time PCR was conducted using a 20 µl reaction volume, which contained 2 µl of template, 10 µl SYBR Premix Ex Taq, 0.4 µl ROX Reference Dye II, 0.4 µl forward primer, 0.4 µl reverse primer, and 6.8 µl ddH2O. PCR amplification was carried out using the following conditions: denaturation at 95°C for 30 s, followed by 40 cycles of amplification where each cycle comprised denaturation at 95°C for 5 s, and annealing and extension at 60°C for 34 s. The quantitative variations in the selected genes in the two growth conditions were evaluated using the relative quantification method (ΔΔCT) [28].

Results

Screening of the specifically expressed genes of *B. xylophilus* when cultured on *B. cinerea* and inoculated into *P. thunbergii*

*B. xylophilus* was separated from *B. cinerea* and *P. thunbergii* seedlings using Baermann funnels. To reduce the likelihood of biological errors, nematodes were collected from five inoculated *P. thunbergii* seedlings that exhibited similar symptoms and combined as one sample. There were three replicates for each treatment. Differences in the transcript abundances of *B. xylophilus* in the two different growth conditions were
detected by genome-wide expression profiling using the Agilent *B. xylophilus* GeneChip microarray containing 31121 probe sets. Over 31000 probe signals were detected from the microarrays and 1310 (4.2%) *B. xylophilus* genes were differentially expressed (Figure 1). Of these 1310 genes, 633 genes were upregulated, whereas 677 genes were downregulated. Of the 633 upregulated genes, 569 genes were upregulated by 2- to 5-fold and 64 genes were upregulated >5-fold. Among the specifically expressed genes, we focused on those related to cell wall degradation, detoxification, and reproduction to identify pathogenesis-related genes.

**Functional annotation**

Functional annotation was performed to assign the genes of *B. xylophilus* with Gene Ontology (GO) terms. The main GO categories included Cellular Component, Molecular Function, and Biological Process. Based on the annotations (Figure 2) for Biological Process, 13.4% and 12.9% of the assignments belonged to the categories “Metabolic Process” (GO: 0008152) and “Cellular Process” (GO: 0009987), respectively, followed by “Developmental Process” (GO: 0032502, 10.5%) and “Multicellular Organismal Process” (GO: 0032501, 10.5%). In addition, the “Binding” (GO: 0005488) and “Catalytic Activity” (GO: 0003824) categories were prominent among the Molecular Function terms with 41.5% and 37.7% of the assignments, respectively, followed by the categories “Transporter Activity” (GO: 0005215, 6.2%), “Transcription Regulator Activity” (GO: 0030528, 3.5%), and “Electron Carrier Activity” (GO: 0009055, 3.5%). Furthermore, the categories of “Cell Part” (GO: 0044464) and “Cell” (GO: 0005623) had the same proportion, followed by “Organelle” (GO: 0043226) and “Macromolecular Complex” (GO: 0032991), which accounted for 9.1% and 3.4% of assignments. Interestingly, a recent study reported a similar classification of the annotated amino acid sequences in *Pinus pinaster* and *Pinus pinea* after inoculation with *B. xylophilus* [28]. In general, the aforementioned GO classes accounted for the majority of the specifically expressed genes.

The gene expression levels of *B. xylophilus* changed dramatically when the nematode was used to inoculate *P. thunbergii* compared with those grown on *B. cinerea*. We found that 520 genes were upregulated at least 3-fold, including 176 annotated genes. The GO analysis showed that these highly expressed genes (changes of >3-fold) were related mainly to metal ion binding, transferase activity, protein binding, nucleic acid binding, nucleotide binding, oxidoreductase activity, and hydrolase activity (Figure 3). The upregulated metal ion binding-related genes may contribute significantly to the enhancement of various enzyme activities and increase the adaptability of *B. xylophilus*. Interestingly, transferases related to metal ions were also upregulated. The protein binding factors associated with regulation probably activated the over-expression of the pathogenicity-related genes of *B. xylophilus* during the PWD process. The high expression levels of nucleic and nucleotide binding-related genes indicated that *B. xylophilus* can reproduce in *P. thunbergii*, which is the causal premise of PWD. In addition, a number of oxidoreductase and hydrolase genes, such as glucose oxidase, pectate lyase, and glutathione S-transferase genes, were detected by the microarrays. According to the functional annotation, we found that some of the cell wall degradation-related genes were upregulated significantly. These genes are considered to be key factors that allow *B. xylophilus* to invade its host.

**qRT-PCR analysis**

To validate the microarray results, 20 genes were selected at random from the highly expressed sequences and analyzed by qRT-PCR. We designed specific-primers for nine downregulated genes (Probe name: nl-p01087, nl-p02937, nl-p03084, nl-p04066, nl-p04652, nl-p15102, nl-p17373, nl-p20437, nl-p24677) and 11 upregulated genes (Probe name: nl-p03314, nl-p04906, nl-p10686, nl-p10889, nl-p12558, nl-p13660, nl-p14243, nl-p14336, nl-p22988, nl-p27181, nl-p29107). The expression patterns of the 20 candidate genes detected by qRT-PCR were similar to those detected by the microarray (Figure 4), which demonstrated the reliability of the microarray data.

**Discussion**

Valuable information was found by identifying specifically expressed genes based on the differential gene expression of...
Figure 2. Classification of the annotated genes for *Bursaphelenchus xylophilus*. The genes that changed significantly were divided into three functional sub-categories based on the Cellular Component (A), Molecular Function (B), and Biological Process (C) categories, according to the Gene Ontology (GO) principles.

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PWNs used to inoculate *P. thunbergii* compared with those grown on *B. cinerea* [26]. The plant cell wall is the primary barrier that a pathogen must penetrate [29]. The cell wall is comprised mainly of pectin, cellulose, and hemicellulose [30]. Cellulose is a polymer of (1–4)-linked β-D-glucose that is interlocked by hemicellulose to form a strong elastic network [31,32]. The network is embedded in pectin, which is the most complex component of the cell wall polysaccharides. The plant cell wall plays a critical role in resisting invasion by *B. xylophilus*. To invade the host successfully, *B. xylophilus* must produce a range of cell wall degradation enzymes that break down this barrier. Therefore, the high level expression of cell wall degradation-related genes is essential to allow *B. xylophilus* to infect its plant host.

Cell wall-degrading enzymes have been proposed to determine the pathogenicity of PWN. Plant parasitic nematodes have a wide range of cell wall-degrading enzymes, such as cellulase, polygalacturonase [33], pectate lyases [34], and xylanases [35], which contribute to the nematode’s ability to feed on plant tissues. Cellulase is one of the key enzymes associated with the pathogenicity of PWN [36]. Only the GHF5 and GHF45 families of cellulases have been found in nematodes and it has been proposed that they were acquired via horizontal gene transfer (HGT) events [37]. Mayer [38] studied the evolution of the cellulase genes of ten nematode species based on a phylogenetic framework and demonstrated that nematode species with a cellulase gene acquired via HGT exhibited cellulase activities. It has been suggested that the cellulase gene was functionally integrated into the nematode’s genome. In the present study, a number of cellulose degradation-related genes were detected by our microarray analysis, such as beta-1,3-endoglucanase, beta-1,4-endoglucanase, and cellulase (Table 1). The expression of beta-1,4-endoglucanase genes did not change greatly (0.9-fold). However, the expression levels of cellulase genes were downregulated significantly. During the early stage of infection, it is possible that cell wall components other than cellulose are degraded primarily by *B. xylophilus*.

Pectin-degrading enzymes, such as polygalacturonases and pectate lyases (PLs), are the primary cell-degrading enzymes secreted by pathogens, which can expose more polymers that need to be degraded by other cell-degrading enzymes [39,40]. PLs are thought to be important pathogenic factors for phytopathogens [41]. A pectate lyase 2 (pel-2) gene was detected in *B. xylophilus* based on the microarray assays (Table 1) and its expression was upregulated >6-fold. The high expression of PLs may enhance the infection capacity of *B.*
xylophilus and facilitate its migration through plant tissues. Kang et al. (2012) showed that the migration activity of B. xylophilus plays an important role in efficient parasitism [42]. The pel-2 gene has also been detected in potato cyst nematodes (PCNs) and experiments demonstrated that the silencing of pel-2 in PCN juveniles reduced the infection efficiency greatly [43].

As one of the primary natural biopolymers, chitin is a major component of fungal cell walls. B. xylophilus can secrete chitinase to digest fungal cell walls throughout its growth cycle [44]. The expression of a chitinase gene (cht-1) was downregulated dramatically when B. xylophilus was used to inoculate P. thunbergii compared with culture on B. cinerea, probably because of the change in the available sources that B. xylophilus could feed upon. The formation and degradation of chitin are essential for nematode oviposition and molting cycle development. Nematode metamorphosis is regulated by the release of edcsyone, which is related to the regulation of chitin synthesis. A previous report showed that edcsyone plays a role in regulating the expression levels of the CHS-1 and CHS-2 genes during Drosophila metamorphosis [45]. However, abnormal chitin synthesis could lead to disorders during nematode development. Several proteins, such as yeast P. thunbergii was inoculated with B. xylophilus. For example, the ptc-1 and rack-1 genes were upregulated by 4.2-fold and 3.6-fold, respectively.

The cytochrome p450 (CYP450) family includes enzymes that play important roles during the biotransformation of secondary metabolites [48]. Studies have shown that C. elegans CYP450s catalyze a series of exogenous and endogenous substrates, which cope with variation in the growth conditions [49]. This phenomenon may also occur in B. xylophilus. Pine trees generate numerous secondary metabolites, such as terpenoids and cyclic aromatics, to combat the invasion by B. xylophilus [26]. B. xylophilus must also mobilize defensive reactions to avoid damage by these complex compounds [50,51]. The presence of chemical compounds and plant secondary metabolites in the environment can induce the expression of CYP450 genes [52,53]. Based on complete genome sequencing data, 80 and 76 CYP450 genes were detected in C. elegans and B. xylophilus, respectively [54]. As shown in Table 2, the expression levels of seven CYP450 genes changed strikingly according to the microarray analysis. Of these, four genes were upregulated by at least 3-fold, whereas three genes were downregulated. It is surprising that the CYP450 genes of B. xylophilus not only generate enzymes to utilize secondary metabolites, but they also produce toxic metabolites that damage P. thunbergii [32]. Thus, the over-expression of CYP450 genes may enhance the pathogenicity of B. xylophilus and play a crucial role in the disease process [55]. However, the pathogenic role of the CYP450 genes in B. xylophilus remains unknown [56]. The gene function annotations showed that the W01A11.1 gene of B. xylophilus, which was upregulated by 6.3-fold, was a response to toxins, thereby suggesting that P. thunbergii generates a range of secondary metabolites as toxins that resist invasion by B. xylophilus. Furthermore, the accumulation of secondary metabolites produced by P. thunbergii may induce the over-expression of B. xylophilus CYP450 genes [57,58].

At present, the detoxification process of B. xylophilus is divided into three phases [54]. First, CYPs are the primary proteins in the first phase, which provide the enzyme substrates for the next stage. Second, the glutathione S-transferases (GSTs) and UDP-glucuronosyl transferases (UGTs) are essential in the second phase. A series of detoxification reactions occurs during this stage, which produce a high level of efflux. As shown in Table 2, one GST and four UGTs were found to be upregulated by at least 3-fold. In the last phase, ATP-binding cassette (ABC) transporters are the main group responsible for the efflux of detoxified molecules. One ABC transporter was found to be highly upregulated according to the microarray analysis.

In particular, the expression levels of genes related to detoxification were upregulated significantly, which suggests that B. xylophilus enhanced its gene expression in response to the secondary metabolites produced by pine trees. One gene, Y52B11A.8, which is related to the lipid catabolism process, was detected by the microarray. The expression of Y52B11A.8 was upregulated by more than 5-fold. This indicated that lipids, which are likely to have nematocidal activity, were produced by pine trees after invasion by B. xylophilus.

The reproductive ability of B. xylophilus in pine trees is a crucial causal factor in PWD [56]. Many reports have suggested that the virulence level of B. xylophilus is associated with its reproductive ability in vitro or in vivo conditions [59,60]. When two PWN isolates (virulent and avirulent) were inoculated into pine trees, the PWN population of the virulent isolate was greater than that of the avirulent isolate. The rate of

| Genes | Function | Fold change |
|-------|----------|-------------|
| ugl-49 | UDP-Glucuronosyl Transferase | 3.1834 |
| ugl-47 | UDP-Glucuronosyl Transferase | 3.5447 |
| ugl-59 | UDP-Glucuronosyl Transferase | 3.3254 |
| ugl-64 | UDP-Glucuronosyl Transferase | 7.509 |
| cyp-31A3 | Monooxygenase activity | 0.3014 |
| cyp-25A5 | Monooxygenase activity | 0.1778 |
| cyp-13A11 | Monooxygenase activity | 3.0041 |
| cyp-33C1 | Monooxygenase activity | 0.3102 |
| cyp-33C4 | Monooxygenase activity | 4.4049 |
| cyp-33C9 | Monooxygenase activity | 6.2141 |
| cyp-33D3 | Monooxygenase activity | 3.2066 |
| abtm-1 | ABC Transporter | 3.8127 |
| gat-33 | Glutathione S-Transferase | 3.3241 |
| W01A11.1 | response to toxin | 6.3557 |
| Y52B11A.8 | lipid catabolic process | 5.1726 |

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The translocation of the virulent isolate into the xylem results indicated that the expression levels of the reproduction-related genes were detected by the microarray analysis (Table 3). The functions of reproduction-related genes involved with egg hatching, germ cell development and meiosis, and the positive regulation of the growth rate, etc. The results indicated that the expression levels of the reproduction-related genes of *B. xylophilus*, such as rpl-24.2, R11A8.2, set-16, and F25H5.6, were upregulated dramatically by growth in *P. thunbergii* compared with those when grown on *B. cinerea*.

In the early stages of PWD, ligno-suberization and wound periderm were observed around the cortex resin canal. In the advanced stage, the number of nematodes increased dramatically and the cambium of pine trees was destroyed [25,66]. The results of previous studies have shown that *B. xylophilus* feeds mainly on the xylem ray parenchyma cells of pine trees [67]. The rapid reproduction rate of *B. xylophilus* in *P. thunbergii* probably leads to more serious destruction of the cortex resin canal. However, more of the cortex parenchyma was fed on by *B. xylophilus*, which induces rapid increases in the cavitation area. The accumulation of the toxic substances produced by *B. xylophilus* also aggravated the wilting of needles. Thus, a high frequency of nematode reproduction is probably an important pathogenicity factor related to PWD.

PWD is a complex disease and its mechanism remains unclear. The specific genes related to cellulose degradation, hydrolase, and detoxification by *B. xylophilus* may play key roles in this disease. Thus, the mechanism of PWD may be involved with variation in the transcript levels of *B. xylophilus* genes. Our microarray analysis demonstrated that the expression levels of *B. xylophilus* genes changed significantly after it was used to inoculate *P. thunbergii* seedlings.

The present study investigated the differential gene expression of *B. xylophilus* cultured on *B. cinerea* and when used to inoculate *P. thunbergii*. The results showed that the gene expression of *B. xylophilus* changed significantly and a number of genes were upregulated dramatically. We found that some of the upregulated genes were related to cell wall degradation and reproduction, which suggests that *B. xylophilus* not only succeeded in invading *P. thunbergii*, but it also reproduced within *P. thunbergii*. In addition, we detected several toxin-related genes, which indicate that *B. xylophilus* may produce toxins and utilize the secondary metabolites of its host, and this process is possibly involved with the wilting of pine trees. These data facilitate a better understanding of the molecular mechanism of PWD and they may help to develop effective control strategies to combat *B. xylophilus*. However, further investigations are required to understand the functions of these specifically expressed genes.

### Supporting Information

Table S1. Primers used in the quantitative real-time PCR (qRT-PCR) analysis.

(LOCX)

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### Author Contributions

Conceived and designed the experiments: JRY LH. Performed the experiments: XWQ LH XQW. Analyzed the data: XWQ. Contributed reagents/materials/analysis tools: JRY XWQ MQT. Wrote the manuscript: XWQ LH.
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