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Molecular Characterization and Functional Analysis of GPCR Gene *Bx-srh-1* in Pinewood Nematode (*Bursaphelenchus xylophilus*)

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Abstract: *Bursaphelenchus xylophilus*, also known as the pine wood nematode (PWD), which causes pine wilt disease (PWD), is one of the most devastating diseases affecting pine forests globally. G protein-coupled receptors (GPCRs) are crucial in many biological processes and serve as privileged points of communication between cells and the surrounding environment. α-pinene has been found to play a crucial role in combating the infection, colonization, and early stages of pathogenesis caused by *B. xylophilus*. In this study, we investigated the molecular characteristics and biological functions of the GPCR gene *Bx-srh-1* in *B. xylophilus*. Fluorescence in situ hybridization (FISH) was performed to determine the spatial expression patterns of *Bx-srh-1* in *B. xylophilus*. The results indicated that *Bx-srh-1* is expressed in the intestine and subcutaneous tissues of J2 and J3 juveniles and in the spicules of adult males and vulvae of adult females. RNA interference (RNAi) was used to analyze *Bx-srh-1* gene function, and we examined the expression patterns of *Bx-srh-1* in *B. xylophilus* under α-pinene stress. The RNA interference indicated that *Bx-srh-1* was involved in the reproductive ability and pathogenicity of *B. xylophilus*; the expression levels of *Bx-srh-1* significantly increased after the exposure to α-pinene for 12 h, and they peaked at 48 h. Silencing *Bx-srh-1* may therefore lead to a reduction in *B. xylophilus* reproduction and pathogenicity. These results demonstrate that *Bx-srh-1* is related to the feeding behavior, reproduction, pathogenicity, and resistance to α-pinene process of *B. xylophilus*.

Keywords: *Bursaphelenchus xylophilus*; pine wilt disease; G protein-coupled receptors; reproduction; RNAi

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1. Introduction

Bursaphelenchus xylophilus (Steiner and Buhrer) Nickle, commonly referred to as the pinewood nematode (PWN), is a highly menacing endoparasitic plant nematode. This nematode, which is indigenous to North America, is responsible for the onset of pine wilt disease (PWD), leading to significant economic and ecological damages in pine forests. [1,2]. Under field conditions, PWNs are transmitted by the *Monochamus* beetle, which emerges from dead pine trees to eat young, succulent, and healthy pine tree branches. When *Monochamus* beetles feed, they cause wounds and provide entry portals (infection courts) for the PWN. Upon penetration of the tree, the PWN proceeds to consume the plant epithelial cells located in the cortex and xylem tissues, thereby disseminating throughout the tree via tissue migration during the phytophagous phase. This ultimately culminates in the manifestation of wilting symptoms, ultimately resulting in the demise of the pine tree. [3]. *B. xylophilus* poses a significant threat to pine forests worldwide, resulting in substantial damage to these forests in North America, Europe, and East Asia [4–9]. In China, this disease was first identified in Nanjing City, Jiangsu Province, in 1982 and has since spread rapidly to 19 provinces, encompassing 731 county administrative regions and

covering an area of 650,000 ha [10–12]. Despite extensive research on the pathogenicity, biological properties, infection cycle, and vector insects [13–17], the high mortality rate of host trees and the rapid spread of the disease continue to pose a significant challenge.

G protein-coupled receptors (GPCRs) constitute the largest family of transmembrane ™ receptors, playing a crucial role in various physiological processes [18]. Prior research has demonstrated the involvement of GPCRs in innate/adaptive immunity, environmental signal detection, and pathogen defense in the model nematode *Caenorhabditis elegans* [19–21]. The prefix "*sr*" designates over 1300 potential chemoreceptor genes identified in *C. elegans*, which belong to the receptor superfamilies *Sra* (*Sra*, *Srb*, *Srab*, *Sre*) and Str (*Srh*, *Str*, *Sri*, *Srd*, *Srj*, *Srm*, *Srn*), and *Srg* (*Srx*, *Srt*, *Srg*, *Sru*, *Srv*, *Srxa*) [22]. Some researchers have identified ~1059 *C. elegans* neuropeptide G-protein coupled receptor (Ce-NP-GPCR), a genome-wide prediction of homologous genes in 10 key parasitic nematodes covering a wide range of phylogenetic clades and lifestyle types [23]. However, there are still only a few studies on *B. xylophilus* with GPCRs. Previously, it was demonstrated that *B. xylophilus* responds to low temperatures through GPCRs; thus, GPCR genes play a crucial role in abiotic stress adaption [24]. Other researchers have found that GPCRs are extremely important for the locomotion, early ontogenesis, and mating behavior in *B. xylophilus* [25]. Therefore, GPCRs are extremely important in *B. xylophilus.* However, it is unknown whether they are involved in the host defense and *B. xylophilus* invasion.

Monoterpenes are important part of terpenoids produced by pine trees, which can provide resistance to external pathogen invasion [26]. The monoterpene secreted by pine trees are largely α-pinene [27], and α-pinene has low toxicity to *B. xylophilus* [28]. Previous studies have proved that low concentration of α-pinene could reduce the reproduction of *B. xylophilus*. The detoxification-related and resistance-related genes of *B. xylophilus* are upregulated when *B. xylophilus* is exposed to α-pinene [29,30]. However, it is unknown whether GPCRs are involved under the stress of α-pinene.

In order to delineate the expression pattern and biological functions of GPCRs in *B. xylophilus*, we cloned the GPCR gene *Bx-srh-1*. Subsequently, we conducted an investigation into the molecular characteristics and biological functions of *Bx-srh-1*. To determine the spatial expression patterns of *Bx-srh-1* in *B. xylophilus*, we utilized fluorescence in situ hybridization (FISH). The identification of *Bx-srh-1* with altered expressions induced by α-pinene was accomplished through the utilization of real-time quantitative reverse-transcription PCR. The objective of this study is to examine the role of *Bx-srh-1* in the reproduction, pathogenicity, and defense against α-pinene stress of *B. xylophilus*.

2. Materials and Methods

2.1. Biological Materials

The highly virulent isolate QH-1 of *B. xylophilus* [31], obtained from infected *P. koraiensis* in Liaoning, China, was cultured on *Botrytis cinerea* Pers. (1794) fungus grown on barley grains at 25 °C for two weeks [32]. The Baermann funnel technique was employed at room temperature to extract mixed stages of nematodes [33], which were subsequently washed using 1 × M9 buffer for the experiments. The counting methods of *B. xylophilus* were based on Wang's methods [34]. After the nematode suspension was collected from cultured *B. cinerea*, the volume of nematode suspension was adjusted to 10 mL with 1 × M9 buffer and mixed. Next, the nematode suspension was retrieved to the counting dish by gentle suction. The number of nematodes in the suspension was counted using stereomicroscope (Olympus, Tokyo, Japan). Four-year-old *P. koraiensis* seedlings were grown in a nursery situated as previous methods [31].

2.2. RNA Extraction and cDNA Synthesis

The nematodes were subjected to RNA extraction using the Tissue/Cell Total RNA Mini Kit (GeneBetter, Beijing, China) in accordance with the manufacturer's guidelines. The total RNA concentration was determined at 260/280 nm using an ultraviolet spectrophotometer (NanoDrop ND-2000, Thermo Fisher, Waltham, MA, USA), and its qual-

ity was assessed via electrophoresis on a 1% agarose gel. Subsequently, the RNA was reverse-transcribed to cDNA using the Scripit III RT Kit with gDNA Eraser (GeneBetter, Beijing, China).

2.3. Bx-srh-1 Gene Cloning

Utilizing transcriptome data from WormBase ParaSite (http://parasite.wormbase.org/index.html, (accessed on 10 October 2022)), the *Bx-srh-1* sequence was obtained for the *B. xylophilus* genome. Subsequently, a specific primer pair (Bx-srh-1-F/R) was designed to target the complete *Bx-srh-1* via polymerase chain reaction (PCR) from cDNA reverse-transcribed from *B. xylophilus* mRNA (Table S1). The PCR protocol begins with 94 °C pre-denaturation for 5 min, then 35 cycles of 28 °C denaturation for 30 s each, 56 °C annealing for 30 s, 72 °C extension for 2 min, and 72 °C extension for 10 s each. The amplified PCR products were subjected to electrophoresis on 1% agarose gels for confirmation and subsequently purified using the Fast-pure Gel DNA Extraction Mini Kit protocol (Vazyme, Nanjing, China). The purified PCR products were then cloned into the pUC57 Vector (TsingKe, Beijing, China) and transformed into competent cells of *Escherichia coli* Trans1-T1 (TsingKe, Beijing, China). We incubated a transformed *E. coli* culture overnight at 37 °C on Luria-Bertani (LB) plates containing ampicillin. Positive transformants were identified using the M13F/R primers by PCR (Table S1).

2.4. Sequence Analysis of Bx-srh-1

In this study, NCBI tools were employed for amino acid prediction and homology analysis, while MEGA7 with the N-J method was utilized to construct the phylogenetic tree for the protein encoded by *Bx-srh-1*. To ensure the reliability of the phylogenetic tree, a self-expansion test was conducted by repeating the sampling test 1000 times. Furthermore, the molecular weight and other molecular function of each protein were predicted through the bioinformatics website ExPASy (SIB Swiss Institute of Bioinformatics, Zurich, Switzerland, https://www.expasy.org/, (accessed on 15 October 2022)). To analyze the signal peptide, transmembrane structure, and tertiary structure of the proteins, the ExPASy, SMART (http://smart.embl-heidelberg.de/, (accessed on 15 October 2022)), TMHMM (Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby Denmark, https://services.healthtech.dtu.dk/services/TMHMM-2.0/, (accessed on 15 October 2022)), and SWISS-MODEL programs (Swiss Institute of Bioinformatics Biozentrum, University of Basel Spitalstrasse, Switzerland, https://swissmodel.expasy.org/, (accessed on 15 October 2022)) were utilized.

2.5. Fluorescence In Situ (FISH)

To determine the spatiotemporal expression patterns of *Bx-srh-1*, mRNA in situ hybridization was performed. A red fluorescence-labeled probe was synthesized from the cloned sequence of *Bx-srh-1*, as outlined in Table S1. The FISH in situ hybridization kit C007 (Gefan, Shanghai, China) was utilized following the manufacturer's instructions, with the sense probe (Table S1) serving as the negative control. Visualization and capture of the samples were conducted using a Zeiss Axio Image M2 microscope (Zeiss, Oberkochen, Germany).

2.6. RNA Interference

By using RNA interference technology, we investigated *Bx-srh-1*'s function in vitro, and green fluorescent protein (GFP) was performed using the T7 RNAi Transcription Kit (Vazyme, Nanjing, China) with the following primers: *Bx-srh-1*T7-F/R, *Bx-srh-1*T7-R/F, and *Bxgfp*-F/R (Table S1). The soaking method was used to analyze the nematodes at mixed developmental stages, as per previous studies [34]. For dsRNA treatment, nematodes were soaked in ddH_2O containing 800 ng/L of dsRNA with intermittent stirring (25 °C, 180 rpm) for 48 h. The control group nematodes were soaked in ddH_2O. After soaking, nematodes were collected by centrifuging and were then thoroughly washed with ddH_2O for subsequent experiments.

2.7. Quantitative Real-Time PCR (qRT-PCR)

The qRT-PCR (20 µL) was performed to detect the gene expression levels using the Taq Pro Universal SYBR qPCR Master Mix for qPCR (Vazyme, Nanjing, China) and CFX96TM Real-Time system (Bio-Rad, Hercules, CA, USA). Primers were designed with Primer Premier 5 software (Premier Biosoft International, San Francisco, CA, USA) for the target gene *Bx-srh-1* and reference gene *Actin* (Table S1).

The three-step reaction procedure for qRT-PCR was executed with the following parameters: denaturing stage at 95 °C for 30 s, PCR stage consisting of 40 cycles of 95 °C for 10 s and 60 °C for 30 s, melting stage at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 10 s, and cooling stage at 50 °C for 30 s. The experiment was conducted with three biological and technical replicates for each sample. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [35].

2.8. Assay for Feeding, Reproduction, and Pathogenicity after RNAi

In order to assess the impact of RNA interference (RNAi) on the reproductive capacity of *B. xylophilus*, approximately 100 nematodes subjected to RNAi treatment were cultivated on potato dextrose agar (PDA) that had been overgrown with *Botrytis cinerea* at a temperature of 25 °C. Daily observations and photographic documentation were made of the nematodes' feeding behavior. After a period of nine days, the nematodes were removed from the cultures, counted, and used to calculate the rate of reproduction. Additionally, to examine the effects of RNAi on the pathogenicity of *B. xylophilus*, 2000 nematodes that had undergone *dsBx-srh-1* treatment were inoculated into four-year-old *P. koraiensis* seedlings. A control group consisting of nematodes soaked in ddH_2O was utilized, while negative controls were treated with *dsgfp*. The infective ability was periodically monitored, and the severity of disease on *P. koraiensis* was classified into five stages (0, I, II, III, and IV) using Xiang's methodology [36]. The experiment was conducted with ten biological replicates. The mortality rate was calculated using the following formula:

$$\text{Motality rate} = \frac{\Sigma\text{Number of dead plants}}{\text{Total number of plants}} \times 100\%$$

2.9. Analysis of Expression Levels of Bx-srh-1 in B. xylophilus under Abiotic Stress

Li's method was employed to administer treatment for a brief duration [29]. Specifically, 500 nematodes were subjected to soaking in a 200 µL solution of α-pinene (56.33 mg/mL) dissolved in 0.5% (*w*/*w*) Triton X-100 and ddH_2O for 6, 12, 24, and 48 h, while Triton X-100 and ddH_2O were utilized as controls. Each treatment was replicated thrice biologically. Following the washing of nematodes from the solution, RNA extraction was carried out, and cDNA was synthesized. Subsequently, gene expression levels of *Bx-srh-1* were determined by quantitative real-time PCR using specific primers (Table S1).

2.10. Data Analysis and Processing

The mean and standard deviation (SD) of triplicate measurements for each group were calculated using Microsoft Excel software (Office excel 2016, Microsoft Corp., Redmond, WA, USA). Statistical analysis was conducted using SPSS17.0 (SPSS Inc., Chicago, IL, USA) and Origin8.0 (OriginLab, Northampton, MA, USA). Significance was determined at $p < 0.05$ using one-way ANOVA with appropriate *t*-tests.

3. Results

3.1. Alignment and Phylogenetic Analysis of Bx-srh-1

PCR amplification of the *Bx-srh-1* sequence was accomplished with success, as depicted in Figure S1. Subsequent sequence analysis revealed that the PCR product encompasses 1080 base pairs, encompassing a full open reading frame (ORF) that encodes 359 amino acids. The coding sequence for *Bx-srh-1* was deposited in the NCBI GenBank, with an assigned accession number of OQ383310. Phylogenetic analysis revealed that the protein

Bx-SRH-1 is relatively conservative in its evolution between plant parasitic nematodes (Figure 1B). Bx-SRH-1 shares a 99% homology with SRH1 from *Bursaphelenchus okinawaensis*. We found that Bx-SRH-1 is not closely related to free-living nematode species (*Caenorhabditis elegans* and *C. briggsae*). The results of the conserved domain analysis by SMART indicated that Bx-SRH-1 belongs to the *srh* family of chemoreceptors of G protein-coupled receptors (GPCRs) (Figure 1C). We analyzed the physicochemical properties of the Bx-SRH-1 protein on the ExPASy, and the results showed that the molecular weight of Bx-SRH-1 is 40.819 kDa, with isoelectric points (pI) of 9.25. Based on the prediction of the signal peptides and transmembrane and tertiary structures, the grand average of the hydropathicity (GRAVY) of Bx-SRH-1 was 0.502, it had no signal peptides, and it had seven transmembrane regions (Figure 1D,E), indicating that Bx-SRH-1 is a typical transmembrane protein.

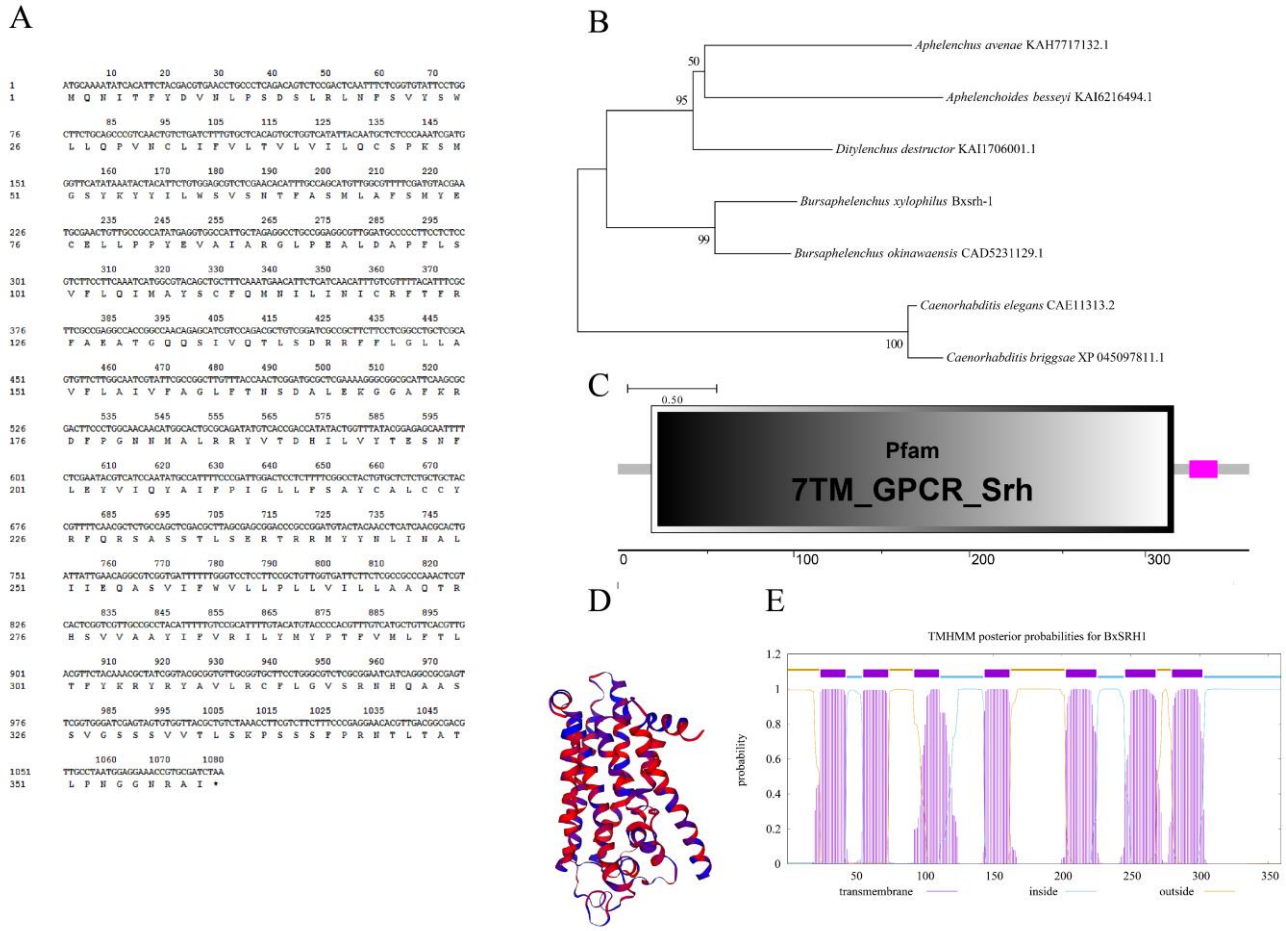

Figure 1. Cloning and sequence analysis of *Bx-srh-1* in *Bursaphelenchus xylophilus*. (**A**) Coding sequences and deduced amino acid sequences of *Bx-srh-1*. (**B**) Phylogenetic relationships of Bx-SRH-1 proteins. Accession numbers of protein sequences are listed in Table S2. (**C**) Results of conserved domain analysis. Domain analysis by SMART Search indicated *Bx-srh-1* belongs to 7TM_GPCR_Srh domain. (**D**) Tertiary structure models of Bx-SRH-1. (**E**) Prediction of transmembrane helices in Bx-SRH-1.

3.2. Gene Expression Pattern of Bx-srh-1 in Bursaphelenchus xylophilus

The spatiotemporal expression of *Bx-srh-1* was identified using FISH. Hybridization signals were observed in both juvenile (J2 and J3) and adult nematodes. In the adult stage, the hybridization signals were predominantly detected in the intestine and subcutaneous tissues, with a notable increase in expression near the spicules of adult males and vulvae of adult females. Specifically, the hybridization signals were observed in close proximity to the spicules of adult males and vulvae of adult females (as depicted in Figure 2A,C).

The hybridization signals were also detected in the intestine and subcutaneous tissues of J2 and J3 juveniles, as illustrated in Figure 2B,D. Conversely, no hybridization signals were observed in the negative controls, as depicted in Figure 2E. It is speculated that that *Bx-srh-1* could play important roles in the sex specificity in adult nematodes and in the feeding in juvenile nematodes.

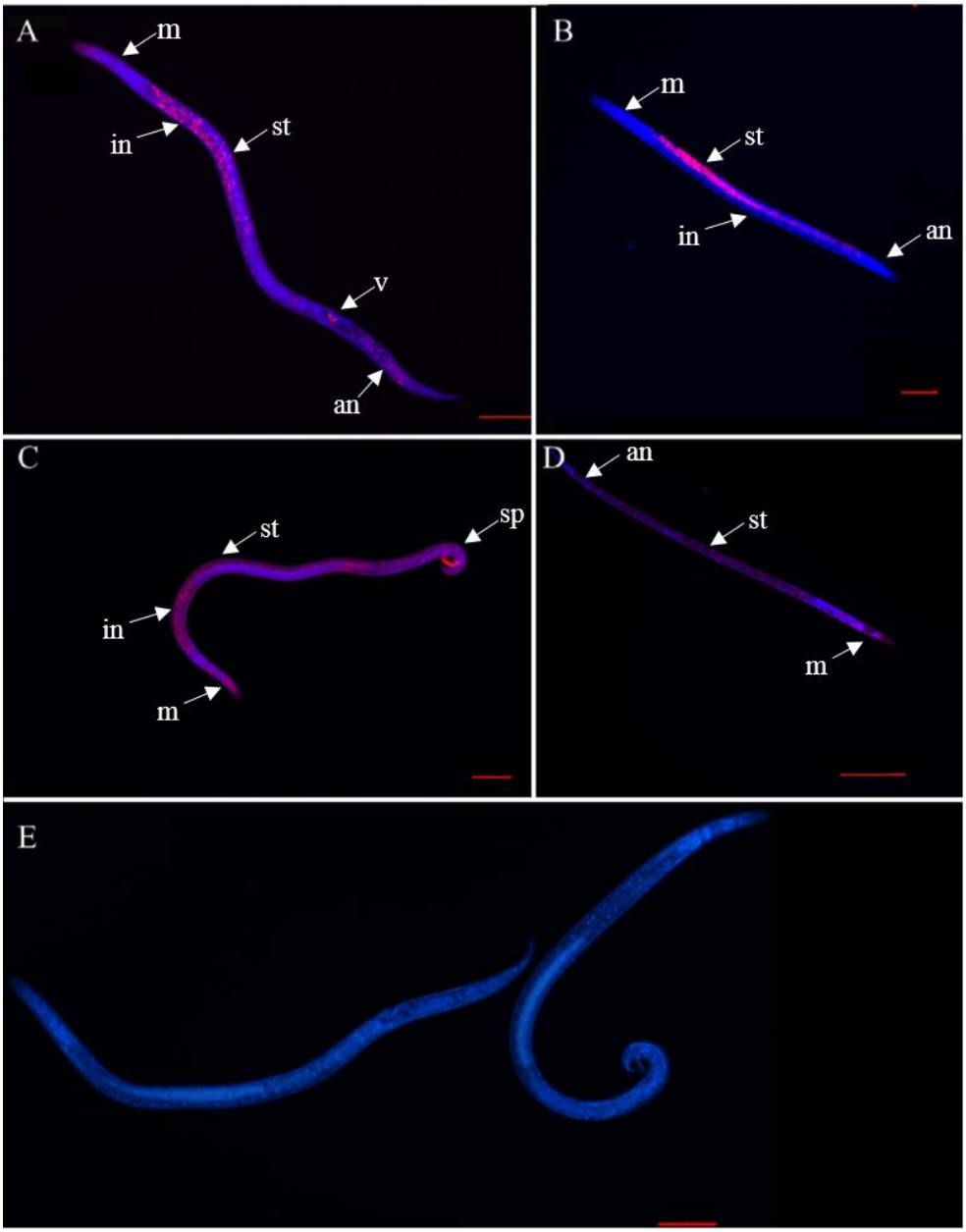

Figure 2. Localization of *Bx-srh-1* in *B. xylophilus* determined through fluorescence in situ hybridization: (**A**) Female adult; (**B**) J2 juvenile; (**C**) Male adult; (**D**) J3 juvenile; (**E**) Negative control. m: metacorpus; an: anus; v: vulva; sp: spicules; in: intestine; st: subcutaneous tissue. Scale bar = 20 μm (**B**,**D**) and 50 μm (**A**,**C**,**E**).

3.3. RNA Interference Effect on B. xylophilus Feeding, Reproduction, and Pathogenicity

In comparison to the non-dsRNA-treated *B. xylophilus*, a significant reduction of 74.9% ($p < 0.0001$) in the expression of *Bx-srh-1* was observed in the ds*Bx-srh-1*-treated *B. xylophilus* (Figure 3A). Additionally, the *Bx-srh-1* expression in the ds*gfp*-treated *B. xylophilus* was similar to that of the non-dsRNA-treated group. The feeding rate of the ds *Bx-srh-1* treatment groups was notably slower than that of the other treated group (Figure 3C).

Within 9 days, almost all the hyphae were consumed by *B. xylophilus* in the water treatment and ds*gfp* treatment. In the group treated with ds*Bx-srh-1*, consumption of hyphae was observed in only one- to two-thirds of the sample. The reproductive rate of *B. xylophilus*, when soaked in ds*Bx-srh-1*, was significantly lower compared to the water treated and ds*gfp* groups (Figure 3B). These findings suggest that *Bx-srh-1* may have significant implications in feeding and reproduction.

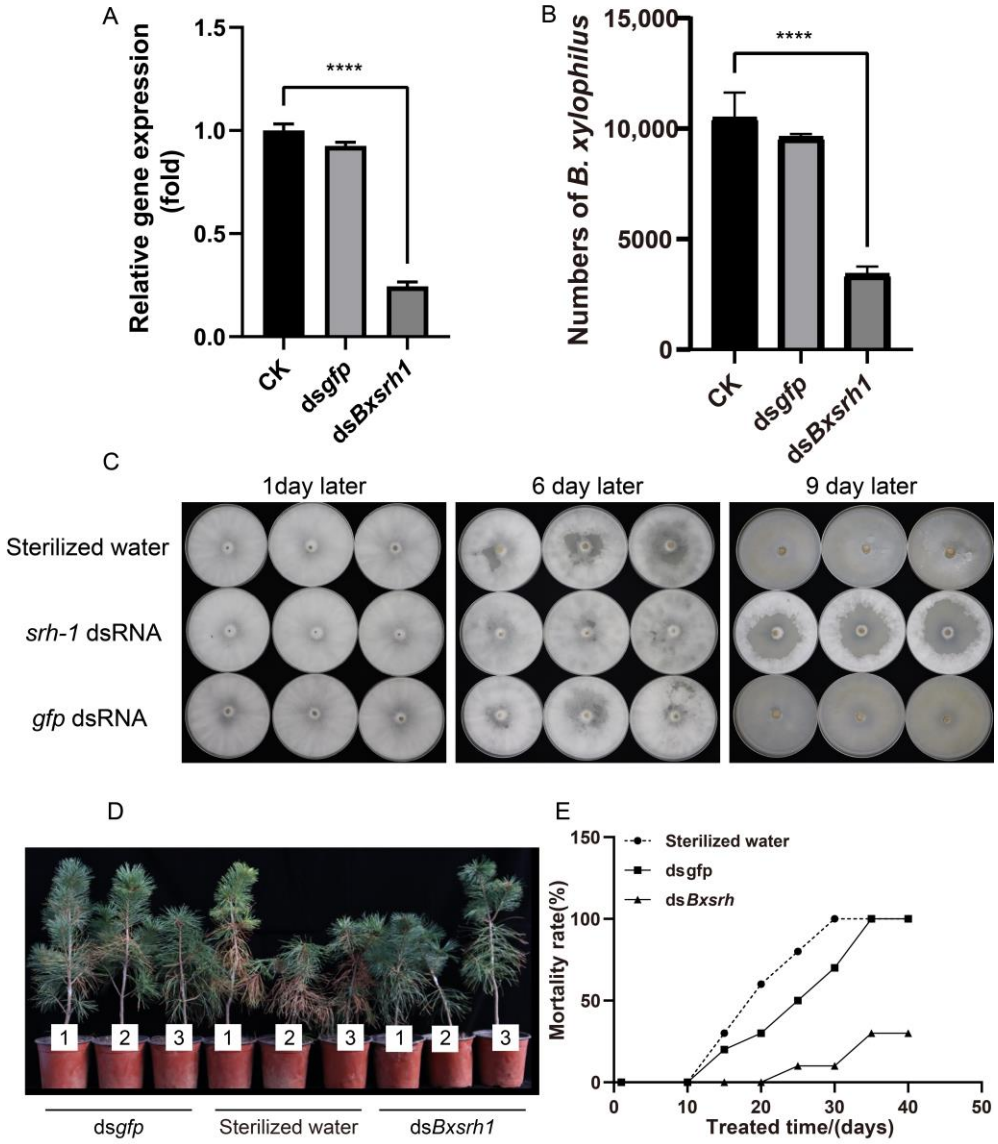

Figure 3. Expression and function analyses of *Bx-srh-1* after RNAi: (**A**) Relative expression levels of *Bx-srh-1* after RNAi; (**B**) Reproductive assay of *B. xylophilus* on *Botrytis cinerea* after RNAi; (**C**) Feeding of *B. cinerea* by *B. xylophilus* soaked in sterilized-water, ds*gfp*, and ds*Bx-srh-1* dsRNA solutions; (**D**) Pathogenicity assay of *B. xylophilus* of *Bx-srh-1* after RNAi, with wilting symptoms in *P. koraiensis* seedlings produced at 10 days (Numbers mean replicates); (**E**) Mortality rates of *P. koraiensis* seedlings with different treatments. Asterisks indicate statistically significant differences (**** $p < 0.0001$).

Within 10 days after inoculation, seedlings inoculated with *B. xylophilus* treated with sterile water displayed initial wilt symptoms, as depicted in Figure 3D. Within 20 days after inoculation, *P. koraiensis* inoculated with ds*Bx-srh-1*-treated *B. xylophilus* exhibited wilt symptoms, with mortality rates of 60% for the sterile-water treatment and 30% for ds*gfp* treatment seedlings, respectively (Figure 3E). By the 30th day after inoculation, all

P. koraiensis seedlings inoculated with sterile-water-treated *B. xylophilus* had perished, while the mortality rate for the ds*Bx-srh-1* treatment seedlings was 10%.

3.4. Expression Levels of Bx-srh-1 in B. xylophilus under α-Pinene Stress

The expression levels of *Bx-srh-1* were compared between the α-pinene treatment and TritonX-100 treatment (Figure 4). The expressions of *Bx-srh-1* gradually increased with the *B. xylophilus* stress with α-pinene for 12 h, with 1.7-fold changes from the TritonX-100 treatment. After treatment for 48 h, the expression levels of *Bx-srh-1* from the α-pinene treatment were upregulated to 4.37-fold changes from the TritonX-100 treatment. These results indicate that the α-pinene exposure directly upregulated the expression levels of *Bx-srh-1.* Therefore, *Bx-srh-1* may play an important role in the nematode resistance to α-pinene stress.

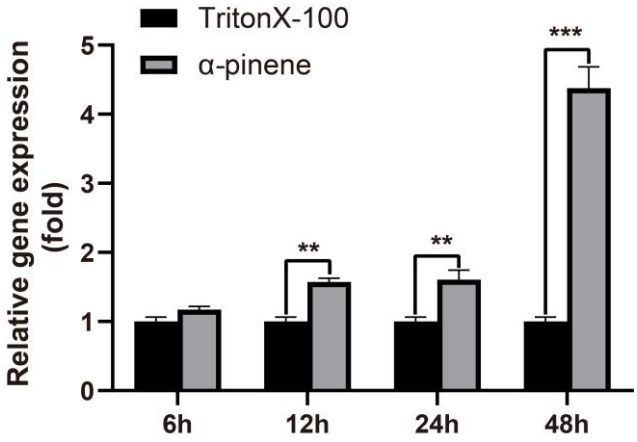

Figure 4. *Bx-srh-1* expression levels in *B. xylophilus* under α-pinene stress. Triton X-100 was used as the control. Asterisks indicate statistically significant differences (** $p < 0.01$, *** $p < 0.001$).

4. Discussion

GPCRs constitute a large protein family with diverse functions, such as autocrine, paracrine, and endocrine functions [37]. GPCR genes transduce extracellular signals in *C. elegans* [38]. The *srh* family is part of the *str* superfamily of chemoreceptors of GPCRs in *C. elegans*, and it is very large, containing 214 genes and 90 pseudogenes [39]. Previous studies have found that the *srh* family genes *srh-34* and *srh-234* are regulated by the integration of the sensory and internal feeding state signals in *C. elegans*. When nematodes are under starvation, *srh-34* and *srh-234* are regulated by multiple pathways [40]. Previous studies have reported that GPCR genes are involved with abiotic stress adaption, locomotion, early ontogenesis, and mating in *B. xylophilus* [24,25]. However, the other roles of GPCRs in *B. xylophilus* are still unclear. In this study, we investigated the phylogenetics of SRH-1 between *B. xylophilus* and six other species of nematodes. The results showed that the SRH-1 protein of *B. xylophilus* had the closest relationship with that of *B. okinawaensis*. It formed a founded cluster with the SRH-1 proteins of other plant parasitic nematodes, and it further clustered with the SRH-1 protein of *C. elegans* and *C. briggsae* as one branch. These results indicate that SRH-1 is evolutionarily conserved across the species of different plant parasitic and free-living nematodes. In free-living nematodes, GPCRs have a larger number of genes; however, the number of GPCRs is reduced in parasitic nematodes. Nearly 50% of the *srh* family genes were lost in *B. xylophilus* compared with *C. elegans* [41]. The mechanism of the gene loss in the *srh* family needs further investigation.

Previous research investigated the expression pattern of the GPCR gene *Bxy-octr-1* in *B. xylophilus* using whole-mount in situ hybridization, and *Bxy-octr-1* is expressed throughout all developmental stages of *B. xylophilus*. Specifically, during the larval stage, *Bxy-octr-1* is expressed in the entire body, while in the sexually mature adult stage, it is expressed in the gonads and other reproductive organs [25]. These findings suggest that

Bxy-octr-1 may play a role in the locomotion and feeding behavior during the larval stage, as well as influence mating behavior at adult stage. Our study utilized FISH analysis to determine that *Bx-srh-1* is expressed in the J2 juvenile, J3 juvenile, and adult stages of *B. xylophilus*. The gene was specifically expressed in the intestine and hypodermis of the J2, J3, and adult stages, with particularly high expression near the spicules of adult males and vulvae of adult females. Our study shows that *Bx-srh-1* has various functions in *B. xylophilus*, which is consistent with the findings of Wang's [25]. However, the regulatory mechanism of *Bx-srh-1* in *B. xylophilus* remains unclear. Further investigation is needed to determine the precise tissue or cells where *Bx-srh-1* is expressed in *B. xylophilus*.

The first instance of RNAi-mediated transcriptional gene silencing in *C. elegans* was reported in 1998 [42], and since then, RNAi has been extensively used in gene-silencing research on many multicellular organisms, including plant parasitic nematodes [43–45]. Several studies have been conducted on *B. xylophilus* using RNAi mediation, revealing that the efficacy and reproducibility of RNAi mediation vary significantly depending on the target gene [46,47]. The study found that *Bx-srh-1* RNAi efficiency in *B. xylophilus* was 74.9%. The knockdown of *Bx-srh-1* through RNAi showed that it plays a crucial role in feeding, reproduction, and pathogenicity of the nematode. The results suggest that RNAi treatment could potentially reduce the population growth and pathogenicity of *B. xylophilus*. However, the mechanism of *Bx-srh-1* remains unknown, and further investigation is required to determine if it regulates the expression of other *srh* family genes.

In *C. elegans*, toxicants can trigger a response by inhibiting or activating GPCRs located on the cytoplasmic membrane. Several GPCRs that control the stress response to toxicants have been identified in studies [48,49]. Previous research on *B. xylophilus* found that GPCRs regulate hormone levels during the invasion of pine trees, resulting in the upregulation of genes associated with food digestion, consumption, and xenobiotic metabolism. Thus, genes involved in xenobiotic metabolism are essential for detoxification and transport of toxicants [41]. α-pinene, a critical volatile monoterpene, is responsible for a significant proportion of the monoterpene production in pine trees [27] and exhibits low toxicity to *B. xylophilus* [28]. Studies have demonstrated that α-pinene effectively inhibits the reproduction of *B. xylophilus* at low concentration and upregulates t detoxification-related genes [29]. Our study revealed a gradual increase in the expression levels of *Bx-srh-1* in response to α-pinene-induced stress in *B. xylophilus*, reaching their peak at 48 h. This finding suggests that *Bx-srh-1* may play a crucial role in the detoxification process. Further investigation is required to determine if other GPCR genes respond to α-pinene.

5. Conclusions

This study mainly focuses on molecular characterization and functional determination of the GPCR gene *Bx-srh-1* in *B. xylophilus*. The results revealed that *Bx-srh-1* plays a crucial role in regulating feeding behavior, reproduction, pathogenicity, and resistance to α-pinene in *B. xylophilus*. The findings suggest that *Bx-srh-1* is involved in the pathogenicity of *B. xylophilus*, which could enhance our understanding of the physiological mechanisms of PWD.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14071282/s1, Figure S1. Agarose gel electrophoresis of the coding sequences of *Bx-srh-1*. M: DL2000 DNA Marker; 1: *Bx-srh-1*; Table S1. PCR primers used in the study; Table S2. Accession number of protein sequences from NCBI.

Author Contributions: Y.C. and X.W. (Xizhuo Wang) conceived the study; Y.C. and X.W. (Xiang Wang) performed the experiments; Y.Y., C.L. and X.C. contributed materials; Y.C. and X.W. (Xizhuo Wang) analyzed the data; X.W. (Xizhuo Wang) and L.W. secured funding; Y.C. and X.W. (Xizhuo Wang) wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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References

1. Zhao, B.G.; Futai, K. *Pine Wilt Disease*; Sutherland, J.R., Ed.; Springer: Berlin/Heidelberg, Germany, 2008; pp. 1–4.
2. Mota, M.M.; Kazuyoshi, F.; Paulo, V. Pine wilt disease and the pinewood nematode, *Bursaphelenchus xylophilus*. In *Integrated Management of Fruit Crops Nematodes*; Mota, M.M., Futai, K., Vieira, P., Eds.; Springer: Dordrecht, The Netherlands, 2009; pp. 253–274.
3. Tanaka, S.E.; Dayi, M.; Maeda, Y.; Tsai, I.J.; Tanaka, R.; Bligh, M.; Takeuchi-Kaneko, Y.; Fukuda, K.; Kanzaki, N.; Kikuchi, T. Stage-specific transcriptome of *Bursaphelenchus xylophilus* reveals temporal regulation of effector genes and roles of the dauer-like stages in the lifecycle. *Sci. Rep.* **2019**, *9*, 6080. [CrossRef] [PubMed]
4. Yang, B.; Wang, Q. Distribution of the pinewood nematode in China and susceptibility of some Chinese and exotic pines to the nematode. *Can. J. For. Res.* **1989**, *19*, 1527–1530.
5. Mota, M.M.; Braasch, H.; Bravo, M.A.; Penas, A.C.; Burgermeister, W.; Metge, K.; Sousa, E. First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology* **1999**, *1*, 727–734. [CrossRef]
6. Mamiya, Y. History of pine wilt disease in Japan. *J. Nematol.* **1988**, *20*, 219. [PubMed]
7. Mamiya, Y. Pathology of the pine wilt disease caused by *Bursaphelenchus xylophilus*. *Annu. Rev. Phytopathol.* **1983**, *21*, 201–220. [CrossRef] [PubMed]
8. Futai, K. Pine wood nematode, *Bursaphelenchus xylophilus*. *Ann. Rev. Phytopathol.* **2013**, *51*, 61–83. [CrossRef]
9. Vicente, C.; Espada, M.; Vieira, P.; Mota, M.M. Pine wilt disease: A threat to European forestry. *Eur. J. Plant Pathol.* **2012**, *133*, 89–99. [CrossRef]
10. Cheng, H.; Lin, M.; Qian, R. A study on morphological diagnosis and pathogenicity of the pine wood nematode. *J. Nanjing Agric. Univ.* **1986**, *2*, 55–59.
11. Hao, Z.; Huang, J.; Li, X.; Sun, H.; Fang, G. A multi-point aggregation trend of the outbreak of pine wilt disease in China over the past 20 years. *For. Ecol. Manag.* **2022**, *505*, 119890. [CrossRef]
12. Wang, R.; Li, Y.; Li, D.; Zhang, W.; Wang, X.; Wen, X.; Liu, Z.; Feng, Y.; Zhang, X. Identification of the Extracellular Nuclease Influencing Soaking RNA Interference Efficiency in *Bursaphelenchus xylophilus*. *Int. J. Mol. Sci.* **2022**, *23*, 12278. [CrossRef]
13. Halik, S.; Bergdahl, D. Survival and Infectivity of *Bursaphelenchus xylophilus* in Wood Chip-Soil Mixtures. *J. Nematol.* **1992**, *24*, 495–503.
14. Zhang, X.; Stamps, W.T.; Linit, M.J. A nondestructive method of determining *Bursaphelenchus xylophilus* infestation of *Monochamus* spp. vectors. *J. Nematol.* **1995**, *27*, 36–41. [PubMed]
15. Hou, Z.; Shi, F.; Ge, S.; Tao, J.; Ren, L.; Wu, H.; Zong, S. Comparative transcriptome analysis of the newly discovered insect vector of the pine wood nematode in China, revealing putative genes related to host plant adaptation. *BMC Genom.* **2021**, *22*, 189. [CrossRef]
16. Gao, M.; Li, Y.; Zhang, W.; Wei, P.; Wang, X.; Feng, Y. *Bx-daf-22* contributes to mate attraction in the gonochoristic nematode *Bursaphelenchus xylophilus*. *Int. J. Mol. Sci.* **2019**, *20*, 4316. [CrossRef]
17. Li, M.; Li, H.; Sheng, R.C.; Sun, H.; Sun, S.H.; Chen, F.M. The first record of *Monochamus saltuarius* (Coleoptera, Cerambycidae) as vector of *Bursaphelenchus xylophilus* and its new potential hosts in China. *Insects* **2020**, *11*, 636. [CrossRef]
18. Howard, A.D.; McAllister, G.; Feighner, S.D.; Liu, Q.; Nargund, R.P.; Van der Ploeg, L.H.; Patchett, A.A. Orphan G-protein-coupled receptors and natural ligand discovery. *Trends Pharmacol. Sci.* **2001**, *22*, 132–140. [CrossRef] [PubMed]
19. Ewbank, J.J.; Pujol, N. Local and long-range activation of innate immunity by infection and damage in *C. elegans*. *Curr. Opin. Immunol.* **2016**, *38*, 1–7. [CrossRef]
20. Pradel, E.; Zhang, Y.; Pujol, N.; Matsuyama, T.; Bargmann, C.I.; Ewbank, J.J. Detection and avoidance of a natural product from the pathogenic bacterium *Serratia marcescens* by *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 2295–2300. [CrossRef] [PubMed]
21. Yang, Y.; Dong, W.; Wu, Q.; Wang, D. Response of G protein-coupled receptor CED-1 in germline to polystyrene nanoparticles in *Caenorhabditis elegans*. *Nanoscale Adv.* **2021**, *3*, 1997–2006. [CrossRef]
22. Troemel, E.R.; Chou, J.H.; Dwyer, N.D.; Colbert, H.A.; Bargmann, C.I. Divergent seven transmembrane receptors are candidate chemosensory receptors in *C. elegans*. *Cell* **1995**, *83*, 207–218. [CrossRef]
23. Atkinson, L.E.; McCoy, C.J.; Crooks, B.A.; McKay, F.M.; McVeigh, P.; McKenzie, D.; Irvine, A.; Harrington, J.; Rosa, B.A.; Mitreva, M. Phylum-spanning neuropeptide GPCR identification and prioritization: Shaping drug target discovery pipelines for nematode parasite control. *Front. Endocrinol.* **2021**, *12*, 718363. [CrossRef] [PubMed]
24. Wang, B.; Hao, X.; Xu, J.; Ma, Y.; Ma, L. Transcriptome-Based Analysis Reveals a Crucial Role of *BxGPCR17454* in Low Temperature Response of Pine Wood Nematode (*Bursaphelenchus xylophilus*). *Int. J. Mol. Sci.* **2019**, *20*, 2898. [CrossRef]
25. Wang, J.; Liu, H.; Guo, K.; Yu, H.; Ye, J.; Hu, J. Molecular characterization and functional analysis of *Bxy-octr-1* in *Bursaphelenchus xylophilus*. *Gene* **2022**, *823*, 146350. [CrossRef]

26. Lewinsohn, E.; Savage, T.J.; Gijzen, M.; Croteau, R. Simultaneous analysis of monoterpenes and diterpenoids of conifer oleoresin. *Phytochem. Anal.* **1993**, *4*, 220–225. [CrossRef]
27. Ning, T.; Fan, J.; Fang, Y.; Sun, J. Changes in contents of host volatile terpenes under different damaged states and electroantennogram response of *Monochamus alternatu* Hope to these volatiles. *Acta Entomol. Sin.* **2006**, *49*, 179–188.
28. Wang, X.; Yu, Y.X.; Ge, J.J.; Xie, B.Y.; Zhu, S.F.; Cheng, X.Y. Effects of α-pinene on the pinewood nematode (*Bursaphelenchus xylophilus*) and its symbiotic bacteria. *PLoS ONE* **2019**, *14*, e0221099. [CrossRef]
29. Li, Y.; Meng, F.; Deng, X.; Wang, X.; Feng, Y.; Zhang, W.; Pan, L.; Zhang, X. Comparative Transcriptome Analysis of the Pinewood Nematode *Bursaphelenchus xylophilus* Reveals the Molecular Mechanism Underlying Its Defense Response to Host-Derived α-Pinene. *Int. J. Mol. Sci.* **2019**, *20*, 911. [CrossRef]
30. Rui, L.; Liu, H.; Liang, R.; Wu, X. Resistance genes mediate differential resistance to pine defensive substances α-Pinene and H_2O_2 in *Bursaphelenchus xylophilus* with different levels of virulence. *J. For. Res.* **2021**, *32*, 1753–1762. [CrossRef]
31. Cao, Y.; Wang, L.; Wang, X.; Wang, X.; Xu, M. Pathogenicity of Three *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle. Isolates in *Pinus koraiensis* (Siebold & Zucc.) Seedlings. *Forests* **2022**, *13*, 1197. [CrossRef]
32. Hooper, D.J. *Extraction of Nematodes from Plant Materials*; Southey, J.F., Ed.; Laboratory Methods for Work with Plant and Soil Nematodes; Her Majesty's Stationery Office: London, UK, 1986; pp. 51–58.
33. Senthilkumar, M.; Amaresan, N.; Sankaranarayanan, A. Extraction of Nematodes from Plant Materials. In *Plant-Microbe Interactions*; Springer: New York, NY, USA, 2021; pp. 275–277. [CrossRef]
34. Wang, Y.; Chen, J.; Chen, F. Transmission of *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae) through feeding activity of *Monochamus alternatus* (Coleoptera: Cerambycidae). *J. Nanjing For. Univ.* **2019**, *43*, 1–10.
35. Urwin, P.E.; Lilley, C.J.; Atkinson, H.J. Ingestion of Double-Stranded RNA by Preparasitic Juvenile Cyst Nematodes Leads to RNA Interference. *Mol. Plant-Microbe Interact.* **2002**, *15*, 747–752. [CrossRef]
36. Xiang, Y.; Wu, X.; Zhou, A. Bacterial diversity and community structure in the pine wood nematode *Bursaphelenchus xylophilus* and *B. mucronatus* with different virulence by high-throughput sequencing of the 16S rDNA. *PLoS ONE* **2015**, *10*, e0137386. [CrossRef]
37. Vassilatis, D.K.; Hohmann, J.G.; Zeng, H.; Li, F.; Ranchalis, J.E.; Mortrud, M.T.; Brown, A.; Rodriguez, S.S.; Weller, J.R.; Wright, A.C. The G protein-coupled receptor repertoires of human and mouse. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 4903–4908. [CrossRef] [PubMed]
38. Bockaert, J.; Pin, J.P. Molecular tinkering of G protein-coupled receptors: An evolutionary success. *EMBO J.* **1999**, *18*, 1723–1729. [CrossRef] [PubMed]
39. Robertson, H.M. The large *srh* family of chemoreceptor genes in *Caenorhabditis* nematodes reveals processes of genome evolution involving large duplications and deletions and intron gains and losses. *Genome Res.* **2000**, *10*, 192–203. [CrossRef]
40. Gruner, M.; Nelson, D.; Winbush, A.; Hintz, R.; Ryu, L. Feeding State, Insulin and NPR-1 Modulate Chemoreceptor Gene Expression via Integration of Sensory and Circuit Inputs. *PLoS Genetics* **2014**, *10*, e1004707. [CrossRef] [PubMed]
41. Zhang, W.; Yu, H.; Lv, Y.; Bushley, K.E.; Wickham, J.D.; Gao, S.; Hu, S.; Zhao, L.; Sun, J. Gene family expansion of pinewood nematode to detoxify its host defense chemicals. *Mol. Ecol.* **2020**, *29*, 940–955. [CrossRef]
42. Fire, A.; Xu, S.; Montgomery, M.K.; Kostas, S.A.; Driver, S.E.; Mello, C.C. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **1998**, *391*, 806–811. [CrossRef]
43. Lilley, C.; Davies, L.; Urwin, P. RNA interference in plant parasitic nematodes: A summary of the current status. *Parasitology* **2012**, *139*, 630–640. [CrossRef]
44. Banerjee, S.; Banerjee, A.; Gill, S.; Gupta, O.; Dahuja, A.; Jain, P.K.; Sirohi, A. RNA Interference: A Novel Source of Resistance to Combat Plant Parasitic Nematodes. *Front. Plant Sci.* **2017**, *8*, 834. [CrossRef]
45. Rajam, M. RNA silencing technology: A boon for crop improvement. *J. Biosci.* **2000**, *45*, 118. [CrossRef]
46. Park, J.; Lee, K.Y.; Lee, S.; Oh, W.; Jeong, P.; Woo, T.; Kim, C.; Paik, Y.; Koo, H. The efficiency of RNA interference in *Bursaphelenchus xylophilus*. *Mol. Cells* **2008**, *26*, 81–86. [PubMed]
47. Liu, X.; Zhou, X.; Zhou, L.; Hu, J.; Guo, K. Application of RNA Interference in the Pinewood Nematode, *Bursaphelenchus xylophilus*. *J. Vis. Exp.* **2022**, *181*, e63645. [CrossRef]
48. Jee, C.; Lee, J.; Lim, J.; Parry, D.; Messing, R.; McIntire, S. SEB-3, a CRF receptor-like GPCR, regulates locomotor activity states, stress responses and ethanol tolerance in *Caenorhabditis elegans*. *Genes. Brain. Behav.* **2013**, *12*, 250–262. [CrossRef]
49. Wang, D. *Target Organ Toxicology in Caenorhabditis elegans*; Springer Nature Press: Singapore, 2019; pp. 247–249. [CrossRef]

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