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Transcriptional and morphological changes in the transition from mycetophagous to phytophagous phase in the plant-parasitic nematode *Bursaphelenchus xylophilus*

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SUMMARY

Drastic physiological and morphological changes in parasites are crucial for the establishment of a successful infection. The nematode *Bursaphelenchus xylophilus* is the pathogenic agent of pine wilt disease, and little is known about the physiology and morphology in this nematode at the initial stage of infection. In this study, we devised an infection system using pine stem cuttings that allowed us to observe transcriptional and morphological changes in the host-infecting phytophagous phase. We found that 60 genes enriched in xenobiotic detoxification were up-regulated in two independent post-inoculation events, whereas down-regulation was observed in multiple members of collagen gene families. After 48 h of inoculation, the tails in some of the adult females exposed to the host changed in morphology. These results suggest that *B. xylophilus* may change its physiology and morphology to protect itself and to adapt to the host pine wood environment.

Keywords: *Bursaphelenchus xylophilus*, collagen, pinewood nematode, RNAseq, tail shape.

INTRODUCTION

Pine wilt disease is one of the most serious forest pests in the world and has been responsible for timber losses of over two million cubic metres in some years (Jones *et al*., 2013). The causal agent of pine wilt disease is the nematode *Bursaphelenchus xylophilus*. This nematode is thought to be indigenous to North America, but has subsequently spread to East Asia and European countries (Fonseca *et al*., 2012; Jones *et al*., 2008; Robertson *et al*., 2011). *Bursaphelenchus xylophilus* has mycetophagous and phytophagous feeding stages in its life cycle. Nematodes are transmitted to healthy trees by a vector insect during maturation feeding of the insect. The nematodes spread through the tree's vascular system and resin canals, feeding on epithelial cells and living parenchyma, and start to reproduce. This phytophagous phase results in the disruption of plant tissues, leading to a lethal wilt within months of infection under certain environmental conditions. At this stage, fungi start to colonize the tree and the mycetophagous phase begins. When fungi become limiting, the nematodes locate pupae of their insect vectors and enter a survival (dauer) stage, which invades the tracheal system of the insects and is subsequently transmitted to the next plant when the adult insect emerges (Jones *et al*., 2008).

The nutrient and environmental conditions encountered by the nematodes at the two phases of the life cycle vary. In the phytophagous phase, the nematodes are resident in living plant material and are exposed to a variety of pre-existing structural and chemical defences, as well as induced responses. These are likely to include phenols, terpenes and reactive oxygen species (ROS) (Abdel-Rahman *et al*., 2013; Holscher *et al*., 2014; Sun *et al*., 2011; Torres *et al*., 2006). These threats are present throughout the phytophagous phase (i.e. until host death) because *B. xylophilus* moves continuously to fresh parts of the plant. To evade such host defence responses, many parasites are known to change their morphology and physiology. This is reflected in changes in transcriptional patterns in a wide range of plant- and animal-parasitic nematodes, such as *Globodera pallida* (Palomares-Rius *et al*., 2012), *Strongyloides stercoralis* (Stoltzfus *et al*., 2012), *Haemonchus contortus* (Jasmer *et al*., 2004) and *Ancylostoma caninum* (Williamson *et al*., 2006). Morphological changes that occur in response to the onset of parasitism can include changes to the surface coats (Akhkha *et al*., 2002; Lopez de Mendoza *et al*., 2000; Proudfoot *et al*., 1993).

In this study, we compared the morphology and transcriptomes of mycetophagous and phytophagous *B. xylophilus* using a stem-cutting inoculation system. We showed that the two phases have distinct morphological characters and identified genes whose

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expression patterns suggest that they may be involved in these morphological changes. In addition, we showed that the nematode undergoes rapid changes in gene expression in response to changes in its environment.

RESULTS

Morphological changes

To imitate the environment of the nematodes in the phytophagous phase (moving and feeding in fresh parts of the tree), we devised a stem-cutting inoculation system as shown in Fig. 1 (see Experimental procedures). Nematodes were observed in the bottom of tubes about 30 min after inoculation and continued to be observed until 3 days post-inoculation (Fig. 2A).

We sought to observe any morphological changes between the two stages. In the first 24 h after inoculation, no morphological changes were observed in the nematodes. However, at 48 and 72 h post-inoculation, we observed that some female fourth-stage larvae (L4) and female adults showed different morphology in the tails (M-shape) (Fig. 3). The proportion of female nematodes showing this change in tail shape increased until 72 h post-inoculation (Fig. 2B). No such change was observed in other stages (males and larvae) of *B. xylophilus* (data not shown) or when the nematodes were incubated on fungi or in water (Fig. 2B).

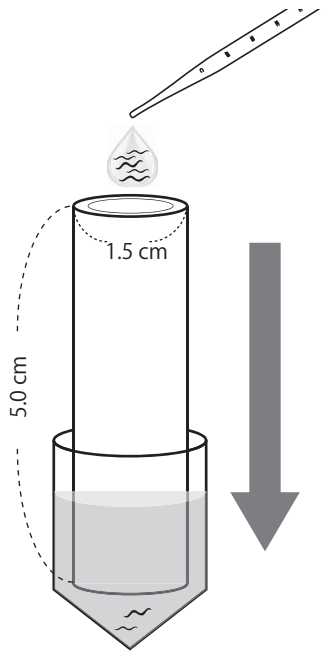

Fig. 1 Stem-cutting inoculation system. *Pinus thunbergii* seedlings were cut (diameter, 1 cm; length, 5 cm) and placed in a plastic tube filled with distilled water. The nematodes were placed on top of the cutting and were collected from the water at the bottom at the appropriate times.

RNA sequencing (RNAseq) of *B. xylophilus*

To identify differentially expressed genes when *B. xylophilus* enters the phytophagous phase, and to account for additional factors associated with different environmental conditions, RNA was obtained from mixed-stage nematodes incubated for 2 h in water and extracted 0.5–2.5 h after inoculation at two different times of the year (August and September). The experimental design therefore used two post-inoculation events and one control with two biological replicates per condition. The relative expression of *B. xylophilus* genes was then quantified by Illumina RNAseq. A total of 144.3 million 100-bp paired-end sequence reads was generated and, on average, 92% of these mapped to the *B. xylophilus* reference genome (Table S1, see Supporting Information). Differential expression of transcripts was calculated between pairwise comparisons of each condition. To avoid false

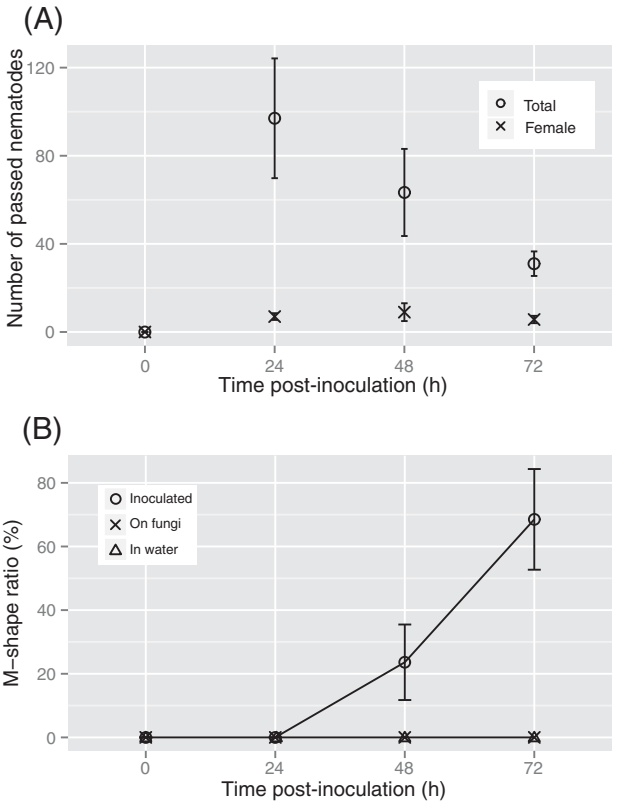

Fig. 2 (A) Number of nematodes isolated from the stem cuttings at different time points after inoculation. Twenty microlitres of water containing 2000 *Bursaphelenchus xylophilus* Ka4 were inoculated on top of the 5-cm-long stem cuttings (Fig. 1). The inoculated stem cuttings were incubated at 25 °C and 100% relative humidity. The numbers of nematodes which came through the stem cuttings were counted every 24 h. The number of females observed in each time interval is also shown in the graph. (B) Change in ratio of mucronated (M-shape) nematodes. Tips of females were observed under a microscope for the same samples as in (A). Nematodes incubated in water or growing on fungi were examined as controls. Error bars represent the standard error of the mean.

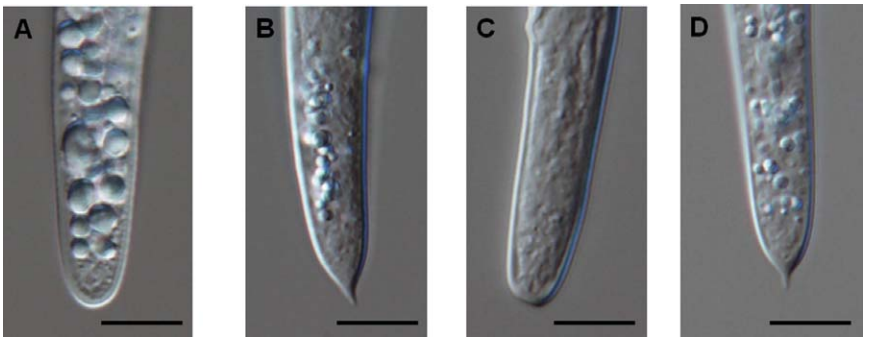

Fig. 3 Tail morphology of *Bursaphelenchus xylophilus* Ka4 before and after inoculation. (A) L4 female with a round (R-shape) tail (in water). (B) L4 female with a mucronated (M-shape) tail (48 h after inoculation). (C) Adult female with a round (R-shape) tail (in water). (D) Adult female with a mucronated (M-shape) tail (48 h after inoculation). All scale bars are 10 μm.

positives on extremely poorly expressed genes, transcripts with reads per kilobase per million mapped reads (RPKM) < 1 in any of the conditions were excluded from further analysis. This still left 12 851 of 17 704 genes available for analysis, and the full list of transcript RPKM counts is shown in Table S2 (see Supporting Information).

The two biological replicates in each condition were clustered together, indicating that the experiments were highly reproducible and gene expression in *B. xylophilus* was indeed influenced by life cycle stage and environmental factors (Fig. S1, see Supporting Information). Our experimental set-up allowed us to distinguish 1143 genes that were up-regulated in post-inoculation samples (Fig. 4). However, many of these genes were up-regulated specifically in one of the two environmental samples (1061 and 22 genes in August and September, respectively). These genes may be differentially regulated as a result of environmental differences, for example, genes only up-regulated in the August post-inoculation event were enriched in embryo development, translation and positive growth rates (Table S3, see Supporting Information), suggesting that nematodes in this particular set-up had a more optimal growth condition compared with the control and the September post-inoculation event.

Sixty and 384 genes were up-regulated or down-regulated, respectively, in both post-inoculation samples when compared with the control (Fig. 4). It is possible that some of these differentially expressed genes underlie the mucronated morphological changes observed here (Fig. 3). Indeed, gene ontology (GO) term enrichment of down-regulated genes reveals a dominant term of 'morphological change' when nematodes enter the phytophagous stage (Table S4, see Supporting Information). Within this term, collagen gene family members are predominant in the list (Table S5, see Supporting Information). Collagens are important in structural formation and modification in a range of species and are the dominant proteins of the cuticle (Johnstone, 2000). Using quantitative polymerase chain reaction (qPCR), we further validated the down-regulation of four *B. xylophilus* collagen genes after infection (Fig. 5). In particular, *col-5* shows a 16.7-fold decrease in expression. Ten heat shock proteins were also down-regulated after infection (Table S5), which may be responsible for

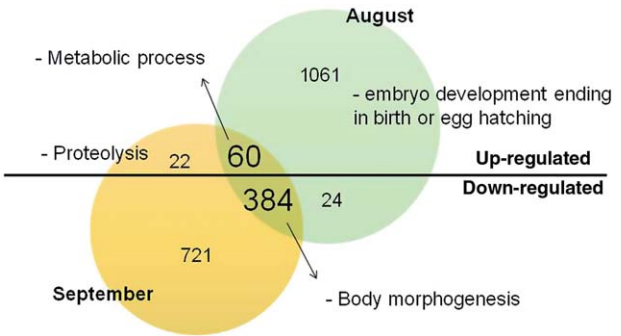

Fig. 4 Venn diagram showing the up-regulated and down-regulated genes after infection with *Bursaphelenchus xylophilus*.

dealing with osmotic stress when the nematodes are incubated solely in water.

For 28 of the 60 up-regulated genes that have functional annotations (Table S6, see Supporting Information), we found that 12 genes were largely involved in different phases of xenobiotic metabolism, including cytochrome P450, short chain dehydrogenase, UDP-glucuronosyl transferase and glutathione *S*-transferase (GST). This increase in expression after inoculation may be involved in dealing with host-derived stress (Lindblom and Dodd, 2006). In addition, we also identified five proteases that were up-regulated and may be involved in other aspects of the parasitic process, such as host tissue penetration. Interestingly, we further identified copies of P450 and proteases up-regulated only in the September post-inoculation event, again suggesting more optimal growth conditions in the August post-inoculation event.

DISCUSSION

Plants produce a variety of chemicals to protect themselves from pathogens as part of the induced defence response (O'Brien *et al*., 2012). ROS are thought to be a major part of these defence mechanisms and are known to be deployed against plant-parasitic nematodes (Bolwell *et al*., 1995; Mehdy, 1994; Torres *et al*., 2006), as are resins and reinforced cell walls. These mechanisms are thought to make nematode survival and movement difficult (Futai, 2013). However, *B. xylophilus* can survive, move and grow under

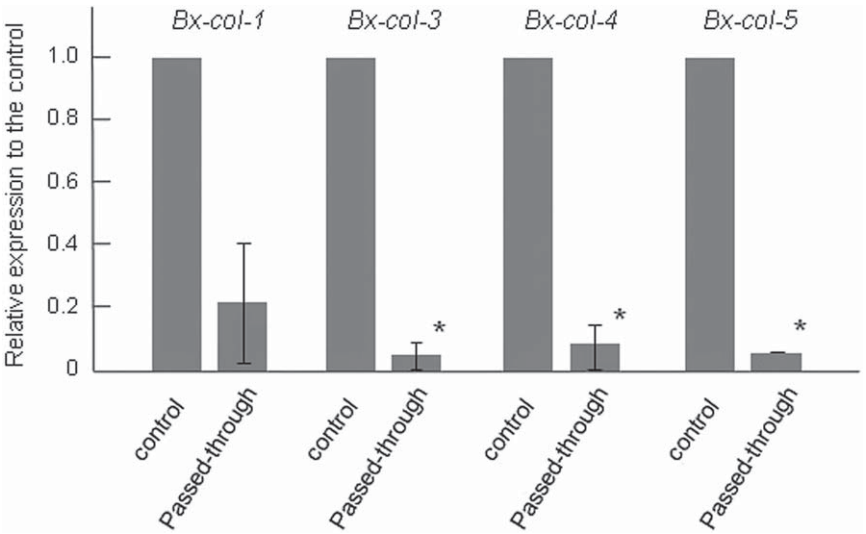

Fig. 5 Relative expression levels of collagen genes measured by quantitative polymerase chain reaction (qPCR). Expression of collagen genes (*Bx-col-1*, *Bx-col-3*, *Bx-col-4* and *Bx-col-5*) was compared between the control and after inoculation (Passed-through). Error bars represent the standard deviations of the biological replicates. Asterisks indicate statistically significant differences between the two conditions (Student's *t*-test, $P < 0.05$).

these conditions in both living host trees (phytophagous) and in the dead host (mycetophagous) (Futai, 2013). In this study, we have employed an experimental set-up to retrieve *B. xylophilus* as it is just entering the phytophagous phase. We have shown that various strategies are employed by *B. xylophilus* in order to establish a successful infection.

Many pathogens are known to change morphology when they infect their hosts. For example, the heteroecious parasites *Toxoplasma*, *Plasmodium* and *Theileria* change their morphology and physiology host by host (Plattner and Soldati-Favre, 2008). For the first time, we also observed morphological changes in *B. xylophilus* in the tails of L4 and adult females 48 h after inoculation, changes that were not seen in nematodes not exposed to the host. This suggests that the presence of the host is required to stimulate this specialized moulting. The change in tail shape in females may be part of a response of the whole body surface as it adapts to the host pine wood environment. Changes in tail structure were not observed across all life stages; in the case of larvae, changes were not clear because of their small tail size. In addition, the adult male has a bursa at the end of its tail and the presence of this structure would obscure the morphological changes seen in females. The observation of these changes is important as the diagnosis of pine wilt disease is performed mainly by direct observation of nematodes isolated from pine trees, although some molecular techniques have been used as alternatives (Kikuchi *et al*., 2009). The discrimination of this nematode from other non-pathogenic nematodes is sometimes difficult. In particular, a non-pathogenic species, *Bursaphelenchus mucronatus*, which is closely related to *B. xylophilus*, shares most morphological characters with the pathogenic nematode, except for female tail morphology, i.e. rounded in *B. xylophilus* and mucronated (tipped) in *B. mucronatus*; such differences have been used as one of the main diagnostic characters (Kanzaki, 2008). *Bursaphelenchus xylophilus* individuals with mucronated tails are sometimes observed in dead tree wood with high nematode population density and in newly killed or dying trees regardless of population density (data not shown). This can be explained by our observations showing that adult *B. xylophilus* females in phytophagous phases also have mucronated tails.

Many members of the collagen gene family were down-regulated in *B. xylophilus* 48 h after inoculation. Low expression levels of collagen genes seem to be a common feature among infective life stages of various plant- and animal-parasitic nematodes (Elling *et al*., 2007; Mitreva *et al*., 2004). As collagens make up the majority of cuticle in many nematodes and the expression of collagen genes leads to changes in nematode morphology (Johnstone, 2000), down-regulation of this gene family may reflect changes in cuticle shape and structure. These changes may reflect the changes in morphology seen here or, alternatively, may reflect the synthesis of new cuticle components designed to cope with the stresses of being inside a host. Clearly, it remains to be determined whether these collagens are expressed in specific parts of *B. xylophilus* and how long these down-regulations continue. This may be a specific response of initial contact to the host plant as, in our preliminary RNAseq data from nematodes at 6 and 15 days post-inoculation, such down-regulations were no longer observed (M. Espada and J. Jones, unpublished results).

A probable explanation for the observation of major differences in gene expression between inoculations in August and September is the differences in the environmental conditions of the trees from which the cuttings were made. As tree cuttings were prepared from trees grown in the experimental nursery, the physiological conditions of the plants, which include immune activity and nutritional status, may be very different as a result of temperature, water and other environmental conditions. Furthermore, because the same procedures were used to prepare the two inocula and there were two replicates in each stage of the RNAseq analysis, it is unlikely that condition differences between the two inocula (e.g.

stage ratio) would have caused the major differences in gene expression between inoculations in August and September. Therefore, our data suggest that, when the nematodes invade the tree, they react flexibly according to the host conditions and may use different strategies to survive. In spite of these conditions, we identified 60 genes that were up-regulated in both August and September. These genes may be essential for the early stages of infection under all conditions and include several xenobiotic genes. Indeed, the expression of genes encoding antioxidant proteins may be a response to host-derived ROS. These genes were identified here and were also expressed in *B. xylophilus* 7 days after infection in pine trees (Qiu *et al*., 2013). *Bursaphelenchus xylophilus* is known to produce surface coat proteins to help protect itself from ROS (Shinya *et al*., 2013). Coping with host-derived ROS is therefore likely to be a key factor underpinning *B. xylophilus* survival in the host tree.

A recent proteomic study in *B. xylophilus* by Shinya *et al*. (2010) compared surface coat proteins of nematodes isolated from plants after 15 days of infection with those grown on fungi, and the results were found to be consistent with our August post-inoculation condition. The authors identified 12 proteins over-represented in the phytophagous stage nematodes, which included GST, 14-3-3b protein and glyceraldehyde-3-phosphate dehydrogenase (Shinya *et al*., 2010), 10 of which were also up-regulated in our August phytophagous nematodes (Table S2). In addition, all four of the surface coat proteins under-represented in the phytophagous stage, including paramyosin and enolase (Shinya *et al*., 2010), were down-regulated in our August samples (Table S2). Conversely, only one of 12 over-represented and three of four under-represented proteins were up- or down-regulated, respectively, in our September RNAseq. These results suggest that the conditions used for the protein comparisons were similar to those of our August samples, and the high degree of consistency between the two studies (protein and RNAseq) emphasizes the reliability of the findings in both studies.

To conclude, we have shown that *B. xylophilus* undergoes physiological changes to protect itself from the host environment in the first stages of infection. In addition, morphological changes in the tails of female nematodes occur after infection. These results will assist in the accurate diagnosis of pine wilt nematodes and the control of pine wilt disease.

EXPERIMENTAL PROCEDURES

Nematodes used in this study

The *B. xylophilus* Ka4 and Ka4C1 strains [maintained in the Pathology Laboratory of the Forestry and Forest Products Research Institute (FFPRI), Tsukuba, Japan] were used in this study. Ka4C1 was generated from Ka4 by inbreeding for genome sequencing and RNAseq (Kikuchi *et al*., 2011). Nematodes were cultured for 7 days at 25 °C on *Botrytis cinerea* grown on autoclaved barley grains with antibiotics (100 μg/mL streptomycin and 25 μg/mL chloramphenicol). The nematodes were then collected using the Baermann funnel method for 1 h at 25 °C. The nematodes were washed three times in 0.5 × phosphate-buffered saline (PBS) before use in the experiments.

Stem-cutting inoculation

Shoots (diameter ≈ 1 cm) obtained from 5-year-old Japanese black pine (*Pinus thunbergii*) were cut into 5-cm-long sections and used immediately for the experiments. Twenty microlitres of water containing 2000 mixed life-stage nematodes were inoculated on the top of the stem cuttings, which were placed in 50-mL plastic tubes containing 4 mL of distilled water (Fig. 1). A small pit was made on the top of each stem cutting prior to nematode inoculation and the solution was carefully applied to this pit. The inoculated stem cuttings were incubated at 25 °C and 100% relative humidity. Nematodes that came through the stem cuttings were collected from the bottom of the 50-mL tubes at various time points (0.5, 1, 2, 3, 6, 12, 24, 48 and 72 h post-inoculation) and were observed under a compound microscope (ECLIPSE 80i, Nikon, Tokyo, Japan). This system allowed nematodes to be obtained easily and quickly from the bottom of the tubes without requiring any disruption or maceration of the pine trees, and we considered that this procedure would minimize any artificial effects during the recovery process. For control, 20 μL of water containing 2000 nematodes were inoculated on *B. cinerea* grown on potato dextrose agar (PDA) or 4 mL of distilled water in a glass tube, and incubated at 25 °C. Nematodes were observed in the same way as in the stem-cutting experiments.

RNA-seq dataset generation

Approximately 40 000 nematodes were recovered from the base of 20 stem cuttings, as described previously, between 0.5 and 2.5 h after inoculation. The nematodes were pooled in a 1.5-mL tube and used for RNA extraction immediately or stored at −80 °C until use. After disruption of nematode bodies using zirconia beads (ø = 0.15–0.40 mm) in 500 μL of TRIzol (Invitrogen, Tokyo, Japan), total RNA was extracted according to a standard Trizol protocol (Invitrogen). Total RNA was also extracted from approximately 40 000 nematodes which were incubated in water at 25 °C for 2 h and used as a control.

After RNA quality and quantity assessment using a Bioanalyser2100 (Agilent, Santa Clara, USA), 1 μg of total RNA was used to construct an Illumina sequencing library employing the TruSeq RNA-seq Sample Prep kit according to the manufacturer's recommended protocols (Illumina, San Diego, USA). The libraries were quantified by qPCR and sequenced for 200 cycles (100-bp paired ends) on an Illumina HiSeq2000 sequencer using the standard protocol (Illumina). RNAseq reads were mapped against the *B. xylophilus* genome reference (v1.3) using Tophat v.2.0.11 (Trapnell *et al*., 2009) and differential expression was called using EdgeR v3.2.4 (Robinson *et al*., 2010). A transcript was identified as differentially expressed in a pairwise comparison if the following criteria were met: false discovery rate (FDR) ≤ 0.01 and fold change ≥ 2.0. RNAseq experiments were conducted in duplicate for the test conditions and in triplicate for the control condition.

qPCR

To confirm the expression levels of collagen genes, 1000 mixed-stage nematodes (Ka4C1), which were either incubated in water (control) or

collected from stem cuttings as described above, were used for qPCR. Total RNA was extracted from these nematodes using Trizol (Invitrogen), treated with DNase (TakaraBio, Otsu, Japan) and dissolved in 20 μL of water. Single-stranded cDNA was synthesized from 5 μL of the total RNA solution using the iScript cDNA Synthesis Kit according to the manufacturer's instructions (Bio-Rad, Tokyo, Japan). Full-length sequences of four collagen genes (*Bx-col-1*, *Bx-col-3*, *Bx-col-4* and *Bx-col-5*) were manually curated using the genome assembly (v1.3; available from http://parasite.wormbase.org/index.html) and the RNAseq data. Primers for target genes were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) (Table S7, see Supporting Information). The qPCRs were performed using StepOnePlus (Applied Biosystems, Foster City, USA) with Power SYBR reagents (Applied Biosystems) in a reaction volume of 10 μL containing 5 μL of Power SYBR Green PCR Master Mix (2X), 0.5 μL of cDNA solution and 0.9 μM of each primer under the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All qPCRs were conducted with two biological replicates, each having three technical measurements. The actin gene (*Bx-act-1*) was used as an endogenous control. Relative transcript levels of the two samples were calculated using StepOne Software v2.3 (Applied Biosystems). Statistical analyses were performed using R packages (http://www.r-project.org/).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1 Heatmap showing the hierarchically clustered correlation matrix by comparison of the transcript expression values for each pair of samples.

Table S1 *Bursaphelenchus xylophilus* RNAseq mapping statistics.

Table S2 Reads per kilobase per million mapped reads (RPKMs) of transcribed genes of *Bursaphelenchus xylophilus* in this study.

Table S3 Enriched gene ontology (GO) terms of the solely up-regulated genes in the August and September phytophagous phase of *Bursaphelenchus xylophilus* nematodes.

Table S4 Enriched gene ontology (GO) terms of the up or down-regulated genes in both phases of *Bursaphelenchus xylophilus* nematodes.

Table S5 Reads per kilobase per million mapped reads (RPKMs) of down-regulated genes in the phytophagous phase of *Bursaphelenchus xylophilus* nematodes. Collagens and heat shock proteins are highlighted in yellow and blue, respectively.

Table S6 Reads per kilobase per million mapped reads (RPKMs) of up-regulated genes in the phytophagous phase of *Bursaphelenchus xylophilus* nematodes.

Table S7 Primers used in the quantitative polymerase chain reaction (qPCR) experiments.