Comparison of the Surface Coat Proteins of the Pine Wood Nematode Appeared During Host Pine Infection and In Vitro Culture by a Proteomic Approach

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ABSTRACT

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Pine wilt disease, caused by the pine wood nematode (PWN), *Bursaphelenchus xylophilus*, has become of worldwide quarantine concern in recent years. Here, we disclosed the surface coat (SC) proteins of the PWN which are thought to be one of the key components in pine wilt development. This is the first report that focused on the SC proteins and thoroughly identified those proteins of a plant-parasitic nematode using the proteomic approach. In this study, SC protein profiles were compared for PWNs grown on the fungus *Botrytis cinerea* and in host pine seedlings. The results demonstrated that the gross amount of PWN SC

proteins drastically increased during infection of the host pine. Thirty-seven protein bands showed significant quantity differences between fungus-grown and host-origin PWNs, and were used for identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis. These included several proteins that are presumed to be involved in the host immune response; for example, regulators of reactive oxygen species (ROS) and a ROS scavenger. These results might suggest that the PWN SC proteins are crucial in modulating or evading host immune response. Our data provide a new insight into the mechanism of pine wilt disease and the biological role of the SC proteins of plant-parasitic nematodes.

Additional keywords: *Pinus thunbergii*, proteome.

Pine wilt disease (PWD), caused by the pine wood nematode (PWN), *Bursaphelenchus xylophilus*, is one of the most serious tree diseases in the world, not only because pine forests are important sources of forest products but also because pine trees are used in wind, tide, and sand breaks; landslide prevention; and beautiful landscapes, especially in East Asia. However, pine forests now face a catastrophe resulting from PWD. The disease was first discovered in the early 20th century in Japan and, in 1971, *B. xylophilus* was shown to cause the disease (20). It is now known that the PWN originated from the United States and introduced into Japan from there. Subsequently, in the 1980s, the pathogen spread to neighboring East Asian countries and, since then, has been found causing pine wilt in Portugal in 1999 (27) and in Spain in 2008. Thus, the disease has become a worldwide threat. As a result of international trade in forest products, the PWN poses a potentially serious problem in both the Southern and Northern Hemispheres. The European and Mediterranean Plant Protection Organization has listed both the PWN and its vector, the *Monochamus* beetle, as quarantine pest A1.

Resulting from research on PWD over the years, several hypotheses have been put forward regarding the pathogenic mechanisms for the disease (e.g., several pathogen-produced molecules have been suggested as playing key roles in PWN–host interactions). These include, for example, cell-wall-degrading enzymes secreted by PWN (17,18,28) and surface coat (SC) proteins of PWN (39). Also, it has been reported that molecules

originating from PWN-associated bacteria are important in PWD development (12,40). Furthermore, it has been shown that the abnormal regulation of reactive oxygen species (ROS) which are produced in large quantities in pine trees during the initial stage of PWD infection are a key factor in the pine wilt mechanism (13). However, just how much each of these factors contributes in the development of PWD still remains unclear. This situation is caused not only by the ecological complexity of PWD but also by the lack of genomic information and tools for doing functional genomic analysis on the organisms involved in PWD.

Here, we analyzed the PWN SC proteins based on the *B. xylophilus* expressed sequence tag (EST) information recently constructed (16) to clarify the detailed profile of the SC proteins by a proteomic approach. Many reports suggest that nematode SC plays various crucial roles in host–parasite interactions (31,36). For example, some of the many functions of SC are regulation of other microbes' adhesion to the nematode's body surface, lubrication, elicitation of the host defense responses, and modulation to help counter host defense responses (3,8,10,11,36). However, to date, little has been discovered about the detailed characteristics of the nematode SC because of difficulties involved in dealing with, for example, insufficient quantity, and heavy glycosylation. In particular, there have been no reports of attempts to thoroughly identify the SC proteins of plant-parasitic nematodes. The reasons for this vary but two major problems occur because of the nematodes are very small and difficult to propagate on a large scale.

In the present study, we identified the series of SC proteins which are specifically regulated in relation to the PWN infection into host pine trees by comparing the SC proteins between PWNs grown on a fungus versus those propagated in pine seedlings.

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Identification of these proteins should provide a new insight into the biological role of SC proteins of plant-parasitic nematodes, and allow us to propose the roles of those molecules in host pine-PWN interactions.

MATERIALS AND METHODS

Nematodes and their propagation. The virulent 'S10' isolate (originally isolated from Shimane Prefecture, Japan) of *B. xylophilus* was used in all the experiments. To compare the profiles of SC proteins, the nematodes were propagated on either the fungus *Botrytis cinerea*, designated "nematodes grown on the fungus" (NGFs), or the host, Japanese black pine (*Pinus thunbergii*) seedlings, designated "nematodes grown on the pine seedlings" (NGPs). The NGFs were cultured on *B. cinerea* growing on autoclaved barley grains and incubated at 25°C in 50-ml Erlenmeyer flasks. After incubation for 18 days, the PWNs were extracted for 12 h from the culture using the Baermann funnel technique (6). PWNs for use in inoculating the pine seedlings were also cultured on *B. cinerea* for 14 days before being inoculated into 3-year-old, potted, Japanese black pine, *P. thunbergii*, seedlings kept outdoors at Kyoto University, Kyoto, Japan, during the summers of 2008 and 2009. After 18 to 22 days from PWN inoculation, the PWN population was extracted from woody pieces of the inoculated seedlings using the Baermann funnel technique for 12 h, to serve as the NGP sample. Both samples of nematodes (NGFs and NGPs) were used for the extraction of SC proteins.

Extraction of SC proteins. The SC proteins of the PWN were extracted using the standard procedure for extraction of the SC of nematodes (24,32), modified as follows. To compare the gross quantities of the proteins extracted from NGF and NGP, 8×10^5 nematodes (mixed life stages) were suspended in 500 μ l of 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) in phosphate-buffered saline (PBS) with 1% protease inhibitor cocktail for mammalian cells (Sigma-Aldrich, St. Louis), and gently agitated for 1 h at 4°C. The adequacy of this protocol was confirmed by electron microscope, and the PWN lived and had high activity even after the extraction of SC protein. The nematodes were then pelletized by centrifugation at $3,000 \times g$ for 5 min at 25°C and the supernatant was collected. Next, for comparative proteomic analysis of SC proteins, 2×10^7 nematodes (mixed life stages) propagated under the two different conditions were suspended in 5 ml of 2% CHAPS in PBS with 1% protease inhibitor cocktail, and the supernatant was collected in the same manner as described above.

Protein sample preparation and quantification. Each protein solution that was obtained was desalted and buffer exchanged on PD-10 columns (GE Healthcare, Piscataway, NJ) with 20 mM Tris-HCl (pH 8.0) according to the manufacturer's protocol. Then, each protein concentration was determined using a Protein Assay Bichinchoninate (BCA) kit (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. The protein concentration data for three replicates were subjected to evaluation for statistical significance with analysis of variance, followed by Tukey's highly significant difference test.

Protein separation by reversed-phase high-performance liquid chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis. The extracted and desalted mass proteins of NGFs and NGPs were separated by reversed-phase high-performance liquid chromatography (RP-HPLC) as a first-dimension protein separation. Before being submitted to RP-HPLC analysis, each protein amount was equally adjusted to 90 μ g for the differential analysis. An HPLC system consisted of an LC-20AD pump (Shimadzu, Kyoto, Japan), 7725 injector (Rheodyne, Cotati, CA), CTO-20A column oven (Shimadzu), and SPD-M20A UV detector (Shimadzu). The chromatographic data were collected and processed by LC solution software (Shi-

madzu). In RP-HPLC analysis for protein separation, a COSMOSIL Protein-R (15 cm long, 4.6 mm i.d.) (Nacalai Tesque) was used at a flow rate of 1.0 ml/min. The gradient was provided by changing the mixing ratio of the two eluents: A, 0.1% (vol/vol) trifluoroacetic acid (TFA), and B, acetonitrile (ACN) containing 0.1% (vol/vol) TFA. The gradient was started with 10% B for 10 min, increased to 60% B for 10 min, and further increased to 100% B within 2 min to wash the column. The fractions including separated proteins were collected from 8 to 11.8 min at 20-s intervals and from 11.8 to 14.75 min at 9-s intervals. Thirty-three fractions (1 to 10, A to W) were collected during RP-HPLC analysis. These fractions were dried by vacuum centrifugation and resuspended in 10 μ l of PBS (pH 7.4). The resuspended samples were mixed with an equal volume of 2 $\times$ sodium dodecyl sulfate (SDS) sample buffer and heated at 100°C for 3 min before being loaded onto a gel for subsequent SDS-polyacrylamide gel electrophoresis (PAGE) analysis.

Tricine SDS-PAGE and Laemmli SDS-PAGE systems were used as the second-dimension protein separations. Fractions 1 to 10 were separated by a Tricine SDS-PAGE system using 15% Tris-tricine gels (e-PAGEL; ATTO, Tokyo) in a standard minislab PAGE apparatus (model AE-6500; ATTO) to separate low molecular weight proteins. Low-Range Rainbow Molecular Weight Markers (GE Healthcare) were used to determine the molecular mass of the proteins. On the other hand, fractions A to W were separated by Laemmli SDS-PAGE system using 5 to 20% gradient gels (e-PAGEL; ATTO). Full-Range Rainbow Molecular Weight Markers (GE Healthcare) were used to determine the molecular mass of the proteins. The proteins were silver stained using a Sil-Best-staining kit (Nacalai Tesque) according to the manufacturer's instructions.

Gel band visualization and relative quantification. ImageJ software (National Institutes of Health, version 1.42) was used for relative quantitative densitometric analysis of gel band intensities. Selected bands were quantified based on their relative intensities. Relative fold-change was calculated for gel band pairs based on the relative intensities between proteins of NGF and NGP. The densitometry value data for three independent replicates of NGF and NGP samples were subjected a Student's *t* test. A *P* value < 0.05 was considered a significant difference for the NGF and NGP samples.

Tryptic in-gel digestion. In-gel digestion was done as follows. Briefly, protein bands with significant differences between samples were excised and destained by a Sil-Best Destain Kit (Nacalai Tesque) according to the manufacturer's protocol. The destained gels were dehydrated in 100 μ l of ACN and completely dried by vacuum centrifugation. The excised gel pieces were incubated in freshly prepared 10 mM dithiothreitol (DTT) per 25 mM ammonium hydrogen carbonate (NH_4HCO_3) at 56°C for 1 h, and then the proteins were alkylated in 55 mM iodoacetamide per 25 mM NH_4HCO_3 in the dark for 45 min at room temperature. The gel pieces were subsequently dehydrated by 200 μ l of 25 mM NH_4HCO_3 in 50% ACN (two times) and dried by vacuum centrifugation for 15 min. The samples were incubated in 20 μ l of 50 mM NH_4HCO_3 containing trypsin (Promega Diagnostics, Madison, WI) at 10 μ g/ml for 12 h at 37°C. Following enzymatic digestion, the resultant peptides were extracted two times with 50 and then 25 μ l of 0.1% TFA in 50% ACN and concentrated to 10 to 15 μ l by vacuum centrifugation. Furthermore, the peptides were desalted using a MonoTip C18 (GL Sciences, Tokyo) and eluted with 0.1% TFA in 50% ACN.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis. A peptide cocktail was mixed with the matrix solution, consisting of α -cyano-4-hydroxycinnamic acid at 10 mg/ml in 50% ACN containing 0.1% TFA. The mixture (1 μ l) was spotted onto a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) plate, followed by drying at room temperature. Mass spectra were obtained on a Voyager RP

mass spectrometer (Applied Biosystems, Foster City). The mass spectrometer was calibrated using the ProteoMass peptide MALDI Calibration Kit (Sigma-Aldrich) with angiotensin II and ACTH peptide fragment.

Database searching. Mass spectrometry (MS) data were interpreted by searching the National Center for Biotechnology Information nonredundant database (NCBIInr) using the MASCOT search engine on the Matrix Science public domain server (<http://www.matrixscience.com>). Taxonomy was first limited to “*Caenorhabditis elegans*” and, subsequently, set to “All entries”. The search parameters were defined as follows: trypsin digest (allowing up to two missed cleavages), cysteine carbamidomethylation, and maximal mass tolerance of 0.2 Da.

Strategies of protein identification. Currently, no protein information is given for *Bursaphelenchus xylophilus* in any database. Also, the present EST database for *B. xylophilus* is not efficient to directly identify proteins in peptide mass fingerprinting (PMF). A specific strategy needed to be developed for identifying the proteins of *B. xylophilus* due to these limitations inherent in the incompletely sequenced or annotated information of *B. xylophilus*. Therefore, we applied the homology-based identification strategy of Mezhdoud et al. (25). When the MS data were obtained, the NCBI protein database was searched for the candidate protein which is homologous to that of *B. xylophilus* using the MASCOT search engine. Then, the amino acid sequence of the candidate protein was submitted for tBLASTn

search in the *B. xylophilus* EST database using ForestGEN server (<http://forestgen.ffpri.affrc.go.jp>). A protein with a significant MOWSE score ($P < 0.05$) in the MASCOT search was regarded as a hit for the candidate protein. However, in some cases, when no significant MOWSE score was observed, the protein was regarded as a hit for the candidate protein only when it fulfilled all of the following conditions: (i) at least four peptide matches were observed, (ii) the experimental molecular weight was consistent with the theoretical one, and (iii) the result of tBLASTn search in the *B. xylophilus* EST database showed a significant (bit score > 100 and e value < 0.001) hit. Then, the amino acid sequence of a candidate protein was submitted for the tBLASTn search, and we determined that the protein was successfully identified only when it showed a cluster of *B. xylophilus* with a significant (bit score > 100 and e value < 0.001) hit. The output of the search was given as a list of clusters from *B. xylophilus* (Tables 1 and 2). The sequence thus obtained was used for BLASTx search in the ForestGEN server to determine its putative functions and the protein family to which it belongs.

Immunohistochemical staining. PWNs propagated on the fungus and in the pine seedlings were collected using the protocol described above and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min at room temperature. The nematodes were pretreated with antigen retrieval solution HistoVT One (Nacalai Tesque) for 20 min at 70°C, and then blocked in a solution of 3% bovine serum albumin in PBS for 30 min at room

TABLE 1. Identification of *Bursaphelenchus xylophilus* surface coat proteins upregulated during infection into host pine by matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Band no.	Cluster ID ^a	<i>Bursaphelenchus xylophilus</i> protein identity ^b	Fold ^c	MW (kDa)	Homologue or similar protein in other organisms	Accession no. ^d	Coverage (%)	Matched peptide	MOWSE score ^e
Fp1	BXC06986_1	Mitochondrial substrate carrier	32.5	39	Mitochondrial substrate carrier (<i>Caenorhabditis briggsae</i>)	CAE65099	21	5	54
Fp2	BXC02719_1	ADP-ribosylation factor	6.5	28	ADP-ribosylation factor (<i>C. elegans</i>)	NP_499178	35	4	55
Fp3	BXC00270_1	DnaJ domain protein	14.6	26	DnaJ domain protein (<i>C. elegans</i>)	NP_498155	28	7	67
Fp4	n/a	n/a	31.9	16	Hypothetical protein R02F2.9 (<i>C. elegans</i>), predicted peptidyl tRNA-hydrolase	NP_498174	21	4	57
Gp1	n/a	n/a	16	27	Hypothetical protein (<i>Branchiostoma floridae</i>)	XP_002239524	37	7	76
Hp2	BXC02927_1	Glyceraldehyde-3-phosphate dehydrogenase	3.5	36	Glyceraldehyde-3-phosphate dehydrogenase (<i>Ascaris suum</i>)	BAB68543	18	6	65
Hp3	...	unidentified	14.9	27	...	...	...	...	...
Ip1	BXC02594_1	Gex interacting protein family member	4	32	Gex interacting protein family member (<i>C. elegans</i>)	NP_497189	20	4	39
Jp1	BXC02131_1	Hypothetical protein	4.3	38.5	Hypothetical protein CBG09794 (<i>C. briggsae</i>)	CAE64960	19	4	66
Mp2	n/a	n/a	4.1	23	Hypothetical protein M04C7.3 (<i>C. elegans</i>)	NP_492314	31	5	58
Np1	BXC02057_1	Hypothetical protein	7.2	80	Hypothetical protein Zk1127.9 (<i>C. elegans</i>)	NP_495444	13	5	40
Np2	n/a	n/a	61.2	33	Nematode astacin protease family member (NAS-33) (<i>C. elegans</i>)	CAD99221	20	5	72
Np4	BXC00126_1	Hypothetical protein	6.4	52.5	Hypothetical protein CBG08717 (<i>C. briggsae</i>), predicted ATP synthase α subunit	CAE64108	21	9	95
Op2	BXC00032_1	Actin	8.3	38.2	Actin (<i>Panagrellus redivivus</i>)	AAM47606	40	11	147
Qp1	BXC01248_1	Glutathione S-transferase 1	2.8	13	Glutathione S-transferase (<i>A. suum</i>)	CAA53218	25	4	52
Rp1	...	Unidentified	9.1	23.5	...	...	...	...	...
Tp1	n/a	n/a	8.5	61	Hypothetical protein W03G9.7 (<i>C. elegans</i>)	NP_491493	16	6	64
Vp1	...	Unidentified	5.5	42	...	...	...	...	...
Vp2	BXC00394_1	14-3-3b protein	6.1	29	14-3-3b protein (<i>Meloidogyne incognita</i>)	AAR85527	33	10	117
Wp1	BXC06049_1	MAFBx homolog family member	6.6	43.5	MAFBx homolog family member (F-box protein) (<i>C. elegans</i>)	NP_492376	12	4	42

^a Cluster ID given in the *B. xylophilus* expressed sequence tag database.

^b Protein identity by homology with other nematode's entry; n/a = not available.

^c Fold difference indicated the ratio of the intensity value of three independent replicates of nematodes grown on the pine seedlings sample to the value of nematodes grown on the fungus sample (control). Bands with significant differences ($P < 0.05$; Student's t test) were selected and marked.

^d GenBank protein accession number.

^e A MOWSE score given by MASCOT search.

temperature. After blocking, the nematodes were incubated with rabbit polyclonal antibodies against actin (A2668; Sigma-Aldrich), glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (G9545; Sigma-Aldrich), or 14-3-3 protein (ab6081; Abcam, Cambridge, UK) diluted at 1/30, 1/100, and 1/40, respectively, in PBS overnight at 4°C. After the incubation, the nematodes were washed three times with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin G (F9887; Sigma-Aldrich) diluted 1/160 in PBS for 1 h at room temperature. They were then washed three times with PBS and observed using a confocal laser-scanning microscope (Zeiss LSM510; Carl Zeiss, Oberkochen, Germany). FITC was excited using a 488-nm argon laser line. Serial-section images were acquired using the LSM510 operation system and software. Single section images were selected from these and exported in TIFF format. To assess nonspecific staining, a negative control experiment was performed by incubating the nematodes without primary antibody or with rabbit preimmune serum diluted at 1/100 in PBS for 1 h at 4°C.

Scanning electron microscope observations. PWNs were propagated on both the fungus and in host pine seedlings and extracted (as previously) and prefixed in 2% formaldehyde plus 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 12 h at 4°C. The nematodes were post-fixed with 2% osmium tetroxide for 30 min, and then dehydrated with an ethanol series (30, 50, 70, 90, and 95%, and twice in 100% ethanol) for 30 min at each concentration. Then, the nematodes were dried by a critical point dryer (JCPD-5; JEOL, Tokyo) using liquid carbon dioxide, and sputter-coated with Au-Pd using a magnetron sputter (MSP-1S; Vacuum Device, Ibaraki, Japan). Scanning electron microscope (SEM) images were taken using a VE-8800 (Keyence, Osaka, Japan).

RESULTS

Quantification of the total SC proteins extracted. The concentration of the gross SC proteins extracted from three replicates of 8×10^5 PWNs (NGF and NGP) was determined by BCA protein assay to be 9.2 ± 3.4 (mean $\pm$ standard deviation) and 81.7 ± 3.9 $\mu\text{g/ml}$, respectively (Fig. 1). No significant difference was noted between the body surface ultrastructure of NGFs and NGPs in SEM images (data not shown). The amount of SC protein covering PWNs extracted from pine seedlings was approximately nine times larger than that of PWNs which reproduced on the fungus.

RP-HPLC and SDS-PAGE analysis. The SC protein samples were separated by a special two-dimensional protein separation

system which combined RP-HPLC with SDS-PAGE. First, the proteins of NGF and NGP extracted were separated by RP-HPLC as a first-dimension protein separation. The chromatograms overlaid showed remarkable differences between the NGF and NGP samples from fractions F to H (retention time: 12.03 to 12.30 min) (Fig. 2). Subsequently, 33 fractions of both samples collected from each RP-HPLC experiment were paired and loaded onto an SDS-PAGE gel for the second-dimension protein separation (Fig. 3). The quantitative densitometric analysis, based on the protein band intensity, showed a significant increase for 29 gel bands in NGPs (from pine seedlings) compared with NGFs ($P < 0.05$; Student's t test). However, eight gel bands appeared significantly less in NGP than NGF ($P < 0.05$; Student's t test). As well, ≈ 100 independent proteins without quantity difference between samples ($P > 0.05$; Student's t test) were clearly observed in the images of SDS-PAGE (Fig. 3).

Overview of SC protein identification. An overview of the identification of the PWN SC proteins is shown in Figure 4. Thirty-seven gel bands which showed a significant difference in quantity between NGF and NGP (Fig. 3) were selected and excised for protein identification by MALDI-TOF/MS analysis. Of these, 29 proteins were upregulated and the other 8 were downregulated in NGP compared with NGF samples. From

Fig. 1. Comparison of the total protein concentration extracted from *Bursaphelenchus xylophilus* grown on the fungus *Botrytis cinerea* (NGF) and on Japanese black pine seedlings (NGP). Concentrations shown are the values per 8×10^5 nematodes averaged from three replicates. The asterisk denotes a significant difference between NGF and NGP ($P < 0.01$, Tukey's test). PWN = pine wood nematode.

TABLE 2. Identification of *Bursaphelenchus xylophilus* surface coat proteins downregulated during infection into host pine by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Band no.	Cluster ID ^a	<i>Bursaphelenchus xylophilus</i> protein identity ^b	Fold ^c	MW (kDa)	Homologue or similar protein in other organisms	Accession no. ^d	Coverage (%)	Matched peptide	MOWSE score ^e
7c1	...	Unidentified	-11.7	13	...	...	...	...	...
8c1	n/a	n/a	-3.1	12.5	Unknown (<i>Picea sitchensis</i>)	ABK26292	21	5	69
Dc1	...	Unidentified	-29.8	30	...	...	...	...	...
Fc1	n/a	n/a	-6.2	63	Hypothetical protein R02D3.3 (<i>Caenorhabditis elegans</i>)	NP_499880	12	6	60
Gc1	BXC00442_1	Paramyosin	-3.9	78	Paramyosin (<i>Onchocerca volvulus</i>)	AAA29431	14	10	61
Hc1	BXC02085_1	Prohibitin-like molecule	-4.4	38.5	Prohibitin-like molecule TC-PRO-1 (<i>Toxocara canis</i>)	AAB53231	35	7	86
Kc1	BXC02983_1	Enolase 2	-4.4	47.5	Enolase (<i>C. elegans</i>)	CAH10783	30	9	147
Pc1	BXC00260_1	Hypothetical protein	-6.8	47.8	Hypothetical protein CBG21173 (<i>C. briggsae</i>)	CAE73664	22	8	88

^a Cluster ID given in the *B. xylophilus* expressed sequence tag database.

^b Protein identity by homology with other nematode's entry; n/a = not available.

^c Fold difference indicated the ratio of the intensity value of three independent replicates of nematodes grown on the pine seedlings sample to the value of nematodes grown on the fungus sample (control). Bands with significant differences ($P < 0.05$; Student's t test) were selected and marked.

^d GenBank protein accession number.

^e A MOWSE score given by MASCOT search.

identification, 9 of 29 proteins upregulated (band no. Hp1, Kp1, Lp1, Mp1, Mp3, Mp4, Op1, Sp1, and Vp3) appeared not to be derived from the nematode *B. xylophilus* but, instead, from limited bacterial species (Table 3). The other 20 upregulated

proteins were considered to be originated from *B. xylophilus*; 17 of them (band no. Fp1, Fp2, Fp3, Fp4, Gp1, Hp2, Ip1, Jp1, Mp2, Np1, Np2, Np4, Op2, Qp1, Tp1, Vp2, and Wp1) successfully corresponded to the similar proteins of other organisms by

Fig. 2. Reversed-phase high-performance liquid chromatography overlay of chromatograms for *Bursaphelenchus xylophilus* grown on the fungus *Botrytis cinerea* (NGF, gray line) and *Bursaphelenchus xylophilus* grown inside Japanese black pine seedlings (NGP, black line). Thirty-three fractions were split and collected from each separate run to serve sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Fig. 3. Comparative analysis with sodium dodecyl sulfate polyacrylamide gel electrophoresis of *Bursaphelenchus xylophilus* protein-derived reversed-phase high-performance liquid chromatography (RP-HPLC) fractions. Fractions of RP-HPLC: A, 1 to 10; B, A to J; C, K to T; and D, U to W. Each fraction was run side by side: *B. xylophilus* grown on the fungus *Botrytis cinerea* (NGF) on the left, C; and *B. xylophilus* grown inside Japanese black pine (NGP) on the right, P. Bands with significant differences ($P < 0.05$; student's t test) in band intensity between NGF and NGP were selected and marked. Broken box shows the downregulated proteins in the Japanese black pine seedlings and solid box shows the upregulated proteins in the Japanese black pine seedlings. Numbers described in the upper position of 'C' or 'P' show the fraction number of RP-HPLC.

MASCOT search (Table 1). In addition, we identified the EST clusters of 12 of 20 upregulated proteins (band no. Fp1, Fp2, Fp3, Hp2, Ip1, Jp1, Np1, Np4, Op2, Qp1, Vp2, and Wp1) by tBLASTn search. However, as for six of eight downregulated proteins (band

no. 8c1, Fc1, Gc1, Kc1, and Pc1), the homologues or similar proteins were successfully detected as being from other organisms by MASCOT search (Table 2). Also, we identified the EST clusters of four of eight downregulated proteins (band no. Gc1, Hc1, Kc1, and Pc1). Five proteins (band no. Hp3, Rp1, Vp1, 7c1, and Dc1) that did not match any data of other organisms by MASCOT search. These five proteins are possibly unique to *B. xylophilus*.

Upregulated proteins of SC of the PWN. As mentioned above, 20 proteins were upregulated in the SC of PWNs during infection into host pine (Table 1). These include mitochondrial substrate carrier, ADP-ribosylation factor, DnaJ domain protein, GAPDH, gex interacting protein family member, nematode astacin protease family member (NAS), actin, glutathione S-transferase (GST), 14-3-3b protein, MAFBx homolog family member, and seven hypothetical proteins that include two predicted proteins (peptidyl tRNA-hydrazase and ATP synthase α subunit). The relative fold-changes were 2.8 to 61.2.

Downregulated proteins of SC of the PWN. Eight downregulated proteins of PWN were observed during PWN infection into host pine seedlings (Table 2). These include paramyosin, a prohibitin-like molecule, enolase, an unknown protein, and two hypothetical proteins. The significant relative fold-changes were -61.2 to -3.1.

Proteins of PWN-associated bacteria. In the present study, nine proteins of bacterial origin were found only in the extracted proteins of NGP (Table 3). All of these were successfully identified: glutamine synthetase, heat shock protein 90, ATP synthase subunit β , nucleoside diphosphate kinase, elongation factor G, elongation factor Tu, and ketol-acid reductoisomerase. These proteins are likely to be surface proteins of bacteria, because the extraction was done with a very mild condition and short period in this study. They all were identified as proteins derived from gram-negative bacteria, and seven of them as proteins of *Pseudomonas* spp. There are several reports that certain bacteria are associated with the surface of PWN reproducing in pine (40). We also clearly observed (using the SEM) bacteria attached to the surface of PWNs that reproduced only in pine seedlings (Fig. 5).

Immunohistochemical localization by confocal laser-scanning microscope. The immunohistochemical localization of actin, GAPDH, and 14-3-3 protein are shown on the body surface of NGPs (Fig. 6A, B, D, E, G, and H) and NGFs (Fig. 6C, F, and I). In NGPs, all antibodies clearly bound to the body surface of nematodes. In NGFs, however, the antibody bindings were often, but not always, observed onto the body surface of nematodes only for actin and GAPDH (Fig. 6C and F), though the fluorescence signal in NGFs tended to be weaker than in NGPs. In addition, no fluorescence signal was observed for 14-3-3 protein in NGFs (Fig. 6I). There was no significant difference observed in staining pattern among the nematode developmental stages. Negative controls with omission of primary antibody (Fig. 6J) or with preimmune serum (Fig. 6K and L) showed no fluorescence signal.

Fig. 4. Overview of the sample process and the results of database searching. (a), Downregulated proteins of the surface coat (SC) proteins of *Bursaphelenchus xylophilus* grown in the Japanese black pine seedlings (NGP). (b), Upregulated proteins of the SC proteins of *B. xylophilus* grown in the Japanese black pine seedlings (NGP). (c), Putative *B. xylophilus* proteins hit to the homologues or similar proteins of other organisms (except for bacteria) by MASCOT search. (d), *B. xylophilus*-associated bacteria significant hit to the proteins derived from gram-negative bacteria with significant MOWSE scores ($P < 0.05$) by MASCOT search. HPLC = high-performance liquid chromatography; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; MALDI-TOF/MS = matrix-assisted laser desorption ionization time of flight mass spectrometry; and EST = expressed sequence tag.

TABLE 3. Identification of proteins derived from *Bursaphelenchus xylophilus*-associated bacteria

Band no.	Accession no. ^a	Protein identity	Species	Theoretical MW (kDa)	Observed MW (kDa)	Coverage (%)	Matched peptides	MOWSE score ^b
Hp1	BAE44186	Glutamine synthetase	<i>Pseudomonas taetrolens</i>	52	54	22	9	102
Kp1	YP_347357	Heat shock protein 90	<i>P. fluorescens</i>	71	76	10	5	58
Lp1	ZP_04616935	Glutamine synthetase	<i>Yersinia ruckeri</i>	52	52.5	18	6	77
Mp1	AAY95404	F0F1 ATP synthase subunit β	<i>P. fluorescens</i>	50	49	34	10	126
Mp3	YP_350333	Nucleoside diphosphate kinase	<i>P. fluorescens</i>	15	13	43	4	70
Mp4	YP_001178464	Elongation factor G	<i>Enterobacter</i> sp.	77	84	17	7	88
Op1	YP_350809	Elongation factor Tu	<i>P. fluorescens</i>	44	45	54	16	212
Sp1	YP_351454	F0F1 ATP synthase subunit β	<i>P. fluorescens</i>	49	39.5	43	12	187
Vp3	YP_262325	Ketol-acid reductoisomerase	<i>P. fluorescens</i>	36	36	20	6	81

^a GenBank protein accession number.

^b A MOWSE score given by MASCOT search.

These results agree with the results of proteome analysis; thus, this immunohistochemical localization authenticated the protein data identified by proteomic approach in this study.

DISCUSSION

Increase in the total amount of SC proteins during host infection. The amount of total proteins extracted from the PWN body surface greatly differed due to the condition where the PWNs grew (i.e., the total proteins of PWNs reproducing in host pine seedling were much more than those of the PWNs cultured on the fungus) (Fig. 1). Although the proteins extracted in the present study contained a small quantity of those derived from certain PWN-associated bacteria, the results clearly show that a PWN secretes and displays a larger quantity of proteins on its outer surface when it inhabits inside host pine. In fact, the gel band intensities of most SC from PWNs which propagated in pine seedlings (NGPs) were stronger than those of NGFs when the number of PWN individuals that served protein extraction and separation was equally adjusted (data not shown). Furthermore, no upregulated proteins identified here had any plant matches as their top hit in MASCOT search. These results indicate that the cause of the increase in total amount of proteins extracted was solely the increase of the amount of PWN SC proteins. This finding supports the idea that the SC of PWNs should be related to the infection process of PWD as the host–parasite interface. In addition, when the ultrastructure of the outer surface of the PWN body was observed by SEM, no clear difference was found between NGFs and NGPs (Fig. 5). Thus, the surface change in the PWN which occurs in entering into the host pine seems to be at the molecular level, not accompanied by any changes in the surface structure.

Overview of SC protein identification results. In the present study, ≈ 100 proteins were successfully separated by a special two-dimensional protein separation system that combines RP-HPLC with SDS-PAGE. As a result, 17 upregulated and 6 downregulated proteins derived from PWN infesting pine seedlings were successfully annotated with protein functions. In addition, nine proteins derived from PWN-associated bacteria were identified. We classified some of the identified proteins into four categories based on their biological properties which may be concerned with PWD—regulation of ROS production, ROS scavenger, protein with an unexpected role, and proteins of PWN-associated bacteria—and advance the following discussions according to these classifications.

Regulation of ROS production. The 14-3-3 proteins have been well known as kinase and phosphatase. However, there are also several reports that 14-3-3 proteins are involved in ROS regulation and apoptosis in recent years (9,19,38). The gene coding 14-3-3 protein has been identified in several unicellular and multicellular parasites. Interestingly, some reports suggested a shift in 14-3-3 gene expression during transition to the successive parasitic stages in some parasites (33). However, little is known about associated functionality or production patterns of the corresponding proteins in these parasites. Regarding plant-parasitic nematodes, it was reported that *Meloidogyne incognita* secreted 14-3-3b protein from the stylet of infective juveniles (2,15). However, in plants, a 14-3-3 protein regulates the plasma membrane H^+ -ATPase in response to pathogen attack, and also regulates the production of ROS in plant–pathogen interactions (9,14).

ROS scavenger. Two SC proteins were identified that could potentially act as ROS scavengers, including GST and GAPDH. GST is a kind of antioxidant enzyme, and it is known that various parasites overexpress GST during the infection process (5,22). Furthermore, GST was reported to localize on the surface of various parasites, and the localization might allow the parasites to protect themselves from oxidative damage on the cuticular

membranes (30). GAPDH is generally regarded as a house-keeping glycolytic enzyme. However, many reports suggest that GAPDH is a multifunctional protein that is involved in various subcellular processes in animals (34,35). One of these functions is cellular redox regulation. Baek et al. (1) suggested that GAPDH had a direct ROS scavenging activity. Thus, there is a fair possibility that GAPDH localized on the surface of PWNs acts as a ROS scavenger, although further study will be needed to confirm the precise function of this molecule.

Protein with an unexplained role. Another interesting PWN SC protein with unexplained roles is the NAS protein. NAS proteins are metalloproteases present in various organisms which serve multiple physiological functions (7,26). However, information about the detailed functions of NAS proteins is very limited. In the animal parasites, several reports indicated that the metalloprotease secreted from parasites was a key molecule in its infection process (4,23,37). Williamson et al. (37) reported that the dog hookworm *Ancylostoma caninum* secretes an astacin-like metalloprotease in the infective stage during host migration, and suggested that this metalloprotease localized on the outer surface of hook worm was critical for its invasion process.

Proteins of PWN-associated bacteria. In upregulated proteins, nine proteins were not identified as PWN proteins because they best matched bacterial proteins by MASCOT search and because no significant tBLASTn similarity was detected to any available amino acid sequences of *B. xylophilus* using ForestGEN server. Also, the deduced origins of the bacterial proteins indicated by the results of MASCOT search were very limited, mostly *Pseudomonas* spp. It has been reported that PWNs are accompanied by some bacteria on their outer surface during reproduction in the pine tree, of which major bacterial species are *Pseudomonas* (12,40). Also, in the present study, the PWN might be accompanied by some bacteria on its outer surface when it was propagated in the pine seedlings and, therefore, the protein derived from the PWN-associated bacteria might be extracted and identified, although the PWN mass collected from the seedlings was vigorously washed many times before extraction of SC protein. In fact, some bacteria attached to the PWN outer surface were observed by SEM (Fig. 5). Zhao et al. (40) and Han et al. (12) suggested that the complex infection of PWNs and their

Fig. 5. Scanning electron microscope images of **A, B, and C**, ultrastructure of the body surface of the pine wood nematode (PWN) grown in the pine seedlings and its associated bacteria attaching to the surface of the PWN body; **D**, ultrastructure of the body surface of PWN grown on the fungus. Scale bars: 1.0 μ m.

Fig. 6. Immunohistochemical localization of actin, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and 14-3-3 protein on the body surface of **C, F, and I**, *Bursaphelenchus xylophilus* grown on the fungus and **A, B, D, E, G, and H**, in the pine seedlings. The *B. xylophilus* were stained with primary antibody against actin, GAPDH, or 14-3-3 protein followed by fluorescein-isothiocyanate-conjugated secondary antibody. Negative control experiments were also carried out by incubating the *B. xylophilus* without primary antibody (**J**) or with rabbit preimmune serum (**K and L**). Fluorescence microscopy images with black background (the far left image in **A, C, D, F, G, I, J, K, and L**); normal light microscope images (the middle image in **A, D, and G** and the right image in **C, F, I, J, K, and L**); fluorescence microscopy images with white background (the far right image in **A, D, G**). Transverse image of *B. xylophilus* treated with anti-actin antibody (**B**); anti-GAPDH antibody (**E**); anti-14-3-3 protein antibody (**H**). Contrast of images **C** and **F** were increased to make it easier to understand, while the contrast of all the other images was same. White arrows show each protein clearly localized on the surface of *B. xylophilus*. Scale bars: 20 μ m; la = lateral field.

accompanying bacteria is responsible. However, it should not be denied at present that these proteins, which have bacterial proteins as their best matches, may possibly be derived from the PWN itself, because the PWN acquired several genes by horizontal gene transfer from bacteria. More studies are needed to determine the exact origins of these proteins.

Possible roles of the PWN SC proteins in the development of PWD. In PWD, in the pine trees infected by PWNs, the excess ROS production and the leakage of electrolytes have been observed at the initial stage of infection (21). Some researchers hypothesized that these abnormal regulations of ROS productions may finally lead the pine trees to death (13,39). That is, it can be speculated that the regulation and evasion of ROS might be the key point in the disease. In the present study, we revealed that the 14-3-3 protein known as a potential regulator of ROS was localized on the surface of PWNs during host pine infection. Furthermore, two potential ROS scavengers, including GST and GAPDH, were identified. These proteins may not be only an antioxidative barrier but also a fundamental factor to establish and let persist the host-parasite relationship. The results of this study suggest that the PWN has a kind of mechanism to evade or modulate the host defense responses, and one of the main sites that serve such an important function can be the SC of PWNs, which stands at the interface with the plant immune response. Further analysis of these interesting proteins will be required to verify our hypotheses.

Conclusions. Here, we provide the first report that focused on the SC proteins and thoroughly identified those of plant-parasitic nematodes by proteome analysis. In the present study, we may say that most of the major SC proteins of the PWN were successfully identified using PMF by improving the protein separation system and collecting a large quantity of samples, although it could enable us to perform large-scale analysis and identify the protein with more accuracy by using tandem MS. In fact, many of the PWN SC proteins identified here corresponded to the surface proteins of the animal parasite *Schistosoma bovis* (29). The PWN SC proteins identified contained a potential regulator of ROS production, and ROS scavengers. These functions of PWN SC proteins seem to be essential for establishing their infection and causing sudden death of host pine trees by the PWD. Further studies are needed to determine whether these SC proteins of the PWN are really involved in pathogenesis. This work will contribute to revealing the biological roles of the SC proteins of plant-parasitic nematodes and solving puzzles behind the PWD mechanisms.

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