Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2016) 17(2), 286–295 DOI: 10.1111/mpp.12280

Identification and characterization of parasitism genes from the pinewood nematode *Bursaphelenchus xylophilus* reveals a multilayered detoxification strategy

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SUMMARY

The migratory endoparasitic nematode *Bursaphelenchus xylophilus*, which is the causal agent of pine wilt disease, has phytophagous and mycetophagous phases during its life cycle. This highly unusual feature distinguishes it from other plant-parasitic nematodes and requires profound changes in biology between modes. During the phytophagous stage, the nematode migrates within pine trees, feeding on the contents of parenchymal cells. Like other plant pathogens, *B. xylophilus* secretes effectors from pharyngeal gland cells into the host during infection. We provide the first description of changes in the morphology of these gland cells between juvenile and adult life stages. Using a comparative transcriptomics approach and an effector identification pipeline, we identify numerous novel parasitism genes which may be important for the mediation of interactions of *B. xylophilus* with its host. In-depth characterization of all parasitism genes using *in situ* hybridization reveals two major categories of detoxification proteins, those specifically expressed in either the pharyngeal gland cells or the digestive system. These data suggest that *B. xylophilus* incorporates effectors in a multilayer detoxification strategy in order to protect itself from host defence responses during phytophagy.

Keywords: *Bursaphelenchus xylophilus*, effectors, gland cells, transcriptome, xenobiotic metabolism.

INTRODUCTION

The pinewood nematode (PWN) *Bursaphelenchus xylophilus* is a migratory plant endoparasitic nematode and is the causal agent of pine wilt disease (PWD). The PWD complex includes the pathogenic agent, its insect vector (cerambycid beetles of the genus *Monochamus*) and the host, which can be one of several different *Pinus* species. *Bursaphelenchus xylophilus* is native to North America and causes little damage to indigenous tree species. However, it was introduced into China and Japan at the start of the 20th century and has caused significant damage in these countries under appropriate environmental conditions (Jones *et al*., 2013). The nematode was found in Europe for the first time in 1999 (Mota *et al*., 1999) and has now been detected in mainland Portugal, Madeira Island and Spain (Fonseca *et al*., 2012; Mota *et al*., 1999; Robertson *et al*., 2011). Pine wood represents a major proportion of the forestry industry and the rapid spread of this disease has become a major problem with the potential to cause significant economic losses and damage to forests on an ecological scale (Mota and Vieira, 2008; Vicente *et al*., 2012a).

The PWN has two different life cycle stages—a phytophagous parasitic stage and a mycetophagous stage. This highly unusual feature distinguishes it from other plant-parasitic nematodes (PPNs) and enables it to reproduce and survive in the host at the later stages of PWD, when healthy plant tissues may be absent, but fungi are abundant (Jones *et al*., 2013; Vicente *et al*., 2012a). Like many other nematode species, *B. xylophilus* has four juvenile stages prior to the mature adult, and all life stages are vermiform. Nematodes can feed on fungi in dead or dying trees and, as nematode numbers increase, and food becomes scarce, a survival and dispersal stage develops (the *dauer* juvenile) that migrates to beetle pupal chambers. When the adult insect emerges, the *dauer* stage of the nematode enters the tracheid and is transported to a new host. The nematode may be transported to a dead or dying tree colonized with fungi, in which case the mycetophagous cycle described above begins again. Alternatively, the nematode can infect healthy host trees through maturation feeding wounds made by the insect. Once inside the pine cortex, the nematode migrates to the xylem resin and ray canals and feeds on parenchyma cells, leading to cell death (Mamiya, 2012). The tree releases polyphenolic compounds (causing browning of the tissues during infection), terpenoids, reactive oxygen species (ROS) and lipid peroxides during the early stages of infection as part of

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a strong defence response (Fukuda, 1997). Nematode numbers increase and water transport through the infected tree is compromised leading to wilt and, consequently, to the death of the tree (Futai, 2013; Jones *et al*., 2008).

Although a genome sequence has been reported for *B. xylophilus* (Kikuchi *et al*., 2011), the details of the mechanisms underlying the interaction between this nematode and its host remain unclear. Although peptides and plant hormones have been suggested to play important roles in the interactions between plants and nematodes, some of the most important nematode-derived factors that manipulate the host are effector proteins, many of which are produced in the pharyngeal gland cells and secreted into the host through the stylet. In aphelenchids, which include *B. xylophilus*, these glands are composed of two subventral and one dorsal gland cell. Despite the morphological similarity of *B. xylophilus* to other PPNs, it is taxonomically unrelated (Van Megen *et al*., 2009) and has a uniquely complex mode of parasitism.

Effectors have been identified from PPNs, including effectors that induce changes in the host cells, facilitate migration and modulate host defences (reviewed by Haegeman *et al*., 2012; Mitchum *et al*., 2013). However, the vast majority of these studies have focused on cyst and root-knot nematodes. Previous studies on PWN have often relied on attempts to identify orthologues of cyst nematode or root-knot nematode effectors from expressed sequence tag (EST) and genomic datasets (Kikuchi *et al*., 2011; Yan *et al*., 2012). This has allowed the identification of a range of cell wall-degrading enzymes that disrupt the plant and fungal cell wall, such as GH45 cellulases, several pectate lyases, expansins and β-1,3-endoglucanases (Kikuchi *et al*., 2004, 2005, 2006, 2009). However, PWN has an entirely different parasitic strategy from cyst nematodes and root-knot nematodes, which does not require the nematode to keep host tissues alive for a prolonged period of biotrophy, and is taxonomically unrelated to these nematodes. It is therefore important to consider alternative approaches which do not make a priori assumptions about the nature of effector molecules. For example, one study has used proteomics analysis of secreted proteins collected from nematodes stimulated with pine extracts and identified cell wall-degrading enzymes, detoxification enzymes and peptidases amongst the secreted proteins (Shinya *et al*., 2013). In an alternative approach, microarray analysis has been used to identify secreted proteins up-regulated during infection (Qiu *et al*., 2013).

Here, we describe a differential expression-based approach for the identification of effectors from PWN. We use RNAseq and bioinformatic analyses to identify a panel of potentially secreted proteins up-regulated after infection. Importantly, and in contrast with other studies of this type, we use *in situ* hybridization to examine the spatial expression profiles of candidate effectors and confirm that some are expressed in the pharyngeal gland cells. We show that detoxification proteins are deployed in a two-layer strategy, most likely in order to counter defence responses of the host. In addition, we examine morphological changes in the PWN pharyngeal gland cells across the life cycle and compare these with the development of these structures in cyst and root-knot nematodes.

RESULTS

Characterization of the pharyngeal gland cells of PWN

Previous studies on effectors of PWN have not attempted to identify the specific gland cells in which different putative effectors are expressed. This is frequently justified on the basis that the pharyngeal gland cells are difficult to distinguish as they are dorsally overlapping and all connect to similar positions in the large median oesophageal bulb (Nickle *et al*., 1981). To rectify this, and to allow the precise site of expression of effectors to be determined, we first undertook a detailed morphological analysis of the structure of the pharyngeal gland cells in juveniles and adults of *B. xylophilus*. The dorsal and subventral gland cells were readily distinguished in both juveniles and adults (Fig. 1). Measurements of the gland cells showed that, although there was no significant difference in the size of the subventral gland cells between juveniles and adults, the dorsal gland is significantly larger ($P \leq 0.05$) in the adult stage than in the juvenile stages (Fig. 1; Table 1).

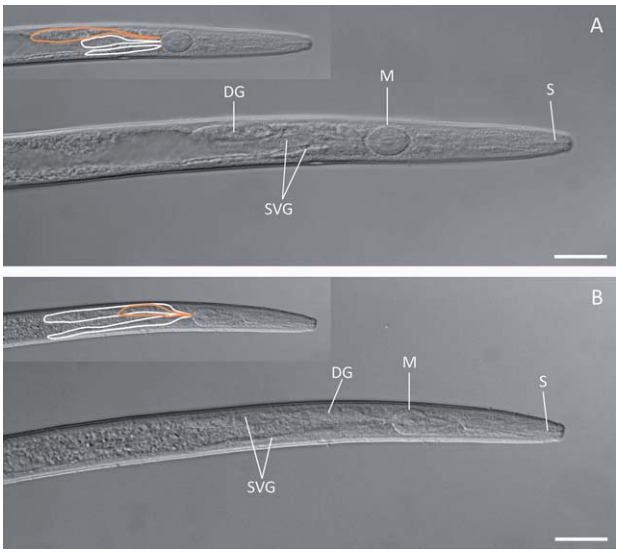


Fig. 1 Positions of pharyngeal gland cells in adult (A) and juvenile (B) *Bursaphelenchus xylophilus*. DG, dorsal glands; M, median bulb; S, stylet; SVG, subventral glands. Subventral glands (white) and dorsal gland (orange) are outlined in the insets. Scale bar, 20 μm.

Table 1 Measurements of the dorsal and subventral pharyngeal gland cells of *Bursaphelenchus xylophilus*, BxPt75OH isolate [in μm and in form: mean ± SD (range)], calculated from 10 individuals for each life stage.

	Juveniles	Adults
Dorsal gland cell	30.9 ± 4.43 (24–38.2)	66.9 ± 6.48 (53.5–73.8)
Subventral gland cell	57.5 ± 8.62 (41.9–72)	41.5 ± 2.26 (39.2–45.1)

Differential gene expression in mycetophagous and phytophagous stages of *B. xylophilus* and identification of candidate effectors

Differential gene expression analysis showed extensive variation between replicates of some life conditions, in particular the fungal feeding (FF) and 15 days post-infection (15-dpi) samples, which failed to cluster in a heat map analysis. This meant that only 29 transcripts were identified as being differentially expressed between the mycetophagous and phytophagous life stages (Fig. S1, see Supporting Information). These genes represent a much lower proportion of the *B. xylophilus* genes than expected, given the very different environments represented by these life stages. In spite of this, genes that may have a role in the host–parasite interaction were included in the sequences identified as differentially expressed after infection, including glutathione *S*-transferase (GST), GHF45 cellulases, peptidases and GH16 endoglucanases (Table S1, see Supporting Information).

An alternative differential expression approach was used in parallel. The top 200 sequences up-regulated in the parasitic life stage of the nematode were identified. These sequences included numerous known effectors from this species (e.g. cell wall-degrading enzymes). The most highly represented gene ontology (GO) terms in this set of 200 genes in the molecular function category were hydrolase, oxidoreductase and lyase activity (Fig. S2, see Supporting Information). Seventy-three of these 200 genes were predicted to have a signal peptide and to lack transmembrane domains. This represents a significant enrichment of potentially secreted proteins compared with the proportion in the whole predicted gene set for this nematode (36.5% versus 12.7%; $P < 0.0001$; χ^2 test analysis). Fewer than one-half (33) of these 73 potentially secreted proteins gave matches in BLAST searches against the non-redundant (NR) database, whereas the other 40 sequences encoded proteins that gave no matches and were therefore considered pioneers. A subset of 46 putatively secreted proteins was subsequently selected for further analysis (Table 2); these were the most highly up-regulated during infection and/or had matches in the database which suggested a potential role in parasitism. These sequences include transcripts encoding several classes of proteases, fatty acid transport proteins, putative V5/TPx1 venom allergen-like proteins (VAPs), a lysozyme, several enzymes involved in the detoxification of xenobiotic compounds and the most highly expressed pioneer genes (Table 2). The pipeline used to generate this list of candidate effectors is summarized in Fig. 2.

Table 2 List of candidate effector genes categorized by predicted function.

Predicted function	Putative protein domain (GeneDB annotation)
PROTEASES (10)	Aspartic protease A1 (5) Cysteine proteases C1A (1); C46 (1) Serine-type protease (2) Metallo-type protease M13 (1)
FATTY ACID METABOLISM (2)	Fatty acid retinoid-binding proteins
DETOXIFICATION OF XENOBIOTIC COMPOUNDS (12)	FMO (flavin monooxygenase) (2) UDP-glucuronosyl transferase (2) Multicopper putative acid oxidase (1) Glutathione *S*-transferase (2) Cytochrome P450 (3) Acid phosphatase (1) Epoxide hydrolase (1)
UNKNOWN PROTEINS DOMAIN (PIONEERS) (16)	None
PROTEIN WITH TOXIN DOMAIN (2)	Metridin-like Sht toxin domain
ALLERGENS (1)	Putative allergen V5/TPx1
GLYCOSYL HYDROLASE CLASSES (2)	GH29 (α-L-fuco domain) GH30-GH2
LYSOZYME ACTIVITY (1)	Lysozyme 7,8

Localization and validation of effectors

In situ hybridization was used to investigate the spatial expression patterns of the 46 putatively secreted proteins in mixed life stage nematodes. The majority of the genes that gave a signal (18 sequences) were expressed in the intestine (Fig. 3), whereas one gene was expressed in the glandular tissues surrounding the anterior sense organs (Fig. 3a) and 17 genes gave no signal in *in situ* hybridization reactions (not shown). Ten genes were expressed in the gland cells; four in the dorsal gland cell and six in the subventral gland cells (Fig. 4). The gland cell genes were similar in sequence to a putative fatty acid and retinoid-binding protein (*BUX.s00422.201*) (Fig. 4a), two pioneer genes (*BUX.s00083.48*, *BUX.s01109.178*) (Fig. 4b, d), one cytochrome P450 (*BUX.s00116.698*) (Fig. 4c), a lysozyme protein (*BUX.s01066.2*) (Fig. 4e) and a predicted VAP protein (*BUX.s00116.606*) (Fig. 4f) expressed in the subventral gland cells. Genes similar in sequence to two putative GSTs (*BUX.s01254.333*, *BUX.s00647.112*) (Fig. 4g, i), one pioneer gene (*BUX.s01144.122*) (Fig. 4h) and a peptidase C1A (*BUX.01147.177*) (Fig. 4j) were expressed in the dorsal gland cell. No signal was detected using sense probes (e.g. Fig. 4k, l). The 10 gland cell-localized sequences represent novel effectors that could be delivered into the host through the stylet during infection.

The expression levels of the 10 putative effectors identified as being expressed in the gland cells were validated by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and compared with the results from the normalized

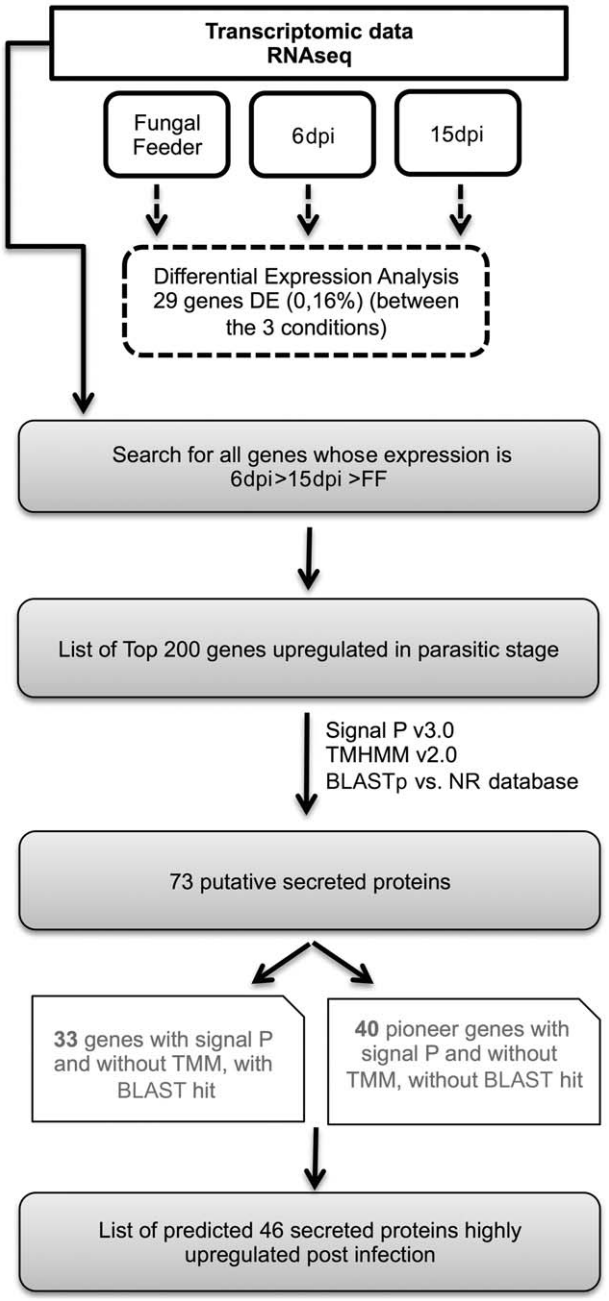


Fig. 2 Bioinformatics pipeline for the identification of candidate effectors from *Bursaphelenchus xylophilus*. DE, differentially expressed; dpi, days post-infection; FF, fungal feeder.

expression values obtained by RNAseq (Fig. S3, see Supporting Information). RT-PCR showed that all 10 putative effector genes were expressed in nematodes after infection of the host. All, with the exception of the putative lysozyme (*BUX.s01066.2*) and cytochrome P450 (*BUX.s00116.698*), were also expressed in the fungal feeder condition. The last two genes were only expressed at 15 and 6 dpi, respectively.

DISCUSSION

A range of morphobiometric, ecological and population genetic studies have been carried out on *B. xylophilus* (Moens and Perry, 2009). Other studies have identified host physiological changes that occur on infection of the nematode (Fukuda, 1997; Hirao *et al*., 2012; Mamiya, 2012). However, compared with cyst and root-knot nematodes, little information is available on the nature of effectors secreted by PWN or the details of the molecular basis by which it parasitizes plants. *Bursaphelenchus xylophilus* has a unique feeding behaviour, a complex life cycle and infests a narrow host range of pine tree species. These features, coupled with the economic damage caused, make further studies on the effector biology of *B. xylophilus* a priority.

The pharyngeal gland cells are the source of the majority of nematode effectors (e.g. Haegeman *et al*., 2012). Like most tylenchid nematodes (including root-knot and cyst nematodes) and other nematode groups, *B. xylophilus* has two subventral gland cells and one dorsal gland cell (Gheysen and Jones, 2006; Haegeman *et al*., 2012; Maule and Curtis, 2011). In *B. xylophilus*, the three pharyngeal gland cells dorsally overlap the intestine and are connected to similar positions in the large median bulb, which can make them difficult to distinguish (Nickle *et al*., 1981). Despite this, we were able to show that the dorsal gland cell in *B. xylophilus* is larger in the adult stages than in juveniles, as is seen in the sedentary stages of root-knot and cyst nematodes, such as *Meloidogyne incognita* and *Heterodera glycines* (Endo, 1987, 1993; Hussey and Mims, 1990). In sedentary nematodes, the subventral gland cells decrease in size after the formation of the feeding structure (Maule and Curtis, 2011). By contrast, the subventral gland cells of *B. xylophilus* remain similar in size in juvenile and adult stages, suggesting a prolonged role in parasitism. Consistent with this, the majority of putative effectors identified here were expressed in the subventral gland. Together our findings align well with a recent study on *B. mucronatus*, a species closely related to *B. xylophilus*, which showed that a larger number of secretory granules are present in the subventral glands during the juvenile stages and in the dorsal gland during the adult stages (Carletti *et al*., 2013).

We generated transcriptomics datasets from mycophagous (pre-invasion of the host) and phytophagous (post-invasion of the host) stages of the nematode. Our first analysis unexpectedly showed extensive variation between replicates of the nematode samples, particularly at the later stages of infection. A similar independent study (Dr. Taisei Kikuchi, Division of Parasitology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan) has shown that the environmental conditions (e.g. time of year) experienced by the host have a profound effect on gene expression in parasitic *B. xylophilus*, and it is likely that the variability seen here reflects a similar process. In order to collect the relatively large numbers of nematodes required for analysis, samples were

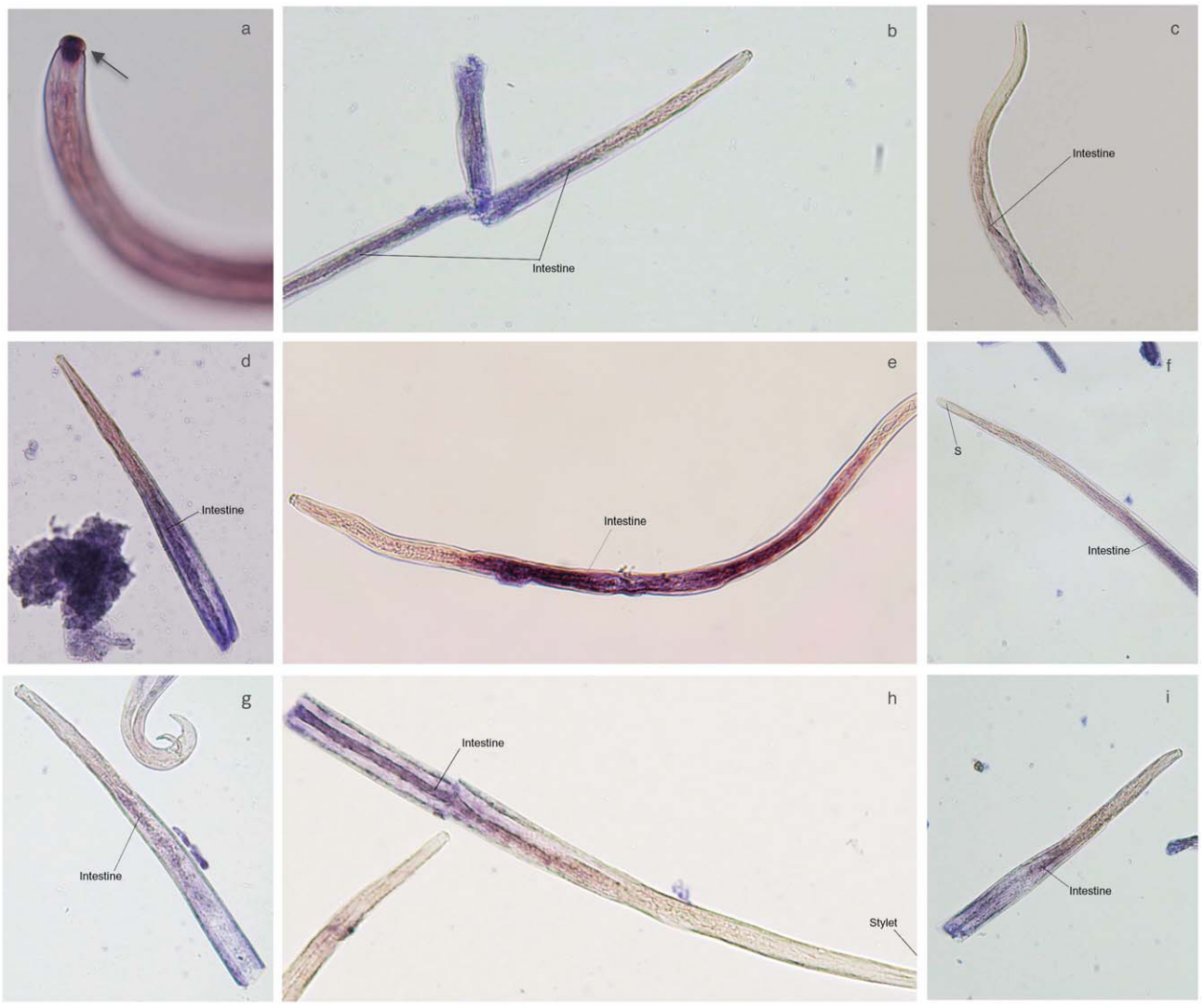


Fig. 3 Localization of the candidate proteases and detoxification enzymes encoding gene expression in the intestine by *in situ* hybridization, with the exception of putative epoxide hydrolase (*BUX.s00298.34*) (a) which was expressed in the glandular tissues surrounding the anterior sense organs. (b) Putative multicopper oxidase (*BUX.s01281.17*). (c) Putative flavin monooxygenase (*BUX.s01337.7*). (d) Putative peptidase C46 (*BUX.s01109.245*). (e) Putative UDP-glucuronosyl transferase (UGT) (*BUX.s00422.680*). (f) Putative CYP33 C-related (*BUX.s01144.121*). (g) Putative peptidase M13 (*BUX.s01661.67*). (h) Putative peptidase A1 (*BUX.s00532.10*). (i) Putative peptidase S28 (*BUX.s01144.130*).

collected from many different trees that may have been exposed to different environmental conditions. In spite of these issues, we were able to identify a panel of genes that were significantly up-regulated after infection and secreted proteins were enriched in these sequences. Subsequent *in situ* hybridization experiments identified 10 putative effector proteins expressed in the gland cells, validating the approach. A comparison of these secreted proteins with the PWN secretome dataset obtained in a previous study, using a proteomics approach (Shinya *et al*., 2013), showed that five of the effectors identified here were also identified in secreted proteins collected from *B. xylophilus* (data not shown). Although there are clearly differences in the results obtained using the two approaches, it is reassuring to see some measure of cross-validation between the two studies.

A significant proportion of the sequences up-regulated during the transition to parasitism, including some of the identified effectors, are likely to have roles in protecting the nematode from host defence responses. Pine trees respond to nematode infection by releasing a range of defence compounds in the areas surrounding the entry wound, including ethylene, terpenoids (α- and β-pinene), ROS and lipid peroxides (Fukuda, 1997). Our study revealed that one secreted cytochrome P450 and two secreted GSTs, up-regulated at the early stages of infection (6 dpi), are expressed in the subventral and dorsal gland cells, respectively

(Fig. 4). These two enzymes are major components of the pathway leading to the metabolism of xenobiotic compounds in the free-living nematode, *Caenorhabditis elegans* (Lindblom and Dodd, 2006). A secreted GST has also been identified that plays an important role in parasitism of plants by root-knot nematodes, and that most likely protects the nematode against host defences (Dubreuil *et al*., 2007; Lindblom and Dodd, 2006). Our results suggest that GST plays a similar role in *B. xylophilus* parasitism.

Our analysis showed that a range of transcripts encoding other enzymes potentially involved in the detoxification of xenobiotic compounds (including epoxide hydrolase, multicopper oxidase, flavin monooxygenase, UGT and cytochrome P450) are up-regulated after infection, but are expressed in the intestine (Fig. 3). A recent study in *C. elegans* has shown that the intestine is the first line of defence against xenobiotic compounds to oxidative stress, and has emphasized the importance of phase 2 detoxification enzymes in this process (Crook-McMahon *et al*., 2014). Our data suggest that *B. xylophilus* uses a two-layered approach to protect itself against host-derived xenobiotic compounds. Some enzymes involved in detoxification pathways are secreted into the host, representing the first layer, whereas others are up-regulated in the digestive system, which will be exposed to ingested host materials, and represent the second.

The other identified effectors have a range of potential roles in the host–parasite interaction. One effector was similar to secreted VAPs from other nematodes and was highly expressed at 6 dpi. Three secreted VAPs have previously been characterized from PWN (Lin *et al*., 2011). It has been suggested that one of these (*Bx-vap-1*) is involved in the migration of PWN inside the host (Kang *et al*., 2012). More recently, a study of the potato cyst nematode *Globodera rostochiensis* has shown that VAPs from this species are required for the suppression of host immunity, possibly through a proteinase inhibition activity (Lozano-Torres *et al*., 2014). VAPs are conserved throughout nematodes and are frequently up-regulated in parasitic nematodes on infection. It is therefore possible that VAPs are widely deployed against host defence responses that require the activity of host proteinases.

The *B. xylophilus* genome encodes hundreds of proteinases (Kikuchi *et al*., 2011). Our RNAseq analysis showed that several, including cysteine, metallo, aspartic and serine catalytic classes, are up-regulated after infection. The majority of these were expressed in the intestine (Fig. 3), consistent with a role in digestion. However, we identified a cysteine proteinase C1A that is expressed in the gland cells and up-regulated at a later stage of infection (15 dpi). This enzyme could play a role in digesting host tissues during migration, or may also target host proteins involved in defence responses, as has been shown in animal-parasitic nematodes (Malagón *et al*., 2013; Sajid and McKerrow, 2002). Consistent with this, plants are known to deploy proteinase inhibitors against pathogens (Xia, 2004).

A secreted fatty acid and retinol-binding protein (FAR) was identified that is expressed in the subventral gland cells during the infection of the host. Most nematode lipid-binding proteins are thought to be important for the internal transport of lipids. However, FAR proteins have been identified in both cyst (*Globodera pallida*) and root-knot nematodes that bind precursors of lipid-based plant defence signalling compounds important in the jasmonate signalling pathway (Iberkleid *et al*., 2013; Prior *et al*., 2001). The role of these pathways in terms of the interaction between *B. xylophilus* and its host remains to be determined.

One effector sequence was similar to lysozymes from a range of nematode species. Nematode lysozymes may have a role in the digestion of host proteins and may also be important in the protection of nematodes against other pathogens. Several lysozymes with antibacterial activity have been described from *C. elegans* (Boehnisch *et al*., 2011) that are thought to play an important role in defence against pathogenic bacteria. It is known that *B. xylophilus* is associated with a range of bacterial species which may form an important component of the infection process (Vicente *et al*., 2012b). The deployment of lysozyme by *B. xylophilus* may restrict bacterial growth in the regions infected by the nematode, reducing competition for food resources.

Our analysis also identified three pioneer genes expressed in the subventral and dorsal gland cells that are highly up-regulated at 6 and 15 dpi. Given the absence of these proteins from other nematodes, they are likely to play key roles in the biology of *B. xylophilus*. Effectors from other nematodes are frequently novel proteins (e.g. Gao *et al*., 2003). The characterization of the function of such sequences in detail is likely to be challenging.

In summary, we have described a transcriptomics approach which has allowed the identification of 10 novel effectors and 18 proteins from the digestive system of *B. xylophilus*. We have also demonstrated that the gland cells of this species, like those of other PPNs, change in structure during the life cycle. Our data suggest that *B. xylophilus* uses a multilayered system of enzymatic detoxification to metabolize host-derived xenobiotics within the host and in the digestive system.

EXPERIMENTAL PROCEDURES

Biological material

The Portuguese isolate of *B. xylophilus*, BxPt75OH, used in this study originated from a symptomatic pine tree in Oliveira do Hospital district in the central region of mainland Portugal. The nematode was identified to species level (Nickle *et al*., 1981) and cultures were maintained in Erlenmeyer flasks containing *Botrytis cinerea* on barley seeds at 25 °C (Evans, 1970). Nematodes were extracted using the Baermann funnel technique (Southey, 1986) for 24 h, followed by sieving (38 μm).

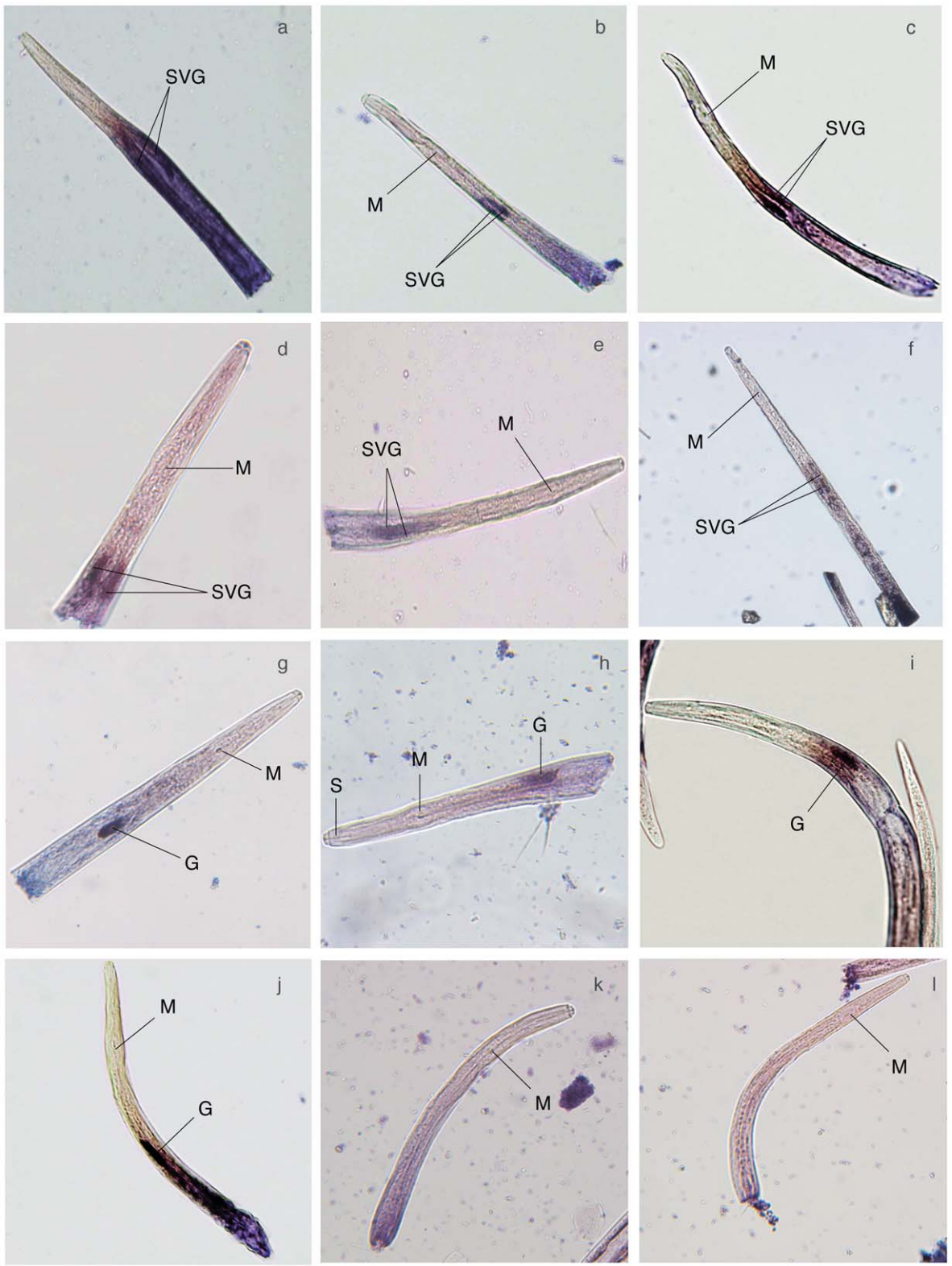


Fig. 4 Localization of candidate effector expression in the pharyngeal gland cells by *in situ* hybridization. (a) *BUX.s00422.201*. (b) *BUX.s00083.48*. (c) *BUX.s00116.698*. (d) *BUX.s01109.178*. (e) *BUX.s01066.2*. (f) *BUX.s00116.606*. (g) *BUX.s01254.333*. (h) *BUX.s01144.122*. (i) *BUX.s00647.112*. (j) *BUX.s01147.177*. k and l are control forward probe. G, dorsal gland cell; M, median bulb; SVG, subventral glands.

Morphometric studies of the pharyngeal gland cells

Mixed life stage nematodes were killed by heat (water bath for approximately 15 min until the temperature reached 60 °C), fixed in 4% formaldehyde and prepared for mounting according to Siddiqi (1964). The nematodes were transferred into lactophenol and incubated for 24 h at 40 °C. The nematodes were then transferred to a solution of 75% glycerine : 25% lactophenol for approximately 24 h at 40 °C, until the lactophenol had evaporated and the nematodes were in pure glycerine. The nematodes were then mounted in glycerine surrounded by a ring of paraffin on a glass slide. A coverslip was placed on the top of the paraffin ring and the preparation was heated until the paraffin had melted. The slides were observed under a laser scanning microscope (Zeiss LSM 710, Germany) using the differential interference contrast (DIC) method.

Measurements of the dorsal and subventral gland cells were performed from 10 individuals for each of the life stages (juveniles and adults), mounted using an agar pad technique, as described by Eisenback (2012). Statistical significance was tested using Mann–Whitney U-test analysis (STATISTICA v12.0) (Mann and Whitney, 1947). Images (measurements) were recorded using an Olympus BX50 light microscope and Cell Software (Olympus, Japan).

PWN inoculation trials

Two-month-old maritime pine trees (*Pinus pinaster*) obtained from a Portuguese nursery were used for inoculation of the PWN isolate. Approximately 2000 mixed life stage nematodes were cultured on fungi as described above and inoculated into a small wound (5 mm) made on the pine stem using a sterilized scalpel. Infections were conducted under controlled conditions (average temperature, 23 °C; humidity, 50%). A subset of the nematodes prepared for each biological replicate was frozen in liquid nitrogen and stored at −80 °C for RNA extraction as the mycetophagous controls. The inoculated nematodes were collected from the trees at 6 and 15 dpi. For this, the pine stems were cut and nematodes were collected by the Baermann funnel technique for approximately 2 h. Nematodes were centrifuged by sucrose flotation (50%), washed three times in 1 × phosphate-buffered saline (PBS) and frozen in liquid nitrogen.

RNA extraction and sequencing

Nematode RNA was extracted from samples corresponding to three different conditions: FF (pre-inoculation), 6 dpi and 15 dpi. RNA extraction was performed using the GeneJET RNA Purification Kit (FermentasThermoScientific, MA, USA) following the manufacturer's instructions. The RNA integrity number (RIN) was assessed using a Bioanalyser (Agilent Technologies, California, USA). The samples (two biological replicates for the FF condition and three biological replicates for the other two conditions), each with a RIN above '7', were used for paired-end sequencing at The Genome Analysis Centre (TGAC), Norwich, Norfolk, UK, on the Illumina HiSeq platform. RNAseq data described in this article are available through the European Nucleotide Archive (ENA) under accession number PRJEB9165.

Differential gene expression analysis

Raw RNA reads were trimmed of adapter sequences and low-quality bases (phred score < 22) using Trimmomatic v0.32 (Bolger *et al*., 2014) (Table S2, see Supporting Information). Remaining high-quality reads (79%) for each library were mapped back to the reference genome (http://www.genedb.org/Homepage/Bxylophilus) (Kikuchi *et al*., 2011) using Tophat2 (Kim *et al*., 2013). Read counts for each gene were determined using bedtools v2.16.2 and normalized (TMM) using Trinity wrapper scripts (Haas *et al*., 2013) for EdgeR (Robinson *et al*., 2010). Two differential expression analyses were carried out on normalized read counts: (i) transcripts with a minimum fold change of four ($P < 0.001$) between conditions were identified using Trinity wrapper scripts for EdgeR, and clustered based on 20% tree height; (ii) all genes were ranked by the ratio of their average normalized expression during all *in planta* stages (6 dpi + 15 dpi) compared with FF. The top 200 most differentially regulated genes were selected for further analyses. Potentially secreted protein sequences were identified using a workflow within a local installation of Galaxy on the basis of the presence of an N-terminal signal peptide (predicted by SignalP 3.0; Bendtsen *et al*., 2004) and the absence of a transmembrane domain (predicted by TMHMM 2.0; Krogh *et al*., 2001) (Cock and Pritchard, 2014). A BLASTp search (using Galaxy version 0.1.01) was performed against the NR database (cut-off value, 1e-03) for all candidates in order to predict their functions based on sequence similarity. The putative protein domain description is based on the annotation of the *B. xylophilus* genome (version 1.2) available on Gene DB (http://www.genedb.org/Homepage/Bxylophilus).

In situ hybridization

In situ hybridization using digoxigenin-labelled probes was performed in order to determine the spatial expression patterns of candidate effectors based on the protocol described by De Boer *et al*. (1998). For each candidate gene, a fragment of approximately 200 base pairs was amplified from the coding region and used as a template for the synthesis of both sense and antisense probes. The primers used for these reactions are shown in Table S3 (see Supporting Information).

Validation of the expression profiles of candidate effectors

The expression profiles of the genes identified as expressed in the gland cells were validated by semi-quantitative PCR as described in Chen *et al*. (2005). Actin was used as a control for all reactions (Table S3). Expression levels of each gene relative to the actin control were determined in the three different conditions (FF, 6 and 15 dpi) using cDNA synthesized from total RNA as a template and after 30–35 cycles. The results were analysed by electrophoresis in agarose gels. The qualitative results were compared with the predicted expression values obtained by RNAseq data.

ACKNOWLEDGEMENTS

This work was supported by the REPHRAME project (KBBE.2010.1.4-09). ME is funded by FCT (Fundação para a Ciência e a Tecnologia, IP) under a PhD grant (SFRH/BD/84541/2012). ME and MM are also funded by FEDER Funds through the Operational Programme for Competitiveness Factors—COMPETE and National Funds through FCT—Foundation for Science and Technology under the Strategic Projects PEst-C/AGR/UI0115/2011 and PEst-OE/AGR/UI0115/2014. AS was funded by an ERASMUS MUNDUS Category B scholarship awarded through project 2008–2102 (EUMAINE).

The James Hutton Institute receives funding from the Scottish Government Rural and Environmental Science and Analytical Services division (RESAS).

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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 Differential expression analysis of the transcripts. The heatmap resulting from the RNAseq analysis, using eight samples in three different conditions—pre-invasive/mycetophagous (fungal feeding, FF) and post-invasive/phytophagous [6 and 15 days post-infection (dpi)].

Fig. S2 Analysis of the most represented molecular function (level 3) in the top 200 set of up-regulated genes obtained by a bioinformatics pipeline.

Fig. S3 Validation of the expression of the secreted effectors by semi-quantitative polymerase chain reaction using actin as housekeeping gene and the primers described in Table S3. The results were analysed by gel electrophoresis and, for each candidate, the results of both actin and the candidate gene are presented. On the right, the bar chart represents the normalized expression values (fragments per kilobase of exon per million mapped fragments, FPKM) predicted by RNAseq for each candidate gene.

Table S1 List of the 29 differentially expressed transcripts between mycetophagous and phytophagous stages. Detailed description of the 29 transcripts includes the presence or absence of putative signal peptide, the putative protein domain (according to Gene DB annotation of version 1.2 of the genome; available at http://www.genedb.org/Homepage/Bxylophilus), the top match and e-value of the BLASTp analysis against the non-redundant (NR) database (cut-off value, 1e-03), and the normalized expression profile in the three different conditions [fungal feeding (FF) nematodes and nematodes 6 and 15 days post-infection (dpi)]. The normalized expression values are in fragments per kilobase of exon per million mapped fragments (FPKM).

Table S2 Summary of RNAseq data.

Table S3 List of pairs of primers used for the amplification of probes for *in situ* hybridization. Gene model according to Kikuchi *et al*. (2011) and sequences available at http://www.genedb.org/Homepage/Bxylophilus. bp, base pair.