Article

Transcriptome Analysis of *Bursaphelenchus xylophilus* Uncovers the Impact of *Stenotrophomonas maltophilia* on Nematode and Pine Wilt Disease

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Abstract: *Stenotrophomonas maltophilia* influences the reproduction, pathogenicity, and gene expression of aseptic *Bursaphelenchus xylophilus* after inoculation of aseptic *Pinus massoniana*. Pine wilt disease is a destructive pine forest disease caused by *B. xylophilus*, and its pathogenesis is unclear. The role of bacteria associated with *B. xylophilus* in pine wilt disease has attracted widespread attention. *S. maltophilia* is one of the most dominant bacteria in *B. xylophilus*, and its effect is ambiguous. This study aims to explore the role of *S. maltophilia* in pine wilt disease. The reproduction and virulence of aseptic *B. xylophilus* and *B. xylophilus* containing *S. maltophilia* were examined by inoculating aseptic *P. massoniana* seedlings. The gene expressions of two nematode treatments were identified by transcriptome sequencing. The reproduction and virulence of *B. xylophilus* containing *S. maltophilia* were stronger than that of aseptic nematodes. There were 4240 differentially expressed genes between aseptic *B. xylophilus* and *B. xylophilus* containing *S. maltophilia* after inoculation of aseptic *P. massoniana*, including 1147 upregulated genes and 2763 downregulated genes. These differentially expressed genes were significantly enriched in some immune-related gene ontology (GO) categories, such as membrane, transporter activity, metabolic processes, and many immune-related pathways, such as the wnt, rap1, PI3K-Akt, cAMP, cGMP-PKG, MAPK, ECM-receptor interaction, and calcium signaling pathways. The polyubiquitin-rich gene, leucine-rich repeat serine/threonine-protein kinase gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, acetyl-CoA carboxylase gene, and heat shock protein genes were the key genes associated with immune resistance. Moreover, there were four cell wall hydrolase genes, thirty-six detoxification- and pathogenesis-related protein genes, one effector gene and ten cathepsin L-like cysteine proteinase genes that were differentially expressed. After inoculation of the host pine, *S. maltophilia* could affect the virulence and reproduction of *B. xylophilus* by regulating the expression of parasitic, immune, and pathogenicity genes of *B. xylophilus*.

Keywords: *Bursaphelenchus xylophilus*; *Stenotrophomonas maltophilia*; *Pinus massoniana*; differentially expressed genes; virulence

1. Introduction

Pine wilt disease (PWD) is a devastating disease in pines caused by the pine wood nematode (PWN), *Bursaphelenchus xylophilus*. It is native to North America [1], and epidemic in East Asian (China, Japan, and Korea) [2–4], causing serious ecological damage and economic loss. Since PWD was reported in 1982 in Nanjing, China, it has spread to 18 provinces with an area of more than 1.1 million hm², and more

than 80 million pine trees have wilted completely [5]. The occurrence and development of the disease involve many factors, such as susceptible pine hosts, a beetle vector, the nematode, associated bacteria and fungi, and the appropriate geographical environment. The pathogenesis of PWD is not clear [4,6].

A large number of bacteria can adhere to the surface of *B. xylophilus*, some of them are random [7–9]. The species of bacteria associated with *B. xylophilus* from different regions and habitats are different [6,10]. *Pseudomonas* were dominant strains in PWNs isolated in China, but *Bacillus* were dominant in Japan and both genera were dominant in Korea [11]. *Ewingella americana*, *Pseudomonas fluorescens*, and *Pantoea agglomerans* are present in PWN obtained from different *Pinus* spp. with distinct geographical locations. *Agrobacterium* sp., *Bordetella* sp., and *Fusobacterium* sp. seem to be particular associated to PWN when cultured in fungus [6]. PWN-Associated bacteria may have putative important effect in PWD. Some PWN-associated bacteria can secrete cellulase, which is extremely important for nematode colonization [12]. *Serratia* spp. may assist the nematode opportunistically under the oxidative stress (OS) resistant in the disease [13]. *Stenotrophomonas* have an important role in α -pinene degradation, which may represent a major protective role in pine defenses [14].

Compared with the surface environment, the internal environment of *B. xylophilus* is relatively stable. Yuan et al. found that the bacteria associated with PWN not only existed on PWN's surface, but also inside of the PWN [15]. The bacteria in *B. xylophilus* from different regions and pine species mainly belong to *Stenotrophomonas*, *Achromatopsia*, *Ewingella*, *Leifsonia*, *Rhizobia*, and *Pseudomonas* [15]. Additionally, relatively abundant bacteria in *B. xylophilus* from different host pine trees were also found to be *Stenotrophomonas*, *Pseudomonas*, and *Elizabethkingia* [16]. Furthermore, the dominant bacterium in *B. xylophilus* individuals with different virulence was *Stenotrophomonas*, followed by *Pseudomonadaceae_unclassified* and *Rhizobiaceae_unclassified* [17]. *Stenotrophomonas* was also abundant in propagative *B. xylophilus* [18]. Eighty percent of culturable bacteria in *B. xylophilus* isolated from *Pinus massoniana* were *S. maltophilia* and *A. xylosoxidans* [19]. Thus, it can be seen that *S. maltophilia* is one of the most dominant bacteria in the *B. xylophilus*.

After treatment with *S. maltophilia*, the reproduction of *B. xylophilus* cultured on *Botrytis cinerea* decreased, while the virulence of *B. xylophilus* increased [20]. He et al. analyzed the genes in aseptic *B. xylophilus* and *B. xylophilus* carrying *S. maltophilia* cultured on *B. cinerea* by transcriptome sequencing and found that a number of reproduction-related genes were downregulated, but many pathogenesis-related genes were upregulated in *B. xylophilus* carrying *S. maltophilia* compared with aseptic *B. xylophilus* [21]. *B. xylophilus* has mycetophagous and phytophagous feeding stages in its life cycle. The nematode feeds on epithelial cells and living parenchyma in living host trees and starts to reproduce. After inoculating pine trees, the reproduction of *B. xylophilus* carrying *S. maltophilia* was lower than that of aseptic *B. xylophilus*. This phenomenon was opposite to that in the individuals cultured on *B. cinerea*. Therefore, according to a previous study reference on nematodes cultured on *B. cinerea*, it is not yet possible to define the role of *S. maltophilia* in the interaction between *B. xylophilus* and pine trees.

For this purpose, the propagative capacity and virulence of aseptic nematodes and nematodes with *S. maltophilia* inoculated into aseptic pine seedlings were examined in this study. Transcriptome sequencing of aseptic nematodes and nematodes with *S. maltophilia* was carried out after inoculation to explore the molecular mechanism by which *S. maltophilia* affects the propagation and virulence of the *B. xylophilus*. The above results will be helpful in revealing the contribution of *S. maltophilia* to PWD and provide a reference for further exploring the mechanism of PWD.

2. Materials and Methods

2.1. Biological Materials

The *B. xylophilus* strain (AMA3) was isolated from pinewood chips of infested *P. massoniana* in Maanshan, Anhui, China. *S. maltophilia* (NSPmBx03) was the bacterium inside *B. xylophilus* extracted from infected *P. massoniana* in Nanjing, Jiangsu, China [18]. Nematodes and bacteria were kept in

the Jiangsu Key Laboratory for Prevention and Management of Invasive Species, Nanjing Forestry University, Nanjing, Jiangsu, China.

2.2. Preparation of Aseptic *B. xylophilus*

Nematodes were cultured on *B. cinerea* grown on potato dextrose agar (PDA) at 25 °C for 5 days and collected with a Baermann funnel [22]. The nematode suspension (1.5 mL) was poured into a petri dish (2–3 cm diam.) at 25 °C for laying eggs. After 4–6 h, eggs were seen adhering to the Petri dish. Sterile water was added to the Petri dish to discard the nematodes, while the eggs were retained. Fresh 15% H₂O₂ was used to asepticize the eggs for 1 h. The bacteria-free eggs were transferred to PDA plates cultured with *B. cinerea* for 5 days. The propagated nematodes were collected under aseptic conditions and stored at 4 °C [23]. At the same time, 1 mL of nematode solution was added to Luria-Bertani (LB) medium and cultured at 25 °C for 3 days to test whether the prepared nematodes were carrying bacteria. Clear LB medium was an indication that aseptic PWNs (Bx_a) had been obtained.

2.3. Preparation of *B. xylophilus* Treated with *S. maltophilia*

A colony of *S. maltophilia* was cultured in an Erlenmeyer flask of 50 mL of LB medium at 28 °C and 200 rpm for 24 h. The concentration of *S. maltophilia* was adjusted to 8.0×10^8 CFUs (colony-forming units), and 200 µL of this suspension was sprayed onto PDA plates containing *B. cinerea*. Each plate was inoculated with 1000 bacteria-free nematodes and incubated for 5 days at 25 °C. The propagated nematodes were collected using a Baermann funnel with 100 mL sterile water under aseptic conditions and stored at 4 °C [21]. A total of 1 µL of the obtained nematode solution was used as the template for 50 µL PCR amplification reaction. The *S. maltophilia* suspension was used as a control. Fragments of 16S rDNA gene were amplified using 2 × TSINGKE Master Mix according to the manufacturer's instructions (Beijing TsingKe Biotech Co., Ltd., Nanjing, China) with bacterial universal primer pairs of 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3'). PCR products were subjected to electrophoresis in 1% agarose gel to verify the size of target fragments and afterwards sent to Beijing TsingKe Biotech Co., Ltd. (Nanjing, China) for sequencing. The determined sequences were then compared with the 16S rDNA of *S. maltophilia* (NSPmBx03) using DNAMAN software (<https://www.lynnon.com/index.html>). Consistent sequences indicated that the nematode only carried *S. maltophilia* and the PWNs carrying *S. maltophilia* (Bx_b) had been obtained.

2.4. Preparation of Aseptic *P. massoniana* Seedlings

Mature seeds of *P. massoniana* were washed with tap water for 1 h, soaked in 30% H₂O₂ for 30 min and rinsed with 3 L sterile water. The aseptic seeds were placed on water agar with 0.1 g/L agar at 25 °C. The germination process was monitored frequently, and uncontaminated germinated seedlings were transferred to aseptic culture bottles containing a paper cup with perlite and 30–50 mL Gresshoff and Doy (GD) liquid medium without any growth regulators at the bottom [24,25]. The aseptic *P. massoniana* seedlings were cultured for 4 months under controlled conditions (temperature 25 °C, 16 h of light at 2000 lx). Clear GD medium was an indication that *P. massoniana* seedlings were aseptic.

2.5. *B. xylophilus* Inoculation

Under aseptic conditions, the shoot tips of four-month-old *P. massoniana* aseptic seedlings were cut away with sterilized scissors, and a piece of aseptic absorbent cotton wool was placed on the wound. A suspension of 200 nematodes was pipetted into the cotton wool. The aseptic pines were inoculated with PWNs Bx_a and Bx_b, respectively. For each treatment 24 seedlings were used, and as control (CK) plants were inoculated with *S. maltophilia* alone. Pines were cultured at 25 °C, with 2000 lx and 16 h of light per day. The disease incidence in the *P. massoniana* was observed and recorded regularly. The infection rate and disease severity index (DSI) of *P. massoniana* were calculated according to Yu et al. [26]. The incidence level of PWD was divided levels 0 to 4: 0 = all needles were green;

1 = 0–25% of needles turned yellow; 2 = 25–50% of needles turned yellow; 3 = 50–75% of needles turned yellow; and 4 = 75–100% of needles turned yellow. The calculation formula was as follows:

$$\text{Infection rate (\%)} = \frac{\sum \text{Number of infected plants with symptoms}}{\text{Total number of plants}} \times 100$$

$$\text{Disease severity index (DSI)} = \frac{\sum \text{Number of diseased plants} \times \text{symptom stage}}{\text{Total number of plants} \times \text{highest symptom stage}} \times 100$$

Seven days post inoculation (dpi), the PWNs of each treatment were separated under aseptic conditions by the Berman funnel method, and aseptic PWNs (PmBx_a) and PWNs with *S. maltophilia* (PmBx_b) were obtained and their reproduction were counted. The ratio of the final population to the initial reproduction of nematodes were presented as means $\pm$ standard deviation (Mean $\pm$ S.D.). All parameters were calculated using Microsoft Excel and GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The statistical significance was determined using SPSS Statistics 24.0 software (IBM China Company Ltd., Beijing, China). A Student's t-test was used to compare two samples. The level of significance was $p < 0.05$. A small amount of nematode solution from each treatment was taken for the sterility test and morphological observation. The rest of the nematodes were rinsed with sterile water 3–5 times and centrifuged, and the supernatant was removed, frozen in liquid nitrogen, and stored in a refrigerator at -80°C for subsequent analyses.

2.6. RNA Extraction and Sequencing

Six samples (PmBx_a_1, PmBx_a_2, PmBx_a_3, PmBx_b_1, PmBx_b_2, and PmBx_b_3, each treatment had three replicates) were used for RNA sequencing. Total RNA was extracted from the nematodes with TRIzol reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. The RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA with a concentration higher than $20.0 \text{ ng}/\mu\text{L}$, a total mass higher than $1.0 \mu\text{g}$, an RNA integrity number (RIN) over 7.0, and 28S/18S no less than 1.0 was used for library preparation. RNA-Seq library construction and paired-end sequencing on an Illumina HiSeq X-Ten was completed at Beijing Genomics Institute (Shenzhen, China). The RNA-Seq experiments have three technical replicates. The data have been submitted to NCBI, and the accession number is SRR11474049- SRR11474054.

2.7. RNA-Seq Analysis

The RNA-Seq data were cleaned using SOAPnuke (<https://github.com/BGI-flexlab/SOAPnuke>) to remove adaptors, reads with unknown bases (N) $> 5\%$, and low-quality sequences (quality score (Q20) < 20). After filtering, high-quality reads were then mapped to the *B. xylophilus* genome (BioProject PRJEA64437) [27] using Hierarchical Indexing for Spliced Alignment of Transcripts (HISAT) [28]. StringTie was used to do transcript reconstruction of each sample [29]. Cuffmerge was used to integrate the reconstruction information of all samples, and then Cuffcompare was used to compare the integrated transcript with the reference annotation information [30]. Transcripts with class code types of “u”, “i”, “o”, and “j” were selected and defined as the new transcripts. The protein coding potential of the new transcripts was predicted by CPC [31], and the new transcripts with predicted protein coding potential were added to the reference gene sequences to obtain a complete reference sequence information, which would be analyzed in the future. Clean reads were compared to the reference sequence to calculate the gene alignment rate by Bowtie 2 [32]. The expression levels of genes and transcripts were calculated using RSEM (RNA-Seq by Expectation Maximization) [33]. Genes with an absolute value of $\log_2$ fold change ≥ 1 and an adjusted p -value (padj) ≤ 0.05 were considered to be differentially expressed genes (DEGs) [34]. The reference genome of *B. xylophilus* was used as the background to determine significantly enriched gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enriched within DEGs. A false discovery rate (FDR) ≤ 0.01 was

used to indicate statistically significant enrichment. DIAMOND [35] software was used to compare the DEGs to the STRING [36] database, resulting in the identification of DEG-encoded protein interactions based on their homology with known proteins. The whole interaction relationship was visualized by Cytoscape software [37].

2.8. qPCR Validation

The real-time quantitative polymerase chain reaction (qPCR) was carried out to confirm the expression of 9 randomly selected DEGs (significantly different expression) using the same RNA samples that were used in sequencing. First-Strand cDNA was synthesized using HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China) following the product manual. The gene-specific primers for q-PCR were designed by Primer Premier 5 (Table S1). The housekeeping *actin* gene of *B. xylophilus* was utilized as a reference gene. The cDNA products were amplified by SYBR Green Master Mix (Vazyme, Nanjing, China) on the ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each q-PCR was conducted in triplicate, and then relative gene expression levels were analyzed by the $2^{-\Delta\Delta C_t}$ method.

3. Results

3.1. Effect of *S. maltophilia* on the Virulence of *B. xylophilus*

At 3 dpi, thirteen *P. massoniana* seedlings inoculated with aseptic PWNs (Bx_a) began to lose water and turn brown; the infection rate was 54.17%, and the disease severity index (DSI) was 13.54. Twenty-one pine seedlings inoculated with the nematode with *S. maltophilia* (Bx_b) showed infection; the infection rate was 87.50%, and the DSI was 21.88. At 5 dpi, 23 pine seedlings inoculated with Bx_a showed symptoms of PWD; the infection rate was 95.83%, and the DSI was 23.96. All of the pines inoculated with Bx_b had symptoms of PWD; the infection rate was 100%, and the DSI was 25. At 7 dpi, all the seedlings of *P. massoniana* inoculated with Bx_a were infected, the infection rate was 100%, the DSI was 37.50; the infection rate of *P. massoniana* inoculated with Bx_b was 100%, and the DSI was 38.54. The *P. massoniana* inoculated with *S. maltophilia* alone did not show any symptoms (Figure 1, Table 1). The seedlings inoculated with Bx_b showed earlier and more visible symptoms than those inoculated with Bx_a. At this time, the number of PmBx_a was 6.74 times of the initial inoculation (Bx_a), and the number of PmBx_b was 7.91 times of the initial inoculation (Bx_b), but the difference between the two treatments was not significant (Figure 2). It is suggested that *S. maltophilia* could enhance the virulence of PWNs.

Figure 1. Disease phenotype of pine seedlings inoculated with aseptic *B. xylophilus* (Bx_a), *B. xylophilus* with *S. maltophilia* (Bx_b), or *S. maltophilia* alone (CK), at 0, 3, 5, and 7 days post inoculation (dpi), respectively.

Table 1. The degree of pine wilt disease after *P. massoniana* seedlings were inoculated with aseptic *B. xylophilus* (Bx_a), *B. xylophilus* with *S. maltophilia* (Bx_b), or *S. maltophilia* alone (CK), at 3, 5, and 7 days post inoculation (dpi), respectively.

Treatment	Infection Rates (%)			Disease Severity Index (DSI)		
	3 dpi	5 dpi	7 dpi	3 dpi	5 dpi	7 dpi
Bx_a	54.17	95.83	100	13.54	23.96	37.50
Bx_b	87.50	100	100	21.88	25	38.54
CK	0	0	0	0	0	0

Figure 2. Population of *B. xylophilus* at 7 days post inoculation (dpi). Aseptic *B. xylophilus* (PmBx_a), *B. xylophilus* with *S. maltophilia* (PmBx_b). The bars indicate S.D., and two samples with a common letter are not significantly different ($p > 0.05$) according to the Student's *t* test.

3.2. Transcriptome Sequencing and Differentially Expressed Genes

RNA sequencing of six independent *B. xylophilus* samples (three PmBx_a and three PmBx_b) was performed on an Illumina HiSeq platform to characterize the transcriptome and measure the differential expression of genes, generating 54.03 to 58.30 Mb of raw reads per sample. After quality filtering, 44.55 (76.43%) to 45.31 (83.78%) Mb of clean reads were obtained, with an average of 6.75 Gb bases. Subsequently, the clean reads were mapped to the *B. xylophilus* reference genome. Approximately 77.12–79.77% of reads matched the sequences in the genome, and 62.13–66.25% of the reads were uniquely mapped. When the clean reads mapped to reference genes, the total mapping ratio was 67.39–69.71%, and the unique ratio was 58.62–60.26% (Table 2). A total of 10,187 new transcripts were detected in the transcripts of the two treatments, of which 8359 were predicted to have coding potential, 7901 were predicted to be new transcripts of known genes with protein coding potential, 458 were new transcripts of new genes with protein coding potential, and 1828 were non-coding transcripts (Table 3). Expression levels of genes and transcripts were calculated using RSEM (RNA-Seq by Expectation Maximization). There were 4240 DEGs (absolute value of $\log_2$ fold change ≥ 1 and adjusted p -value ≤ 0.05) between the bacteria-treated and bacteria-free *B. xylophilus*, including 2763 downregulated and 1477 upregulated DEGs (Figure 3). The number of no-DEGs (absolute value of $\log_2$ fold change < 1 or adjusted p -value > 0.05) was 11,942.

Table 2. Statistical data for RNA sequencing.

Sample	Total Raw Reads (Mb)	Total Clean Reads (Mb)	Total Clean Bases (Gb)	Clean Reads Ratio (%)	Total Mapping Ratio (%)	Uniquely Mapping Ratio (%)
PmBx_a_1	58.30	44.55	6.68	76.43	77.12%	62.13%
PmBx_a_2	56.68	45.31	6.80	79.95	78.46%	62.67%
PmBx_a_3	55.06	44.57	6.69	80.96	79.77%	63.87%
PmBx_b_1	54.04	45.27	6.79	83.78	78.97%	65.49%
PmBx_b_2	54.03	45.2	6.78	83.66	79.72%	66.25%
PmBx_b_3	54.04	45.13	6.77	83.53	79.51%	66.12%

Table 3. New transcripts in the transcriptomes of aseptic *B. xylophilus* and *B. xylophilus* with *S. maltophilia* after inoculation.

Total Novel Transcript	Coding Transcript	Noncoding Transcript	Novel Isoform	Novel Gene
10,187	8359	1828	7901	458

Figure 3. Volcano-Plot of differentially expressed genes (DEGs) of pine wood nematodes in aseptic condition and with *S. maltophilia*. The number of upregulated DEGs ($\log_2$ fold change ≥ 1 , adjusted p -value ≤ 0.05) is 1477, the number of downregulated DEGs ($\log_2$ fold change ≤ -1 , adjusted p -value ≤ 0.05) is 2763, the number of no-DEGs (absolute value of $\log_2$ fold change < 1 or adjusted p -value > 0.05) is 11,942.

3.3. qPCR Verification of Differentially Expressed Genes

Nine genes (7 upregulated genes and 2 downregulated genes) with high expression differences were randomly selected from PmBx_a and PmBx_b transcripts for qPCR verification. The expression trends of gene transcripts were consistent with the results of qPCR verification, indicating that the sequencing data were reliable (Figure 4).

Figure 4. Relative expression of differentially expressed genes of *B. xylophilus* in aseptic condition (PmBx_a) or with *S. maltophilia* (PmBx_b). Relative expression of differentially expressed genes by sequencing (A) and the real-time quantitative polymerase chain reaction (qPCR) (B), the bars indicate S.D.

3.4. Characterization of Differentially Expressed Transcripts

All the transcripts were further functionally characterized into three GO categories: biological process, cellular component, and molecular function. Among the set of 4240 DEGs, 356 genes were associated with at least one GO term, and a total of 50 different GO terms were found. Twenty-four GO terms were involved in biological processes, 16 GO terms were associated with cellular components, and 10 GO terms had various molecular functions (Figure 5). Among the biological process terms, the cellular process, single-organism process, and metabolic process terms were the three terms containing the greatest number of DEGs. In the cellular component category, the top three GO terms were cell, cell part, and membrane, and for the molecular function category, catalytic activity, binding, and transporter activity were the top three terms (Figure 5).

Figure 5. GO categories of differentially expressed genes of *B. xylophilus* in aseptic condition and with *S. maltophilia*.

The putative functions of DEGs were also analyzed with KEGG classification. The 871 DEGs could be mapped to 301 different pathways that might be affected by *S. maltophilia*. KEGG Orthology (KO) analysis showed that most DEGs were assigned to human diseases, metabolism, and organismal systems. In the human diseases class, “cancers: overview”, “endocrine and metabolic diseases”, and “infectious diseases: bacterial” had many DEGs. “Global and overview maps”, “lipid metabolism”, “carbohydrate metabolism”, and “amino acid metabolism” were the dominant terms in the metabolism class. Few transcripts were assigned to “xenobiotic biodegradation and metabolism” (23 transcripts) and “metabolism of terpenoids and polyketides” (6 transcripts) (Figure 6). This result showed that

S. maltophilia mainly affected the growth and development of nematodes. In the organismal systems category, “immune system”, “endocrine system”, and “digestive system” had more transcripts. The top five enriched groups among the KEGG categories included signal transduction, cancers: overview, global and overview maps, cellular community-eukaryotes, and immune system.

Figure 6. Kyoto Encyclopedia of Genes and Genomes (KEGG) classifications of differentially expressed genes of *B. xylophilus* in aseptic condition and with *S. maltophilia*.

A list of the top 30 pathways and number of transcripts mapped to those pathways are shown in Figure 7. The most highly active pathway was cancer (ko05200), with 72 active transcripts, followed by protein digestion and absorption (ko04974, 59 transcripts). A number of signaling pathways that are known to be involved in development and diverse functions were active in PmBx_a, such as the wnt signaling pathway (ko04310), rap1 signaling pathway (ko04015), PI3K-Akt signaling pathway (ko04151), cAMP signaling pathway (ko04024), cGMP-PKG signaling pathway (ko04022), ECM-receptor interaction, MAPK signaling pathway (ko04010), and calcium signaling pathway (ko04020). Thus, it can be concluded that *S. maltophilia* affects the immune system of *B. xylophilus*.

Figure 7. The top 30 pathways of differentially expressed genes of *B. xylophilus* between aseptic condition and with *S. maltophilia*.

3.5. Nematode Response Gene Network

The 4240 DEGs were compared to the STRING database, 2196 of which could be mapped to 2007 protein clusters (Table S1). Cytoscape software was used to analyze and visualize these DEGs, resulting in many interaction networks (Figure 8). The genes central to a highly connected network have been shown to be more likely to have essential functions. Thus, we hypothesized that genes central to the identified network would be fundamental to the *B. xylophilus* response to the host pine seedlings. Table 4 lists the top 20 central genes (hubs) in the network and the number of genes connected to the listed gene. There were 238 genes encoding proteins interacting with the gene *BUX.s01653.391* (polyubiquitin). Second, genes *BUX.s00397.130* and *BUX.s00397.132* encoded the leucine-rich repeat serine/threonine protein kinase and interacted with 116 different genes. Other genes, including *BUX.s01281.46* (Glyceraldehyde-3-phosphate dehydrogenase), *BUX.s00138.1* (DNA topoisomerase 2), *BUX.s00422.590* (1-phosphatidylinositol 3-phosphate 5-kinase), *BUX.s00813.122* (Acetyl-CoA carboxylase), five heat shock protein genes *BUX.s00116.357*, *BUX.s00961.158*, *BUX.s01281.74*, *BUX.s00422.612*, *BUX.s01644.6*, and two myosin genes *BUX.s01281.438* and *BUX.s00083.40*, also interacted with many different DEGs (Table 4).

Figure 8. Interactions of proteins encoded by differentially expressed genes of *B. xylophilus* between aseptic condition and with *S. maltophilia*. Protein interactions of all differentially expressed genes (A); enlarged view of protein interactions (B). The larger the circle and the redder the color, the more proteins interacted with the protein, and the smaller and yellow the fewer interactions.

Table 4. Genes that interacted with many differentially expressed genes of *B. xylophilus* between aseptic condition and with *S. maltophilia*.

Gene ID	N	Predicted Protein
BUX.s01653.391	238	Polyubiquitin
BUX.s00397.130	116	Leucine-rich repeat serine/threonine-protein kinase
BUX.s00397.132	116	Leucine-rich repeat serine threonine-protein kinase 1
BUX.s01281.46	99	Glyceraldehyde-3-phosphate dehydrogenase
BUX.s00138.1	92	DNA topoisomerase 2
BUX.s00422.590	87	1-phosphatidylinositol 3-phosphate 5-kinase
BUX.c03835.1	86	NA
BUX.s00813.122	86	Acetyl-CoA carboxylase
BUX.s01109.1	84	DNA-directed RNA polymerase I subunit RPA1
BUX.s01254.380	80	Neur_chan_LBD domain-containing protein
BUX.s01198.7	75	Glutamyl(E) Amino-acyl tRNA Synthetase
BUX.s00364.24	74	cell differentiation family, Rcd1
BUX.s00116.357	73	Heat shock protein 70
BUX.s00961.158	73	Heat shock protein 70
BUX.s01281.74	73	Heat shock protein 70
BUX.s00422.612	72	Heat shock protein 75 kDa, mitochondrial
BUX.s01281.437	72	Myosin
BUX.s01281.438	72	Myosin
BUX.s00083.40	71	NA
BUX.s01644.6	70	Heat Shock Protein 110

Note: number of DEGs interacting with this gene (N).

3.6. Differential Expression of Virulence Genes in *B. xylophilus*

According to the annotation information, a series of virulence genes were found to be differentially expressed in PmBx_a and PmBx_b (Table 5). Four genes were related to cell wall hydrolysis (including the pectinase gene and cellulase gene), one of which was upregulated, and the other three were downregulated. Thirty-Six detoxification-related genes (including the cytochrome P450 gene, toxic anaphylactic protein gene, glutathione S-transferase gene, ABC transporter gene, retinol dehydrogenase gene, carboxylesterase gene, etc.) were differentially expressed, including ten upregulated and twenty-six downregulated genes. Ten cathepsin L-cysteine protease genes were differentially expressed, including five upregulated and five downregulated genes. In addition, an effector gene (*BxSapB2*) of PWN was upregulated.

Table 5. Differentially expressed genes associated with virulence of *B. xylophilus* in *P. massoniana* inoculated with *B. xylophilus* alone (PmBx_a), or *B. xylophilus* with *S. maltophilia* (PmBx_b).

Gene ID	log ₂ Fold Change (PmBx_b/PmBx_a)	Up/Down-Regulation
	Pectate lyase	
BUX.s01259.20	−4.074762625	Down
BUX.s01259.23	−4.182790139	Down
BUX.s01259.21	−4.510202593	Down
	Cellulase	
BUX.s01066.64	4.618501404	Up
BUX.s01288.37	−4.09111163	Down
	Glutathiones-transferase	
BUX.c09083.1	1.669839508	Up
BUX.s01513.258	−1.139784616	Down
BGL_novel_G000432	−1.250104347	Down

Table 5. Cont.

Gene ID	log ₂ Fold Change (PmBx_b/PmBx_a)	Up/Down-Regulation
Cytochrome P450, CYP		
BUX.s01661.6	1.557847634	Up
BUX.s01144.121	−1.000995452	Down
BUX.s00116.502	−1.063038854	Down
BUX.s00116.734	−1.208684047	Down
BUX.s00298.246	−1.442207328	Down
BUX.s00116.879	−1.480500102	Down
BUX.s00460.194	−1.575995247	Down
BUX.s01038.7	−1.821369766	Down
ABC transporter		
BUX.s01254.42	−2.29841047	Down
Venom allergen-like protein VAP1		
BUX.s00579.80	1.052640318	Up
BGI_novel_G000391	−1.028089184	Down
BUX.s00116.787	−1.218428765	Down
BUX.s01147.77	−1.646314785	Down
Retinol dehydrogenase		
BUX.s00579.178	1.316943271	Up
BUX.s00974.41	−1.492689553	Down
Carboxylesterase		
BUX.s01226.30	1.181734176	Up
BUX.s00713.1002	1.17453585	Up
BUX.s00579.243	1.135072687	Up
BUX.s01145.139	−1.065237837	Down
BUX.s00364.64	−1.137734342	Down
BUX.s00110.90	−1.143247115	Down
BUX.s01147.205	−1.666055127	Down
BUX.s00983.9	−2.183381707	Down
BUX.s00110.48	−2.753293911	Down
UDP-glucuronosyl transferase, UGT		
BUX.s00460.320	1.796726932	
BUX.s00139.166	1.068078732	Up
BUX.s00351.21	1.057762705	Up
BUX.s00649.18	−1.186354129	Down
BUX.s01438.42	−1.193137892	Down
BUX.s00116.87	−1.341914185	Down
BUX.s01092.146	−1.490960439	Down
BUX.s01109.36	−1.580165084	Down
BUX.s00460.69	−2.061031941	Down
BxSapB2		
BUX.s01063.105	1.016870986	Up
Cathepsin L-like cysteine protease		
BUX.s01656.41	6.784683942	Up
BGI_novel_G000167	3.834868069	Up
BUX.s00813.51	3.395018418	Up
BUX.s00813.52	3.153518163	Up
BUX.s00116.604	2.076060588	Up
BUX.s00497.2	−1.048755246	Down
BUX.s01288.15	−1.301409481	Down
BGI_novel_G000040	−1.423476978	Down
BUX.s00713.1009	−2.220301991	Down
BUX.s00713.1011	−2.901975575	Down

4. Discussion

S. maltophilia is a kind of Gram-negative bacteria that is widely distributed in nature [38]. This species is also widely found in different PWN strains, and its relative abundance is high [15,17,19].

He et al. found that wild-type three-year-old *P. massoniana* inoculated with nematodes containing *S. maltophilia* wilted faster than those inoculated with aseptic pine wood nematodes [20]. Since wild pine carries a variety of microorganisms, four-month-old aseptic *P. massoniana* seedlings were used for the inoculation experiment in our study. The results provided more direct evidence that *S. maltophilia* can enhance the reproduction and virulence of *B. xylophilus*.

To explore the mechanism of the effect of *S. maltophilia* on PWNs in PWD, transcriptome sequencing of aseptic PWNs and those carrying *S. maltophilia* after inoculation was carried out. The results showed that 4240 genes were differentially expressed between the two treatments, including 1147 upregulated genes and 2763 downregulated genes. Compared with the transcriptomes of aseptic PWNs and the PWNs carrying *S. maltophilia* cultured on *B. cinerea*, which have 891 significantly DEGs, including 61 upregulated genes and 830 downregulated genes [21], the number of DEGs in the comparative transcriptome of aseptic PWNs and PWNs with *S. maltophilia* in pines was much higher. This suggests that different habitats could affect the gene expression of PWN, which is also reflected in the studies of Qiu et al. [39] and Tsai et al. [40].

Furthermore, the potential functions of DEGs in the comparative transcriptome of PWNs after inoculation were analyzed with gene ontology (GO) enrichment. The GO functional analysis showed that the DEGs were significantly enriched in the cell membrane, transport activity, and metabolic process. These GO classes are believed to be related to the digestion and innate immunity of nematodes [41,42]. White et al. studied the interaction between *Caenorhabditis elegans* and *S. maltophilia* and found that the DEGs were significantly enriched in these GO categories, such as components of membrane, transport, oxidation, and reduction and metabolic process [43]. It was suggested that several DEGs in the comparative transcriptome of PWNs after inoculation might be related to the immunity of PWN.

KEGG classification was also performed to estimate the functions of the DEGs. We found that the DEGs between two PWN treatments were enriched in the Wnt, Rap1, PI3K Akt, cAMP, cGMP PKG, ECM-receptor, MAPK, and calcium signaling pathways. It has been reported that ECM-receptor interaction, Wnt, PI3K Akt, and MAPK signaling pathways play immunomodulatory roles when organisms interact with bacteria or fight against other stresses [44–47]. In the interaction between *Meloidogyne incognita* and bacteria, the expression of many genes involved in Wnt, PI3K Akt, MAPK, and other immune-related pathways, as well as cAMP, cGMP PKG, calcium, and other pathways changed significantly [48,49]. Compared with aseptic PWNs, it could be considered that the PWNs carrying bacteria changed significantly in various immune regulatory pathways after being inoculated pines. This also meant that *S. maltophilia* might affect the immune process of *B. xylophilus* in infected *P. massoniana*.

According to the analysis of the protein interactions of the DEGs, a number of key genes emerged from our study, including polyubiquitin gene, leucine rich repeated serine/threonine protein kinase gene, glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), acetyl CoA carboxylase gene, and heat shock protein gene. One of these genes, Acetyl CoA carboxylase gene of *B. xylophilus* is related to the propagation, migration, and movement behavior of *B. xylophilus* [50,51]. For the HSP genes, the proteins they encode are conserved and related to biotic and abiotic stresses [52,53]. Following interference with the HSP70 gene, the reproductive ability and amount of oviposition of *B. xylophilus* decrease [54]. The Hsp90 gene of *B. xylophilus* is necessary for maintaining body temperature and ensuring reproduction in nematodes [55]. However, little is known about other HSP genes of *B. xylophilus*, such as the Hsp 75 and Hsp 110 genes. Similarly, the functions of the ubiquitin genes, leucine-rich repetitive serine/threonine protein kinase genes, and GAPDH genes of *B. xylophilus* still need to be studied and verified. The ubiquitination pathway of nematodes could help them resist the infection of pathogenic factors [56–59]. Two ubiquitin genes of *B. xylophilus* were also cloned, but their functions remain to be further studied [60,61]. Leucine-rich repetitive serine/threonine protein kinase genes are often associated with plant resistance [62–64]. The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) is a gene with a wide range of functions, including gene expression control, membrane transport, cell signal transmission, interaction with RNA and other proteins, and avoidance of host

immunity [65]. It was speculated that these key genes might affect the survival, reproduction, immunity, and many other processes of *B. xylophilus*, although their characterization remains to be explored.

In addition, many cell wall degrading enzyme genes, detoxification-related genes, and virulence-related genes, for example, pectate lyase genes, cellulase genes, cytochrome p450 genes, and venom allergen-like protein genes, were differentially expressed under the two nematode treatments in our study. Cellulose, hemicellulose, and pectin are important components of the plant cell wall. *B. xylophilus* can secrete pectinase, and cellulase allows the colonization and migration of PWNs in pine [66–69]. Cytochrome P450 genes were associated with the vitality, reproduction, virulence, and detoxification of *B. xylophilus* [70,71]. The venom allergen-like protein gene was related to the migration, parasitism, and virulence of *B. xylophilus* [72]. The silencing of cathepsin L-cysteine protease genes in *B. xylophilus* reduces the feeding, reproduction, and virulence of *B. xylophilus* [73]. The glutathione S-transferase gene, ABC transporter gene, uridine diphosphate glucose transferase related gene, retinol dehydrogenase gene, carboxylesterase gene, etc., are also related to the reproduction and virulence of nematodes [39]. The number of these DEGs in nematodes after inoculation of pine was much greater than that in nematodes cultured on *B. cinerea* [21]. Therefore, it can be concluded that *S. maltophilia* may affect the invasion, parasitism, and virulence of *B. xylophilus*, whether on *B. cinerea* or in trees.

5. Conclusions

One of the most dominant bacteria in the *B. xylophilus*, *S. maltophilia*, can promote the propagation of nematodes and cause pine wilt disease faster. At the level of transcription, we found that 4240 genes were differentially expressed between aseptic *B. xylophilus* and *B. xylophilus* containing *S. maltophilia* after inoculation of aseptic *P. massoniana*. These differentially expressed genes were significantly enriched in some immune-related GO categories and KEGG pathways. The polyubiquitin-rich gene, leucine-rich repeat serine/threonine-protein kinase gene, and heat shock protein genes, etc., may play the key roles on immune resistance. In addition, *S. maltophilia* also make four cell wall hydrolase genes, and several detoxification- and pathogenesis-related protein genes differentially expressed. These results indicate that *S. maltophilia* can affect the expression of some pathogenesis- and immune-related genes, thus enhancing the virulence of *B. xylophilus* and aggravating the pine wilt disease.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4907/11/9/908/s1>, Table S1: Interaction between differentially expressed genes and proteins.

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