View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Genomics 94 (2009) 70–77

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Construction and characterization of subtractive stage-specific expressed sequence tag (EST) libraries of the pinewood nematode *Bursaphelenchus xylophilus*

Jae Soon Kang [a], Hyoungseok Lee [b], Il Sung Moon [c], Yi Lee [d], Young Ho Koh [e], Yeon Ho Je [a], Kook-Jin Lim [f], Si Hyeock Lee [a,g,*]

[a] Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, South Korea
[b] Polar Bio Center, Korea Polar Research Institute, KORDI, Incheon 406-840, South Korea
[c] Nambu Forest Experiment Station, Korea Forest Research Institute, Chinju 660-300, South Korea
[d] School of Plant Science and Bio-Resource, Chungbuk National University, Cheongju 361-763, South Korea
[e] Ilsong Institute of Life Science, Hallym University, Anyang 431-060, South Korea
[f] Division of Diagnostic Research and Development, LG Life Sciences, Daejeon 305-738, South Korea
[g] Research Institute for Agriculture and Life Science, Seoul National University, Seoul 151-921, South Korea

ARTICLE INFO

Article history:
Received 21 November 2008
Accepted 6 March 2009
Available online 13 March 2009

Keywords:
Bursaphelenchus xylophilus
Dispersal 4th larva stage
Pine-grown propagative mixed stage
Suppressed subtractive hybridization
ESTs
Gene ontology

ABSTRACT

To establish expressed sequence tag databases of the two life stages (the dispersal and propagative stages) of pinewood nematode *Bursaphelenchus xylophilus*, subtractive EST libraries that were specific to the dispersal 4th larval stage (D4S) and the pine-grown propagative mixed (PGPS) stage were constructed by suppressed subtractive hybridization, and annotated by BLASTx and Gene Ontology (GO). A total of 1112 (57.7%) contigs from the D4S-cDNA library and 1215 (46.7%) contigs from the PGPS-specific cDNA libraries had matched BLASTx hits ($E \leq 10^{-2}$), among which 913 (47.4%) and 960 (36.9%) contigs, respectively, were classified into three GO categories. A total of 14 genes were selected on the basis of stage-specific abundances and GO subcategories, and their transcription levels were analyzed by quantitative real-time PCR. We discussed the potentials of some stage-specific genes, such as sorbitol dehydrogenase, cysteine protease, venom allergen-like protein, and FMRFamide-like peptide, as diagnosis markers and novel control targets.

© 2009 Elsevier Inc. All rights reserved.

Introduction

The pinewood nematode, *Bursaphlenechus xylophilus* (Steinner and Buhrer) Nickle, is a serious pathogen in several countries including Japan, China, and Korea [1–3]. *B. xylophilus* has two different life stages, a propagative stage for reproduction and parasitization, and a dispersal stage for dauer formation and migration by transfer vector [4]. The propagative stage of *B. xylophilus* is composed of egg, larval, and adult stages, and is the stage during which most host–nematode interactions, such as parasitic infestation, defense by the host, and counter-defense by the parasite, take place. In contrast, the dispersal stage has only 3rd and 4th dispersal larvae which have a thicker cuticle and external cortical layer and suppressed gonad formation compared to the propagative stage larvae [5–7]. Chemical cues provided by *Monochamus* vectors induce the dispersal 3rd larvae to aggregate and to molt to the dispersal 4th larvae [8,9]. Because both stages of *B. xylophilus* are very different in morphology and physiology, the gene expression profiles are also expected to be very different. To study stage-specific biology, physiology, and pathogenicity of *B. xylophilus*, it is necessary to construct a database of genes that are differentially expressed during the two life stages of this organism.

Nematode genome sequencing projects have been conducted in various nematode species ranging from free-living nematodes to animal- or plant-parasitic nematodes. *Caenorhabditis elegans* is one of the most widely-used model organisms, and its genome sequence was completed in 1998 [10]. Whole genome sequencing provides the most abundant information on an organism's genetic make-up, but the process is expensive and takes relatively long time [11]. As an alternative, expressed sequence tag (EST), which are generated by one-shot random sequencing of a cDNA library, are used to obtain essential information about the functional genome. ESTs projects for more than 40 nematode species including animal- and plant-parasitic nematodes have recently been completed, and the total number of EST submissions in the Nematode Genome Sequencing Center exceeds over 500,000 (Nematode.net, http://www.nematode.net). ESTs,

Abbreviations: Ald, allergen Lep d; ASM, acid sphingomyelinase; Cab, cathepsin b; CBG23351, hypothetical protein CBG23351 containing cysteine protease domain; Cyps, cysteine protease; D4S, dispersal 4th larval stage; Ephx, epoxide hydrolase; ESTs, expressed sequence tags; FAD, fatty acid desaturase; FLP, FMRFamide-like peptide; GDP, Glycerophosphoryl diester phosphodiesterase; HSP, heat shock protein; MGPS, media-grown propagative mixed stage; PGPS, pine-grown propagative mixed stage; SEP, SCP-Like extracellular protein; Sodh, sorbitol dehydrogenase; TTL, transthyretin like protein; UGT, UDP-Glucuronosyl transferase; Vap, venom allergen-like protein.

* Corresponding author. Department of Agricultural Biotechnology, Seoul National University, Seoul 151-921, South Korea. Fax: +82 2 873 2319.
E-mail address: shlee22@snu.ac.kr (S.H. Lee).

0888-7543/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygeno.2009.03.001

Table 1
Generation of ESTs in two subtractive stage-specific cDNA libraries.

Library name	Suppressed subtractive hybridization		Clones	High quality sequence (%)	Contigs		Average length (bp)
	Tester	Driver			Multiple sequences	Singleton	
D4S	Dispersal 4th larval stage	Pine-grown propagative mixed stage	3072	2853 (92.9%)	371	1556	456
PGPS	Pine-grown propagative mixed stage	Media-grown propagative mixed stage	3840	3569 (93.0%)	390	2214	385

because of their derivation from mRNAs, provide an efficient and powerful molecular tool for the exploration of putative gene functions under specific conditions and for searching for candidate genes. A total of 13,327 ESTs from *B. xylophilus* were generated from the mixed stage (10,670 ESTs) and dauer-like (dispersal 4th larva) stages (2657 ESTs) [12]. To construct stage-specific libraries, the propagative mixed stage nematode was obtained from fungi cultures or pine callus cell cultures, whereas the dauer-like stage nematode was separated from a pine log using the artificial pupal chamber method or vector beetles. However, since the majority (86.2%) of the mixed stage ESTs was generated from nematodes grown on fungal cultures, these EST libraries may not be specific enough to detect genes that are expressed only during the propagative stage inside the pine tree host.

In this study, we constructed subtractive *B. xylophilus* cDNA libraries specific to the dispersal 4th larva stage (D4S) and pine-grown propagative mixed stage (PGPS) by suppressed subtractive hybridization (SSH) [13–15]. We focused on enriching genes specifically related to the pine tree–nematode interaction by subtracting the PGPS cDNAs with the media (fungi)-grown propagative mixed stage (MGPS) cDNAs. We generated ESTs to search for stage-specific genes, and characterized the properties of several stage-specific genes. We discuss the potential use of certain stage-specific genes as candidate diagnosis markers and novel targets for the control of *B. xylophilus*.

Results

EST generation and annotation

Two subtractive cDNA libraries of *B. xylophilus* were constructed by SSH (Table 1). A total of 3072 and 3840 clones from the D4S- and PGPS-specific subtractive cDNA libraries were sequenced, respectively, to generate ESTs. Trimming of the ambiguous sequences ($N>5\%$), short sequences (<100 bp), and vector sequences resulted in 2853 high quality sequences, which were subsequently clustered into 371 multiple sequences (containing two or more ESTs) and 1556 singletons (containing only one EST) in the subtractive D4S library. Through the same procedures, 390 multiple sequences and 2214 singletons were obtained from 3569 high quality sequences in the subtractive PGPS library. The average lengths of ESTs in the subtractive D4S and PGPS libraries were 456 bp and 385 bp, respectively.

Each contig was used for BLAST search on the GenBank non-redundant database. Among the 1927 contigs in the D4S library, 785 (40.7%), 327 (17.0%), and 567 (29.4%) contigs exhibited high ($E\text{-value}\leq 10^{-14}$), medium ($10^{-14}<E\text{-value}\leq 10^{-2}$) and low ($E\text{-value}>10^{-2}$) identities to known genes in the database, respectively, and the remaining 248 (12.9%) contigs had no significant matches in any of the databases searched. Similarly, among the 2604 contigs in the PGPS library, 746 (28.6%), 469 (18.0%), and 789 (30.3%) contigs showed high, medium and low identities to known genes, respectively, while 600 (23.1%) contigs did not match any known genes.

Among the genes abundantly expressed (contigs composed of 6–70 ESTs), those showing medium to high levels of identity to known genes ($E\text{-value}\leq 10^{-2}$) in the D4S and PGPS are listed in Tables 2 and 3, respectively. In D4S, the most abundant contig composed of 48 ESTs (BXsDS0892) was similar to a gene from bacteria, suggesting that it is likely originated from symbiotic microorganisms. Interestingly, the next most abundant contigs (BXsDS0066 and BXsDS0257; 17 and 13 ESTs, respectively) were homologous to the *C. elegans* HSP16.1 gene that is known to be associated with stress response during the dispersal stage of this organism. BXsDS0373 and BXsDS0762 (12 and 9 ESTs, respectively) showed high identities to a *Caenorhabditis briggsae* hypothetical protein CBG22607 and CBG06065. Other abundant genes containing 9 ESTs (BXsDS0898 and BXsDS1088) were annotated as Sodh 1 [12] and GDP that are known to be overexpressed in the dauer stage of *C. elegance* (Dauer Metabolic Database, http://dauerdb.org).

Table 2
The contigs with the most abundant ESTs that have BLAST matches in the subtractive D4S library of *B. xylophilus*.

Contig ID	Count	Length (bp)	BLAST match							
			Accession number	Description	Species	E-value	Score	Query Coverage	Subject Coverage	Identity (%)
BXsDS0892	48	745	YP_001159312.1	Hypothetical protein Strop_2488	*Salinispora tropica* CNB-440 (bacteria)	4.00E−49	197	89.8	80.0	47
BXsDS0066	30	573	AAA28066.1	Hsp 16.1	*Caenorhabditis elegans*	4.00E−10	60.8	51.8	63.7	31
BXsDS0480	15	674	CAD88796.1	Cytochrome oxidase subunit III	*Necator americanus*	3.00E−66	254	87.7	77.3	63
BXsDS0373	12	448	XP_001675265.1	Hypothetical protein CBG22607	*C. briggsae* AF16	3.00E−22	107	91.9	49.0	41
BXsDS0762	9	373	XP_001670285.1	Hypothetical protein CBG06065	*C. briggsae* AF16	3.00E−16	87	97.3	40.7	33
BXsDS0898	9	708	NP_505991.1	Sorbitol dehydrogenase family member (sodh1)	*C. elegans*	3.00E−54	214	84.7	57.3	52
BXsDS1088	9	352	NP_501176.1	Glycerophosphoryl diester phosphodiesterase	*C. elegans*	4.00E−12	73.6	82.7	27.2	45
BXsDS0578	8	661	NP_006949.1	Cytochrome c oxidase subunit I	*Ascaris suum*	1.00E−84	315	93.5	39.2	73
BXsDS0196	7	655	EDP36463.1	Polyadenylate-binding protein 1, putative	*Brugia malayi*	2.00E−86	91	84.8	39.7	71
BXsDS0343	7	398	Q3KSM3	RDRP_PRV RNA-directed RNA polymerase (RdRp)	–	1.00E−21	105	89.7	11.7	46
BXsDS1099	6	595	YP_001620022.1	IS-10 transposase	*Acholeplasma laidlawii* PG-8A (bacteria)	1.00E−79	251	81.2	40.0	77
BXsDS1239	6	349	NP_504512.1	p-Nitrophenyl phosphatase	*C. elegans*	1.00E−15	85.5	46.4	16.1	72
BXsDS1344	6	397	NP_001024192.1	Receptor mediated endocytosis family member (rme-1)	*C. elegans*	8.00E−42	172	96.0	16.2	63

Table 3
The contigs with the most abundant ESTs that have BLAST matches in the subtractive PGPS library of *B. xylophilus*.

Contig ID	Count	Length (bp)	BLAST match							
			Accession number	Description	Species	E-value	Score	Query Coverage	Subject Coverage	Identity (%)
BXsPS0980	10	577	AAF90133.1	DNA polymerase epsilon, subunit C	*Caenorhabditis elegans*	7.00E−03	55	68.1	86.4	34
BXsPS0255	10	621	XP_001526050.1	Conserved hypothetical protein	*Lodderomyces elongisporus* (yeast)	3.00E−33	144	69.6	72.4	40
BXsPS0181	10	389	XP_001671506.1	Hypothetical protein CBG04431	*C. briggsae AF16*	2.00E−39	135	98.7	25.8	54
BXsPS0062	9	324	CAD88796.1	Cytochrome oxidase subunit III	*Necator americanus*	4.00E−62	131	74.1	31.4	75
BXsPS0256	9	498	BAD12484.1	Alcohol dehydrogenase	*Candida boidinii* (yeast)	3.00E−33	73	50.0	22.4	54
BXsPS0312	7	271	XP_001671506.1	Hypothetical protein CBG04431	*C. briggsae AF16*	1.00E−30	92	97.4	17.6	52
BXsPS0243	7	610	XP_001673640.1	Hypothetical protein CBG23351	*C. briggsae AF16*	8.00E−35	149	88.5	54.4	41
BXsPS0140	7	662	NP_505215.2	Temporarily assigned gene name family member (tag-196)	*C. elegans*	7.00E−44	178	86.1	41.4	48
BXsPS0697	7	448	XP_001670464.1	Hypothetical protein CBG05849	*C. briggsae AF16*	2.00E−15	88	59.6	25.0	47
BXsPS0765	7	355	NP_498081.2	Aldehyde dehydrogenase family member (alh-1)	*C. elegans*	8.00E−13	76	38.9	9.0	78
BXsPS0207	7	476	EDP39371.1	Fatty acid elongation protein 3	*Brugia malayi*	1.00E−37	158	97.1	57.2	50
BXsPS0883	7	471	AAB35044.1	Filarial antigen	*B. malayi*	4.00E−09	71	59.2	20.2	44
BXsPS0750	6	469	XP_001383403.2	Hypothetical protein PICST_30667	*Pichia stipitis* CBS 6054 (yeast)	2.00E−13	37	24.3	21.2	41
BXsPS0595	6	462	XP_001673335.1	UDP-Glucuronosyl transferase	*C. briggsae AF16*	6.00E−19	80.1	92.2	26.9	32
BXsPS0221	6	804	AAA18215.1	Cysteine protease	*Trypanosoma congolense*	9.00E−37	157	95.5	57.7	36
BXsPS0230	6	358	YP_001504334.1	Cytochrome c oxidase subunit III	*C. briggsae*	7.00E−36	152	88.0	41.2	69
BXsPS0456	6	647	XP_001667782.1	Hypothetical protein CBG14867	*C. briggsae AF16*	1.00E−26	123	99.2	22.2	37

Contrasts to the D4S library, genes that are putatively related with parasitism were found in a high abundance in the PGPS library. Cysteine protease (BXsPS0243 and BXsPS0221; 7 ESTs and 6 ESTs, respectively) is known to be an important gene for parasitism [16,17] and UGT (BXsPS0595; 6 ESTs) is known to play an important role in the conjugation and elimination of foreign substances in various organisms, including nematodes [18].

Gene ontology

To obtain additional insights into the putative functions of clustered genes, further analysis based on GO classification was performed. When a total of 1927 and 2604 contigs were annotated from the subtractive D4S and PGPS libraries using GO terms, 913 (47.4%) and 960 (36.9%) contigs were classified into the three major categories of 'cellular component', 'molecular function', and 'biological process', respectively. The remaining 1014 (52.6%) and 1644 (63.1%) contigs from the subtractive D4S and PGPS libraries, respectively, showed no hits. The contigs in the three major categories were further divided into several level 1 subcategories. The 'cellular component' was composed of 8 subcategories, the 'molecular function' of 11 and 12 subcategories, and the 'biological process' of 17 and 15 subcategories in the subtractive D4S and PGPS libraries, respectively (Table 4).

Among these, we focused on the subcategories of 'extracellular region/matrix', 'antioxidant activity', 'response to stimulus', and 'immune system process' as these categories are likely to be involved in the interaction of *B. xylophilus* with its pine host and *Monochamus* vector. The frequency of contigs belonging to the 'extracellular region/matrix' category, 'antioxidant activity', and 'immune system process' of D4S was lower compared to PGPS although not statistically significant ($p = 0.077–0.10$, 0.251, and 1.00). The proportion of contigs belonging to the 'response to stimulus' was slightly larger in D4S (70 contigs) than in PGPS (52 contigs) although not significantly different ($p = 0.103$).

In the subtractive D4S library, 11 contigs containing TTLs were categorized as 'extracellular region/matrix' genes, 5 contigs were classified as having 'antioxidant activity', and 43 contigs, including HSP family genes, were classified as 'response to stimulus' genes. In the PGPS library, 22 contigs containing TTLs and a few allergens were categorized as 'extracellular region/matrix' genes, 4 contigs were classified as 'antioxidant activity' genes, and 30 contigs including HSPs and FAD families were classified as 'response to stimulus' genes.

Identity analysis between subtractive D4S and PGPS libraries

Only 223 (7.8%) ESTs of the subtractive D4S library were found in the subtractive PGPS library, whereas 177 (5.0%) ESTs of the subtractive PGPS library were found in the D4S library (Table 5). This low percentage of overlapping ESTs in both libraries suggests that the two subtractive libraries are highly stage-specific.

Relative transcript levels of stage-specific genes

To verify the stage-specific expression patterns, the relative transcript level of 7 putative D4S-specific genes, 7 putative PGPS-specific genes (Table 6) and two Vaps were determined by qrtPCR. Among the 7 D4S-specific genes, HSP16.1, Sodh1, GDP and FLP3 were either composed of abundant ESTs or expected to have functions related to dauer formation or transmission, whereas TTL46, ASM1, and Ephx1 were chosen since they belong to the GO categories of 'extracellular region' and 'response to stimulus'. CBG23351, UGT, Cysp, and FAD6 were chosen as putative PGPS-specific genes because of their relative abundance in PGPS. In addition, CBG23351, Cysp, and Cab were expected to have cysteine protease activity, which is known to be important in parasitism, and UGT is thought to be associated with xenobiotic metabolism. FAD6 was the most abundant gene among the FAD families (Fad3, 6 and 7) categorized as 'stimulus to response' gene (data not shown). Ald1.02, SEP, and Cab were chosen because they appeared to be secretory proteins, as judged by GO analysis or signal peptide analysis, and involved in parasitism. Two other allegen-like proteins (Vaps), similar to Ald1.02 and known to play a role in the parasitic mechanism of animal- and plant-parasitic nematodes, were also included.

All the 7 putative D4S-specific genes showed higher transcript levels in D4S than in PGPS. Five genes, with the exception of *HSP16.1* and *Ephx1*, showed more than 2-fold higher transcript levels in D4S compared with PGPS, with Sodh1 highly and specifically over-expressed in D4S ($>2.2 \times 10^5$ fold) (Fig. 1A). Likewise, all the nine putative PGPS-specific genes exhibited PGPS-specific transcription patterns. Among these, the transcript levels of Cysp, Ald1.02,

Table 4
The list and proportion of GO categories from two libraries.

Major category	Level 1 subcategory	D4S	PGPS	p-value[a]
		Count (Proportion, %)	Count (Proportion, %)	
Cellular component	Cell	868 (59.4)	1041 (51.0)	7.51E−05
	Envelope	59 (4.0)	45 (2.2)	0.1698
	Extracellular matrix	1 (0.1)	5 (0.2)	0.1025
	Extracellular region	17 (1.2)	29 (1.4)	0.07684
	Macromolecular complex	178 (12.2)	391 (19.1)	<2.2E−16
	Membrane-enclosed lumen	10 (0.7)	9 (0.4)	0.8185
	Organelle	324 (22.2)	519 (25.4)	1.87E−11
	Virion	4 (0.3)	3 (0.1)	0.7055
	Subtotal	1461 (100.0)	2042 (100.0)	
Molecular function	Antioxidant activity	7 (0.4)	12 (0.6)	0.2513
	Binding	753 (40.7)	668 (32.7)	0.02414
	Catalytic activity	807 (43.6)	982 (48.0)	3.51E−05
	Chaperone regulator activity	2 (0.1)	1 (0.0)	0.5637
	Enzyme regulator activity	20 (1.1)	7 (0.3)	0.01235
	Molecular transducer activity	31 (1.7)	10 (0.5)	0.00104
	Motor activity	7 (0.4)	6 (0.3)	0.7815
	Nutrient reservoir activity	0 (0.0)	2 (0.1)	0.1573
	Structural molecule activity	54 (2.9)	245 (12.0)	<2.2E−16
	Transcription regulator activity	12 (0.6)	5 (0.2)	0.08956
	Translation regulator activity	22 (1.2)	7 (0.3)	0.00535
	Transporter activity	135 (7.3)	100 (4.9)	0.02242
	Subtotal	1850 (100.0)	2045 (100.0)	
Biological process	Biological adhesion	7 (0.3)	4 (0.1)	0.3657
	Biological regulation	124 (5.0)	107 (3.9)	0.2633
	Cellular process	502 (20.3)	626 (22.8)	0.00022
	Developmental process	209 (8.5)	221 (8.0)	0.5628
	Establishment of localization	231 (9.4)	119 (4.3)	2.14E−09
	Gene expression	122 (4.9)	253 (9.2)	1.34E−11
	Growth	102 (4.1)	107 (3.9)	0.7295
	Immune system process	1 (0.0)	1 (0.0)	1
	Localization	235 (9.5)	121 (4.4)	1.52E−09
	Locomotion	3 (0.1)	6 (0.2)	0.3173
	Maintenance of localization	1 (0.0)	0 (0.0)	0.3173
	Metabolic process	548 (22.2)	842 (30.6)	3.13E−15
	Multicellular organismal process	209 (8.5)	217 (7.9)	0.6983
	Reproduction	78 (3.2)	63 (2.3)	0.2065
	Reproductive process	26 (1.1)	11 (0.4)	0.01366
	Response to stimulus	70 (2.8)	52 (1.9)	0.1032
	Viral reproduction	2 (0.1)	0 (0.0)	0.1573
	Subtotal	2470 (100.0)	2750 (100.0)	

[a] The p-value was calculated by chi-square test. If p-value is <0.05, it indicates that the proportion of subcategory between two libraries is statistically different at the 95% confidence level.

CBG23351 (a hypothetical protein containing a cysteine protease domain), and Vap2 in PGPS were ca. 5166, 4554, 3938, and 1489-fold higher compared with those in D4S (Fig. 1B). Other genes also showed 11.2–91.8-fold higher transcript levels in PGPS than in D4S. When the transcript levels were compared between PGPS and MGPS, Ald1.02 showed the most selective transcription profile in PGPS (2034 fold), followed by Vap1 (390 fold) and Cysp (102.3 fold). Other genes, including Vap2, Cab and UGT, also exhibited selective transcription profiles (46.1–63.0 fold) in PGPS than in MGPS.

Five D4S-specific genes, including HSP16.1, Sodh1, GDP, FLP3, and Ald1.02, showed relatively higher transcript levels (>1) compared to the reference gene in D4S (Fig. 2A). Likewise, three PGPS-specific genes, including CBG23351, Cysp, and FAD 6, exhibited relatively higher transcript levels (>1) than the reference gene in PGPS (Fig. 2B).

Discussion

We constructed two subtractive stage-specific cDNA libraries using SSH to investigate differential gene expression profiles in each stage of *B. xylophilus* and to search for useful genes that can be exploited as stage-specific markers for rapid and accurate diagnosis of *B. xylophilus* infestations and as novel targets for the control of *B. xylophilus.* We focused on annotating the relatively more abundant genes in each subtractive library based on the number of EST counts (>6) and on examining the GO subcategories of ‘extracellular region/matrix’, ‘antioxidant activity’, ‘response to stimulus’, and ‘immune system process’ because of their potential association with plant–parasite interactions, parasite–insect vector interactions, and migration.

D4S-specific genes

Abundant D4S-specific contigs include genes that showed high sequence identities to HSP16.1, Sodh1 and GDP. HSPs are a group of proteins whose expression is increased by temperature or other external stresses. Because D4S is a stage during which *B. xylophilus* is exposed to various environmental stresses, HSP16.1 in D4S appears to be involved in the defense against external stresses. Moreover, some small molecular HSPs were reported to play a role in transfer from free-living stages to host animal [19,20]. Sodh1 was also determined to be highly specific to D4S based on its abundance, consistent with a previous report [12]. Its specificity was confirmed by extremely high levels of transcript level ($>2\times10^5$) in D4S (Fig. 1A), which suggests

Table 5
The analysis of EST distribution between the subtractive D4S and PGPS libraries.

Library name	Non overlapping sequences	Overlapping sequences with		Sum
		D4S	PGPS	
D4S	2630 (92.2%)	–	223 (7.8%)	2853
PGPS	3392 (95.0%)	177 (5.0%)	–	3569

Table 6
The gene list selected from two libraries for quantitative real-time PCR.

Library	Contig ID	BLAST match					GO term
		Accession number	Description	Abbreviations	Species	E-value	
D4S	BXsDS0066	XP_973442.1	Heat shock protein 16.1	HSP16.1	*Caenorhabditis elegans*	4.00E−10	Response to stimulus
	BXsDS0898	NP_505991.1	Sorbitol dehydrogenase 1	Sodh1	*C. elegans*	3.00E−54	Catalytic activity
	BXsDS1088	NP_501176.1	Glycerophosphoryl diester phosphodiesterase	GDP	*C. elegans*	8.00E−18	Catalytic activity
	BXsDS1507	EDP30939.1	Transthyretin-like proteins 46	TTL46	*C. elegans*	2.00E−25	Extracellular region
	BXsDS1602	NP_495415.2	Acid SphingoMyelinase family member 1	ASP1	*C. elegans*	3.00E−74	Extracellular region
	BXsDS1611	XP_001675807.1	FMRF-Like peptide 3	FLP3	*C. elegans*	3.00E−20	Extracellular region
	BXsDS1822	XP_419497.1	Ephx1 protein	Ephx1	*Bombyx mori*	1.00E−30	Response to stimulus
PGPS	BXsPS0243	XP_001673640.1	Hypothetical protein CBG23351	CGB23351	*C. briggsae AF16*	8.00E−35	–
	BXsPS0595	XP_001673335.1	UDP-Glucuronosyl transferase	UGT	*C. briggsae AF16*	6.00E−19	Cellular metabolic process
	BXsPS0221	AAA18215.1	Cysteine protease	Cyps	*Trypanosoma congolense*	9.00E−37	Response to stimulus
	BXsPS0414	2118249B	Allergen Lep d 1.02	Ald1.02	*Lepidoglyphus destructor*	1.00E−28	Extracellular region
	BXsPS0885	NP_507793.1	SCP-Like extracellular protein	SEP	*C. elegans*	3.00E−09	Extracellular region
	BXsPS0860	ABX75522.1	Cathepsin b	Cab	*C. elegans*	9.00E−10	Response to stimulus
	BXsPS0028	AAC15586.1	Fatty acid desaturase 6	FAD6	*C. elegans*	3.00E−79	Response to stimulus

that Sodh1 plays a critical role in D4S-specific biology. Because Sodh family enzymes convert sorbitol to fructose [21], this protein may be involved in energy generation during D4S.

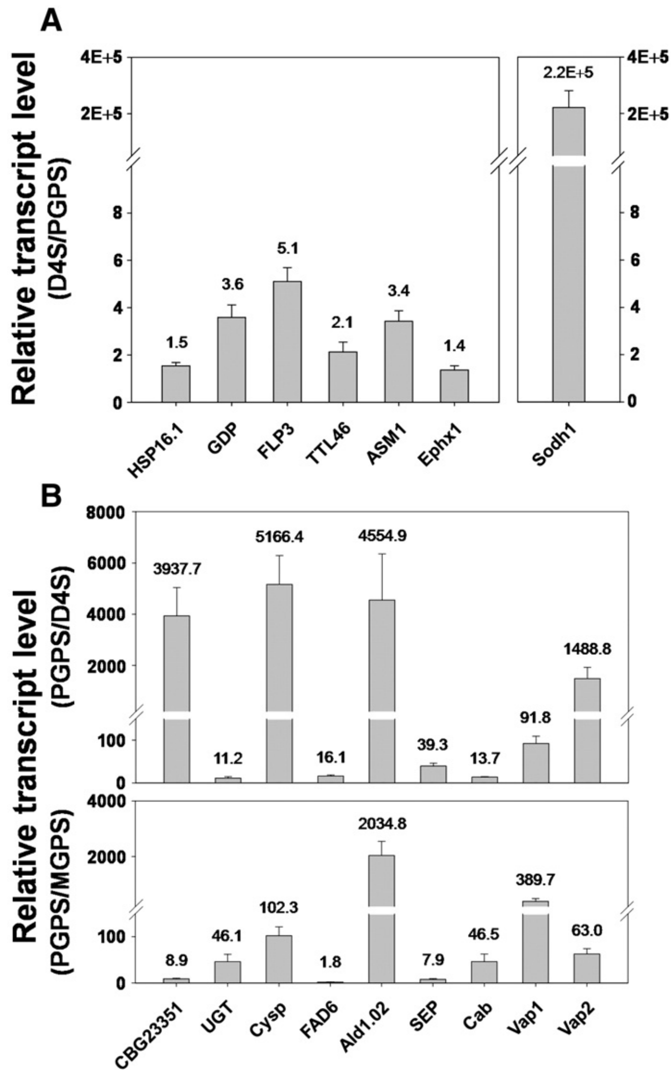

Fig. 1. Relative transcript levels of stage-specific genes using quantitative real-time PCR. The relative transcript levels of D4S-specific genes were compared between D4S and PGPS (A). The relative transcript levels of PGPS-specific genes were compared between PGPS and D4S (up) or between PGPS and MGPS (down) (B).

Because the interaction between *B. xylophilus* and the transfer vector insect, which is stimulated by several volatile chemicals generated from the vector insect [9], appears to be mediated by several secretory proteins of *B. xylophilus*, we also focused on ASM1 in the subcategory of 'extracellular region/matrix' and Ephx1 in the subcategory of 'response to stimulus'. ASM is a hydrolase responsible for breaking sphingomyelin down into phosphocholine and ceramide.

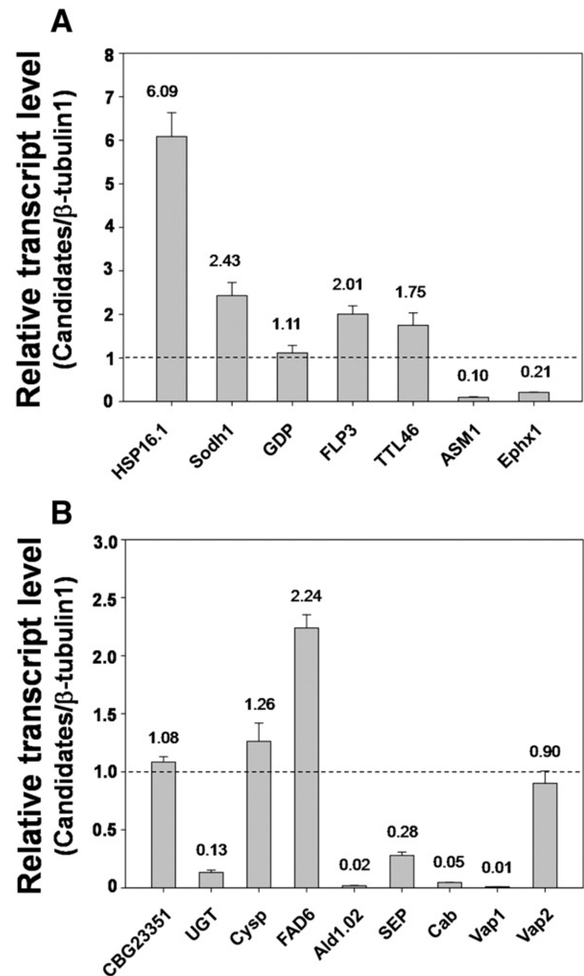

Fig. 2. Relative transcript levels of stage-specific genes compared with a reference gene (β-tubulin 1). The relative transcript levels of D4S-specific genes (A) and PGPS-specific genes (B) were estimated compared with a reference gene. The dotted line indicates that the transcript levels of target genes are the same as that of the reference gene.

ASM is ubiquitous in all mammalian cells but only two ASMs have been reported in *C. elegans* [22]. Since ASM has been known to be activated for the production of ceramide in response to cellular stresses [23], its high abundance in D4S suggests that it is also involved in the stress response pathway during D4S. Ephx1, an enzyme that hydrolyzes epoxide, is known to be associated with the activation or detoxification of exogenous chemicals such as aromatic compounds [24,25]. Although studies on nematode Ephx1 are limited, this protein appears to be related to the detoxification of xenobiotics in parasitic nematodes [26], including *B. xylophilus*, particularly during the dauer stage.

PGPS-specific genes

Cyps and CBG23351 were highly specific to PGPS compared to D4S. Among these, Cysp, known to play a key role in the degradation of host proteins [17], was approximately 100-fold more abundant in PGPS than MGPS (Fig. 1B), suggesting that Cysp is also associated with the parasitic biology of *B. xylophilus* during its propagation inside the host pine tree. In contrast, the transcription of CBG23351 (a hypothetical protein containing a cysteine protease domain) was not as specific to the pine-grown stage as Cyst, although its basal transcription level is similar to that of Cyst (Fig. 2B). This finding implies that CBG23351 is less likely involved in the parasitic interaction between *B. xylophilus* and the pine host.

UGT also showed approximately 50-fold higher transcript levels in PGPS than in MGPS, but its transcription level was ca. 4-fold higher also in D4S than in MGPS (Fig. 1B), indicating that UGT is relatively more specifically expressed in both PGPS and D4S compared to MGPS. UGT is known to be involved in phase II metabolism by conjugating sugars to apolar xenobiotics and endogenous toxins, thereby facilitating excretion [27], and it is therefore also likely to be involved in xenobiotic detoxification in *B. xylophilus*.

Many plant-parasitic nematodes secrete various proteins that play an important role in the parasitism against host plants. These secretory proteins have been detected at the invasion site of the host plant and the inside of the invading nematode [28–30]. *B. xylophilus* Ald1.02 appears to be a secretory protein, as judged by its GO categorization as an 'extracellular region/matrix' gene, and its expression was substantially elevated in PGPS compared to MGPS (ca. 2035 fold) and D4S (ca. 4555 fold), suggesting that it plays an important role in the propagative stage of growth on the pine tree but not on fungal media. Ald1.02 was also found as an allergen protein in the non-pyroglyphid dust mite, *Lepidoglyphus destructor* [31]. Likewise, two other allergen-like proteins, Vap1 and Vap2, have been reported from plant-parasitic nematodes such as *Meloidogyne incognita* and *Heterodera glycines* [29,32,33]. Vaps, a cystein-rich secretory protein, is known to be secreted from esophagus gland cells during infection and parasitism, and proposed to be involved in the early stages of parasitism, although its key function is unknown. Our qrtPCR experiment demonstrated that the Vaps proteins are overexpressed in PGPS of *B. xylophilus*, but the expression pattern of the two Vaps was different. The transcript level of Vap1 was ca. 389- and 92-fold higher in PGPS than MGPS and D4S, respectively, indicating that its expression is more specific to the stage grown on pine or for dispersal (Fig. 1B), but its expression level is almost negligible compared to the reference gene (Fig. 2B). On the contrary, Vap2 exhibited ca. 1489- and 63-fold higher transcript levels in PGPS than in D4S and MGPS, respectively. This finding suggests that Vap2 is specifically expressed in the propagative stage of *B. xylophilus*, particularly in the stage of growth in pine trees. Taken together, Vap2 appears to play a crucial role in the parasitic process when *B. xylophilus* propagates in the host pine tree.

Potential candidate genes for the diagnosis of B. xylophilus

Because early and accurate diagnosis of *B. xylophilus* in infected pine trees is essential for more efficient and long-term management of this serious pest, it is important to search for candidate genes that can be utilized as diagnosis markers of *B. xylophilus* infection. All the diagnosis protocols for the detection of *B. xylophilus* that have been developed to date are based on DNA markers and require PCR amplification of target DNA, which limits its applicability as an on-site diagnosis tool. Stage-specific secretory proteins with relatively high levels of expression can be exploited as protein markers to develop stage-specific on-site diagnosis kits based on the rapid protocol using colloidal gold [34]. The Sodh1 in D4S and the Cysp and Vap2 in PGPS have not only 50-fold higher transcript levels than compared stage, but also similar transcript levels of β-tubulin 1. Because the bioactivity and population density of *B. xylophilus* during the dispersal stage is much lower than during the propagative stage, Sodh1 is a good candidate protein for the diagnosis of the dispersal stage of *B. xylophilus* due to its very high expression. Similarly, the Cysp and Vap2 that are highly expressed in propagative stage appear to be good candidate proteins for the diagnosis of *B. xylophilus* in propagative stage.

Potential candidate genes as novel targets for the control of B. xylophilus

FLPs are the largest family of neuropeptides in invertebrates [35] and are known to play a central role in motor activities [36]: they have therefore been proposed to be a novel drug target [36,37]. The *C. elegans* genome contains at least 30 FLPs [38], and several FLPs from plant-parasitic nematodes, including the potato cyst nematode, have been characterized [39]. Functional studies based on RNA*i* revealed that the silencing of FLP genes caused aberrant behaviors and male sexual turning behavior in *C. elegans* [36], or motor dysfunction and unusual neuronal sensitivity in plant-parasitic nematodes [38].

FLP3 was also identified from *B. xylophilus* in this study. It showed 5.1-fold higher expression in D4S than in PGPS. The basal transcription level of FLP3 was ca. 2-fold higher than that of the reference gene, suggesting that its expression is relatively uniform throughout the life stages of *B. xylophilus*. As recent studies on the FLP neuropeptides of the animal- or plant-parasitic nematodes suggests the possibility of FLP as a novel target of nematode control, the FLP3 from *B. xylophilus* has a merit to be exploited as a potential target for the control of this pathogen.

In summary, we constructed two subtractive ESTs libraries that are specific to D4S and PGPW, and conducted functional annotation and characterization of several stage-specific genes. Our stage-specific EST data, together with the previously published *B. xylophilus* EST database [12], will be useful in understanding the dauer and transmission biologies in the dispersal stage and the parasitic and pathogenic processes in the propagative stages. In particular, identification of genes that are expressed specifically during the pine host growth stage will help elucidate the molecular basis of the host–nematode interaction. Certain highly stage-specific genes annotated in this study can also be exploited as novel markers for stage-specific diagnosis of *B. xylophilus* in the field.

Materials and methods

Nematode species

The pinewood nematodes used in this study were collected from the Jinju area in the Gyeongnam province, Korea. The dispersal 4th larvae were separated from newly emerged *Monochamus alternatus* adults by dissection, and the larvae were combined (total ca. 1.2×10^6 individuals). The identity of nematodes isolated as *B. xylophilus* was confirmed by species-specific PCR [40]. A portion of isolated nematodes (ca. 100 per plate) were reared on a lawn of *Botrytis cinerea* cultured on PDA plates at 28 °C for a week to induce them to the propagative stage. Six to seven year-old *Pinus thunbergii* pine trees were infected with media-grown pinewood nematodes (ca. 5000 ea in

a stump) in early August, and the nematode progenies in the pine trees were harvested using Baermann methods in mid-September [41]. The pinewood nematodes of D4S, MGPS, and PGPS were stored in water at 4 °C until use.

Total RNA extraction and double-strand cDNA synthesis

Nematodes of respective stages (ca. 1.2×10^6 individuals) were rinsed with 0.1 M NaCl and subsequently with an ample amount of distilled water by repetitive centrifugation at 3000 ×g for 5 min. Following centrifugation, ca. 30 mg nematodes were obtained from the bottom of a tube, and then ground in liquid nitrogen with a mortar and pestle. Total RNA was extracted using 3 ml TRI reagent (MRC, Cincinnati, OH) according to the manufacturer's protocol. The double-strand cDNA was synthesized from 1 μg total RNA using the Super SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA), following the manufacturer's instructions.

Construction of B. xylophilus subtractive stage-specific cDNA libraries and generation of ESTs

Two sets of SSH reactions were conducted using the PCR-Select cDNA Subtraction Kit (Clontech) following the manufacturer's instructions. The D4S (tester1) cDNA was subtracted by the PGPS (driver1) cDNA in one set, whereas the PGPS (tester2) cDNA was subtracted by the MGPS (driver2) cDNA. The tester and driver double-strand cDNAs were digested with *Rsa*I to obtain short, blunt-end fragments. The digested tester cDNA was divided into two parts and ligated with Adaptor 1 and Adaptor 2R (provided in the kit), respectively. Each adapter-ligated tester cDNA was mixed with an excess amount of driver cDNA and hybridized at 68 °C for 8 h (1st hybridization). The two hybridized samples were mixed together and fresh denatured driver cDNA was added, and then incubated at 68 °C overnight to further enrich the differentially expressed cDNAs (2nd hybridization). After filling the sticky ends, double-stranded cDNAs with different adaptor sequences (Adaptor 1 and Adaptor 2R) on each end were selectively amplified by PCR using Advantage2 DNA polymerase (Clontech). The subtractive cDNA library specific to each stage of *B. xylophilus* was constructed by direct cloning of the PCR-amplified fragments into the pGEM-T easy vector (Promega, Madison, WI). A total of 3072 and 3840 clones were sequenced with SP6 primers from the subtractive cDNA libraries specific to D4S and PGPS, respectively (NICEM, SNU, Seoul, Korea; Macrogen, Seoul, Korea).

EST annotation

After the trimming of vector and adaptor sequences, short and/or chimeric ESTs were removed. ESTs were clustered and assembled using the Phrap DNA assembly program (http://www.phrap.org). The clustered sequences (contigs) were compared to non-redundant protein databases using BLASTx searches. Functional annotation of putative proteins from the ESTs was also conducted according to the Gene Ontology (GO) database (http://www.geneontology.org). Frequency of GO categories from two libraries was compared by chi-square test.

Identity analysis between the D4S and PGPS libraries

A total of 6912 sequences from the D4S and PGPS libraries were trimmed, clustered, and assembled using Phrap. The identity between two ESTs libraries was estimated by analyzing the origins of entire ESTs assembled into contigs. All contigs were divided into three groups. The first group contained ESTs specific only to D4S, the second group contained ESTs specific only to PGPS, while the third group contained ESTs found in both D4S and PGPS. The number of ESTs in each group was counted and compared to determine the EST distribution in the two subtractive libraries.

Analysis of the relative transcript level using quantitative real-time PCR (qrtPCR)

The transcript levels of several putative stage-specific genes were estimated by qrtPCR. Four reference genes including 18S ribosomal DNA, elongation factor 1α, actin, and β-tubulin 1 were tested, among which β-tubulin 1 was determined to be the most suitable reference gene as it showed the least variation between *B. xylophilus* stages (data not shown).

Total RNA was isolated using RNeasy plus mini kit (Qiagen, Germantown, MD) from three stages (D4S, MGPS, and PGPS). Single-strand cDNA was individually synthesized from total RNA using the SuperScript first-strand cDNA synthesis system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and was diluted to 2 ng/μl. The optimized qrtPCR reaction mixture (20 μl) comprised 10 ng cDNA (5 μl), 0.25 μM specific-primers and 2× DyNAmo™ HS SYBR® Green qPCR master mix (Finnzymes, Espoo, Finland). The cycling profile used for qRT-PCR was as follows: a preheating step for enzyme activation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 58 °C for 10 s, and 72 °C for 20 s. The relative transcript level of each D4S-specific gene was compared between D4S and PGPS. The relative transcript level of each PGPS-specific gene was compared among PGPS, D4S and MGPS. The relative transcript level of target genes was calculated using the $2^{-\Delta Ct}$ method [42], where the ratio $= 2^{-\Delta Ct[\text{target} - \text{reference}]}$ under the condition that E_{target} (amplification efficiency of target gene) $= E_{\text{reference}}$ (amplification efficiency of reference gene) ≈ 2. The relative transcript levels of the D4S-specific genes were compared between D4S and PGPS. The relative transcript levels of the PGPS-specific genes were compared between PGPS and MGPS as well as between PGPS and D4S.

Acknowledgments

This work was supported by grant No. 500-20060168 from Korea Forest Service. Jae Soon Kang was supported in part by the Brain Korea 21 program. The authors thank Dr. Sang Chul Shin (Korea Forest Research Institute) who supported the plan for this project. The authors would also like to thank the anonymous reviewers for their critical comments and suggestions.

References

[1] S. Nagashima, Y. Hayashi, H. Fujiwara, Distribution of *Bursaphelenchus mucronatus* and damage of pine forest by the nematode in Yamaguchi Prefecture, Transactions of the Annual Meeting of the Kansai Branch of Japanese Forestry Society 26 (1975) 271–274.
[2] B. Yang, Q. Wang, Distribution of the pinewood nematode in China and susceptibility of some Chinese and exotic pines to the nematode, Can. J. For. Res. 19 (1989) 1527–1530.
[3] C.K. Yi, B.H. Byun, J.D. Park, S.I. Yang, K.H. Chang, First finding of the pine wood nematode, *Bursaphelenchus xylophilus* and its insect vector in Korea, Res. Rep. For. Res. Inst. 38 (1989) 141–149.
[4] Y. Mamiya, The life history of the pine wood nematode, *Bursaphelenchus lignicolus*, Jpn. J. Nematol. 5 (1975) 16–25.
[5] Y. Kishi, The Pine Wood Nematode and the Japanese Pine Sawyer, Thomas Company Limited, Tokyo, 1995.
[6] E. Kondo, N. Ishibashi, Ultrastructural differences between the propagative and dispersal forms in pine wood nematode, *Bursaphelenchus lignicolus*, with reference to the survival, Appl. Entomol. Zool. 13 (1978) 1–11.
[7] N. Ishibashi, M. Aoyagi, E. Kondo, Comparison of the gonad development between the propagative and dispersal forms of pine wood nematode, *Bursaphelenchus lignicolus* (Aphelenchoididae), Jpn. J. Nematol. 8 (1978) 28–31.
[8] M.J. Linit, Nematode vector relationships in the pine wilt disease system, J. Nematol. 20 (1988) 227–235.
[9] L.L. Zhao, W. Wei, L. Kang, J.H. Sun, Chemotaxis of the pinewood nematode, *Bursaphelenchus xylophilus*, to volatiles associated with host pine, *Pinus massoniana*, and its vector *Monochamus alternatus*, J. Chem. Ecol. 33 (2007) 1207–1216.
[10] T.C.e.S. Consortium, Genome sequence of the nematode *C. elegans*: a platform for investigating biology, Science 282 (1998) 2012–2018.

[11] S. Rudd, Expressed sequence tags: alternative or complement to whole genome sequences? Trends Plant Sci. 8 (2003) 321–329.
[12] T. Kikuchi, et al., Expressed sequence tag (EST) analysis of the pine wood nematode *Bursaphelenchus xylophilus* and *B. mucronatus*, Mol. Biochem. Parasitol. 155 (2007) 9–17.
[13] L. Diatchenko, et al., Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 6025–6030.
[14] N.G. Gurskaya, et al., Equalizing cDNA subtraction based on selective suppression of polymerase chain reaction: cloning of Jurkat cell transcripts induced by phytohemaglutinin and phorbol 12-myristate 13-acetate, Anal. Biochem. 240 (1996) 90–97.
[15] M.V. Matz, et al., Suppression subtractive hybridization: a new method for cDNA subtraction of differentially expressed genes, FASEB J. 10 (1996) 748.
[16] L. Murray, P. Geldhof, D. Clark, D.P. Knox, C. Britton, Expression and purification of an active cysteine protease of *Haemonchus contortus* using *Caenorhabditis elegans*, Int. J. Parasitol. 37 (2007) 1117–1125.
[17] T.C. O'Brien, et al., A parasite cysteine protease is key to host protein degradation and iron acquisition, J. Biol. Chem. (2008) 28934–28943.
[18] C.D. King, G.R. Rios, M.D. Green, T.R. Tephly, UDP-glucuronosyltransferases, Curr. Drug Metab. 1 (2000) 143–161.
[19] N. Raghavan, I. Ghosh, W.S. Eisinger, D. Pastrana, A.L. Scott, Developmentally regulated expression of a unique small heat shock protein in *Brugia malayi*, Mol. Biochem. Parasitol. 104 (1999) 233–246.
[20] D. Hartman, et al., *Haemonchus contortus*: molecular characterisation of a small heat shock protein, Exp. Parasitol. 104 (2003) 96–103.
[21] O. El-Kabbani, C. Darmanin, R.P. Chung, Sorbitol dehydrogenase: structure, function and ligand design, Curr. Med. Chem. 11 (2004) 465–476.
[22] X.H. Lin, M.O. Hengartner, R. Kolesnick, *Caenorhabditis elegans* contains two distinct acid sphingomyelinases, J. Biol. Chem. 273 (1998) 14374–14379.
[23] Y.A. Hannun, L.M. Obeid, The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind, J. Biol. Chem. 277 (2002) 25847–25850.
[24] M. Arand, A. Cronin, M. Adamska, F. Oesch, Epoxide hydrolases: structure, function, mechanism, and assay, Methods Enzymol. 400 (2005) 569–588.
[25] T. Shimada, Xenobiotic-metabolizing enzymes involved in activation and detoxification of carcinogenic polycyclic aromatic hydrocarbons, Drug Metab. Pharmacokinet. 21 (2006) 257–276.
[26] K.D. Pemberton, J. Barrett, The detoxification of xenobiotic compounds by *Onchocerca gutturosa* (Nematoda: Filarioidea), Int. J. Parasitol. 19 (1989) 875–878.
[27] J. Zhou, J. Zhang, W. Xie, Xenobiotic nuclear receptor-mediated regulation of UDP-glucuronosyl-transferases, Curr. Drug Metab. 6 (2005) 289–298.
[28] R.H.C. Curtis, Plant parasitic nematode protein and the host–parasite interaction, Brief. Funct. Genomic Proteomic (2007) 1–9.
[29] B. Gao, et al., Molecular characterisation and expression of two venom allergen-like protein genes in *Heterodera glycines*, Int. J. Parasitol. 31 (2001) 1617–1625.
[30] K. Henkle-Duhrsen, A. Kampkotter, Antioxidant enzyme families in parasitic nematodes, Mol. Biochem. Parasitol. 114 (2001) 129–142.
[31] M. Schmidt, S. Olsson, I. van der Ploeg, M. van Hage-Hamsten, cDNA analysis of the mite allergen Lep d 1 identifies two different isoallergens and variants, FEBS Lett. 370 (1995) 11–14.
[32] X. Ding, J. Shields, R. Allen, R.S. Hussey, Molecular cloning and characterisation of a venom allergen AG5-like cDNA from *Meloidogyne incognita*, Int. J. Parasitol. 30 (2000) 77–81.
[33] X. Wang, H.M. Li, Y.J. Hu, P. Fu, H.H. Xu, Molecular cloning and analysis of a new venom allergen-like protein gene from the root-knot nematode *Meloidogyne incognita*, Exp. Parasitol. 117 (2007) 133–140.
[34] S. Cui, G. Tong, A chromatographic strip test for rapid detection of one lineage of the H5 subtype of highly pathogenic avian influenza, J. Vet. Diagn. Invest. 20 (2008) 567–571.
[35] P. McVeigh, et al., Analysis of FMRFamide-like peptide (FLP) diversity in phylum Nematoda, Int. J. Parasitol. 35 (2005) 1043–1060.
[36] M.J. Kimber, et al., *flp* gene disruption in a parasitic nematode reveals motor dysfunction and unusual neuronal sensitivity to RNA interference, FASEB J. (2007) 1233–1243.
[37] J. Jacob, B. Vanholme, A. Haegeman, G. Gheysen, Four transthyretin-like genes of the migratory plant-parasitic nematode *Radopholus similis*: members of an extensive nematode-specific family, Gene 402 (2007) 9–19.
[38] T.W. Liu, K. Kim, C. Li, M.M. Barr, FMRFamide-like neuropeptides and mechanosensory touch receptor neurons regulate male sexual turning behavior in *Caenorhabditis elegans*, J. Neurosci. 27 (2007) 7174–7182.
[39] M.J. Kimber, C.C. Fleming, A.J. Bjourson, D.W. Halton, A.G. Maule, FMRFamide-related peptides in potato cyst nematodes, Mol. Biochem. Parasitol. 116 (2001) 199–208.
[40] J.S. Kang, et al., Development of an efficient PCR-based diagnosis protocol for the identification of the pinewood nematode, *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoididae), Nematology 6 (2004) 279–285.
[41] D.R. Viglierchio, R.V. Schmitt, On the methodology of nematode extraction from field samples — Baermann funnel modifications, J. Nematol. 15 (1983) 438–444.
[42] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) e45.