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A COMPARATIVE PROTEOMICS ANALYSIS OF *PINUS MASSONIANA* INOCULATED WITH *BURSAPHELENCHUS XYLOPHILUS*

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Abstract

Pine wilt disease caused by *Bursaphelenchus xylophilus*, also known as pine wood nematode (PWN), is the most devastating disease of pine trees. Test results from different geographical provenance of *P. massoniana* inoculated with nematodes, indicated that GuangDong$_5$ (GD_5) has strong resistance to PWN. This article used GD_5 as the experimental material, and sensitive SX_1 as comparison. Total proteins were extracted and separated by 2-DE and MALDI-TOF-TOF MS technology from the pine needles of GD_5 and SX_1, respectively. Differentially expressed proteins in the provenance between inoculated and un-inoculated with PWN for two weeks, were analyzed. At last, 87 differentially expressed proteins were successfully identified by MALDI-TOF-TOF. The results also showed that there were six proteins involved in hydrogen peroxide scavenging capacity and protecting the redox homeostasis system from being damaged. Their up-regulation may be the main cause of the provenance GD_5 resistant to PWN.

Key words: Pine wood nematode, Pine wilt, Proteomics, MALDI-TOF-TOF.

Introduction

Pine wilt disease caused by *Bursaphelenchus xylophilus*, also known as the pine wood nematode (PWN), is one of the important biological disasters on Pinus (*Pinus* spp.) tree. It can cause massive death of pines, do serious damage to the ecological and forestry economy, and is listed by countries around the world as an important forest plant quarantine object (Xu *et al.,* 2011). Pine wilt disease is a complex system which is influenced by nematodes, host pine, environmental conditions, associated fungi, vector insects and other factors. Its pathogenesis is not clear, which causes great difficulty for prevention and control of the disease.

Through irregularly scheduled experiments of observation in 16 years, we have chosen three resistant *Pinus massoniana* provenances GD_5 \ GX_2 \ GX_3 based on *P. massoniana* provenance gene pool from 40 provinces in China planted in 1983. To understand the mechanisms of resistant *P. massoniana* provenance in response to PWN, we have carried out a number of experiments. Part of the resistance mechanism of *P. massoniana* has been analyzed, such as in the phenotype and physiological and biochemical level determination of disease resistance from the growth characteristics differences and inclusions of different *P. massoniana* provenances (Xu *et al.,* 2011).

Proteomics is a technological system developed rapidly in "post-genome era". It can illustrate all the expression laws and biological functions of protein which is the real execution of life activities expressed by the genome.

The proteomic method which are being used to study the stress tolerant mechanism of plant including drought, salinity, heavy metal, heat, anoxia and elicitor, have become one of research hotspots in the field of genetics and plant protection at home and abroad. Nevertheless, plant proteomic analysis is still not used in *P. massoniana* in response to PWN.

It seems necessary to use proteomics strategy to reveal a mechanism that allows us to further understand the possible management strategy of cellular activities occurring in *P. massoniana* to handle PWN invasion. In the present study, a proteomic approach was applied to two *P. massoniana* provenances, GD_5 (*B. xylophilus*-resistant) and SX_1 (*B. xylophilus*-sensitive). We initiated to investigate the protein expression pattern and identify the differentially expressed proteins response to PWN invasion, and understand the pathways related with these proteins in the *P. massoniana*. Based on the proteomic data, the mechanism of resistant *P. massoniana* provenance in responses to PWN invasion was discussed.

Materials and Methods

Plant material: Two-year-old seedling needles of two *P. massoniana* provenances (*B. xylophilus*-resistant provenance GD_5 and *B. xylophilus*-sensitive provenance SX_1) inoculated with PWN (every seedling was inoculated with 1000 nematodes) for two weeks, were harvested and frozen in liquid nitrogen and stored at -70°C. The needles from the un-inoculated plants in both provenances were also collected and used as control.

Total protein extraction and 2-DE: To minimize errors, three biological repeats were conducted for proteome analysis at each sample. For each biological repeat sample, 0.5g *P. massoniana* needles were pooled. The needles were extracted with the method of acetone/TCA precipitation according to BioRad 2-D manual with some modifications. Needle sample powder was suspended in 10% w/v TCA/acetone containing 1 mM PMSF and 0.07 v/v β-mercaptoethanol, and held at -20°C for 1 h. After centrifugation and rinse, the vacuum dried pellets were dissolved in 800 µl lysis solution containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM DTT, 1 mM PMSF and 0.5% v/v biolytes (Bio-Rad). Insoluble

materials were removed by centrifugation and the protein concentration of the sample quantified using the Bradford method. About 350 µg protein was separated by loading the sample on a 24 cm pH 4-7 nonlinear gradient IPG strip (Bio-Rad), and was subjected to eletrophoresis at 19 ^{o}C for a total of 110,000 VH. The second electrophoretic dimension was by 12% SDS-PAGE. The signal was visualised by silver. Gel image was digitalized with a gel scanner (Powerlook 2100XL, UMAX), and analyzed with PDQuest^{TM} software package (Version 7.2.0; Bio-Rad). Spots were detected, matched, and normalized on the basis of total density of gels with the parameter of percent volume according to the software guide. To find the differentially expressed protein spots, we compared the abundance differences between treated and control samples for both provenances. For each spot, the mean relative volume (RV) was computed at every stage, and the spots showing a mean RV that changed more than 2.0 fold or less than 0.5 fold and $p<0.05$ in different stages were considered differentially expressed proteins (Ma *et al.,* 2015).

In-gel digestion and MALDI-TOF-TOF analysis: Protein spots with differential expression patterns on gels were manually excised from gels, washed with Millipore pure water for three times, destained twice with 30 mM $K_3Fe(CN)_6$ for silver staining spots, reduced with 10 mM DTT in 50 mM NH_4HCO_3, alkylated with 40 mM iodoacetamide in 50 mM NH_4HCO_3, dried twice with 100% acetonitrile and digested overnight at 37^{o}C with sequencing grade modified trypsin (Promega, Madison, WI, USA) in 50 mM NH_4HCO_3. The peptides were extracted twice with 0.1% TFA in 50% acetonitrile. Extracts were pooled and lyophilized. The resulting lyophilized tryptic peptides were dissolved in 5 mg/ml CHCA containing 0.1% TFA and 50% acetonitrile. MALDI-TOF-TOF MS analyses were conducted using 4800 *Plus* MALDI-TOF-TOF^{TM} analyzer (Applied Biosystems, Foster City, CA, USA).

All spectra of proteins were submitted to database searching using online MASCOT program (http://www.matrixscience.com), against NCBInr databases. The searching parameters were as follows: 0.15 Da mass tolerance for peptides and 0.25 Da mass tolerance of TOF-TOF fragments, one allowed trypsin miscleavage, carbamidomethyl of Cys as fixed modification, and oxidation of Met, pyro-Glu formation of N-terminal Gln and Glu as variable modification. Only significant hits, as defined by the MASCOT probability analysis ($p<0.05$), were accepted (Ma *et al.,* 2012).

Results and Discussion

2-DE analysis and identification and functional classification of differentially expressed proteins: In this study, 2-DE maps were obtained using IEF on 24 cm pH 4-7 nonlinear IPG gels followed by SDS-PAGE on 12% polyacrylamide gels. The 2-DE proteome profiles of each sample were stained by silver. The representative map 1s shown in Fig. 1. The results revealed a consistent protein pattern in their expression levels on the gels. The 2-DE profile showed that approximately 800 protein spots could be reproducibly detected mainly in the range of pH 4-7 and relative molecular masses of 15-120 kDa (Fig. 1). Quantitative image analysis of three biological replicates of each sample by PDQuest 7.2 saftware revealed that a total of 143 protein spots showed a more than 2.0-fold or less than 0.5-fold difference ($p<0.05$) in expression values at least in one provenance compared to the control. The 143 spots from 2-DE gels were excised and in-gel digested using trypsin and subjected to mass spectrometry analysis. Finally, 87 proteins, as displayed in Fig. 1, were confidently identified according to NCBI database (Table 1).

The 87 identified proteins with more than 2.0-fold ($p<0.05$) or less than 0.5-fold ($p<0.05$) quantitative differences were divided into 9 functional classes according to KEGG (http://www.kegg.jp/kegg/pathway.htm) and literatures, and the distributions of differentially expressed proteins into putative functional categories are shown in Fig. 2 and Table 1. The identified proteins were found to be involved in different metabolic pathways and processes, including cell rescue/defense (4.6%), redox homeostasis (12.6%), photosynthesis (29.9%), carbohydrate metabolism (21.8%), amino acid and nitrogen metabolism (10.3%), protein biosynthesis (1.2%), protein folding and assembly (12.6%), protein degradation (1.2%), and unclassified (5.8%). The class of 'unclassified' proteins included proteins that successfully matched in database but with unknown function.

Identified proteins were involved in stress-regulated proteins in *P. massoniana*: Stress-regulated proteins can be classified into two groups: proteins that take part in signal transduction and proteins that directly play a role in plant survival under invasion conditions. Proteins of the first group include transcription factors, RNA-binding proteins, protein kinases and phosphatases (Porankiewicz *et al.,* 1999). The second group contains proteins whose activity enables the plant to survive under stress conditions and includes proteins involved in ion homoeostasis (Dunford *et al.,* 1998), enzymes involved in scavenging of activated oxygen specie (Tishkov & Popov, 2004), protective proteins (Masaaki *et al.,* 2001), and so on. In this study, we identified 15 proteins classified into the second group. Of them, four proteins (spots 1, 2, 3, and 4) which were identified as abscisic stress ripening protein, pathogenesis-related protein 10-3-3, ABA and WDS induced 3 protein, and betaine-aldehyde dehydrogenase, were involved in cell rescue/defense and showed a significantly ($p<0.05$) down-regulated expression pattern inoculated with PWN in both GD_5 and SX_1. The four identified proteins were all involved in abio-stress, but decreased their expression in *P. massoniana* inoculated with PWN for two weeks. The results indicated that cell rescue/defense ability was decreased in both GD_5 and SX_1 after PWN invasion.

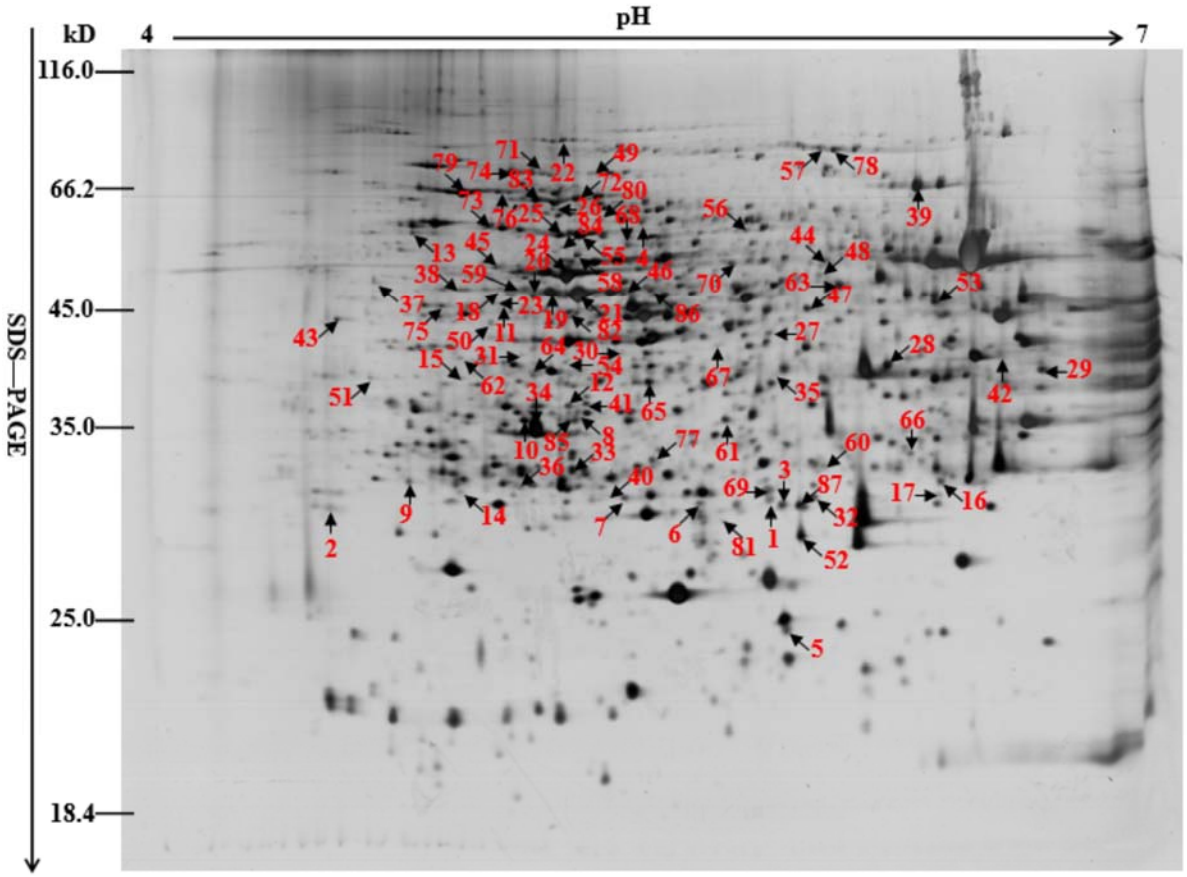

Fig. 1. Identification of 87 protein spots from provenances GD_5 and SX_1 inoculated with *B. xylophilus* for two weeks by 2-DE and MALDI-TOF-TOF MS analysis.The numbers with arrows indicate the differentially expressed and identified protein spots.

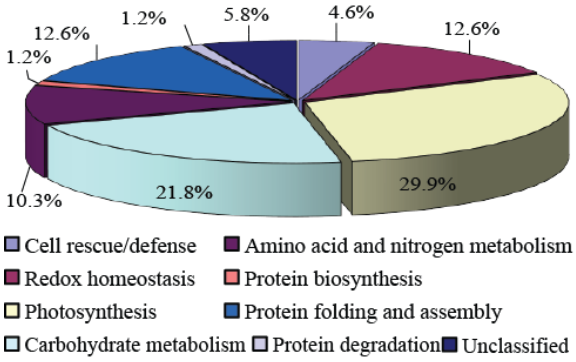

Fig. 2. Functional groups of differentially expressed proteins identified in *P. massoniana* provenances GD_5 and SX_1 inoculated with *B. xylophilus* for two weeks.

On the other hand, antioxidant proteins which related to reactive oxygen species metabolism during plant growth, are regulated by bio-stress and abio-stress (Sandra *et al.*, 1992). In the present study, a total of 11 identities were involved in scavenging of activated oxygen species. They were Cu-Zn-superoxide dismutase precursor (spot 5), two glutathione S-transferase (spots 6, and 7), three ascorbate peroxidase (spots 8, 9, and 10), glyoxalase I homolog 2 (spot 11), two lactoylglutathione lyase (spots 12, and 13) and two oxidoreductase (spots 14 and 15). These proteins are all involved in redox homeostasis which has an important role in the process of *P. massoniana* resist of being attacked by PWN. In the results, oxidoreductase NAD-binding protein (spot 14) and quinone oxidoreductase, chloroplastic (spot 15) were found to be significantly ($p<0.05$) down-regulated in both GD_5 and SX_1 after PWN invasion (Hoshida *et al.*, 2000). Glyoxalase (spot 11) and lactoylglutathione lyase (spots 12, and 13) (also known as glyoxalase) were significantly ($p<0.5$) up-regulated in SX_1 but didn't change their expression in GD_5 after PWN invasion. Glyoxalase can convert toxic 2-oxoaldehydes into less reactive 2-hydroxyacids using glutathione as a cofactor (Hoelzle *et al.*, 1992). Proteins such as Cu-Zn-superoxide dismutase (spot 5), glutathione S-transferase (spots 6, and 7), and ascorbate peroxidase (spots 8, 9, and 10), were significantly ($p<0.5$) up-regulated in GD_5 inoculated with PWN, but down-regulated or didn't change their expression in SX_1. These proteins are major ROS-scavenging proteins, providing plant cells with highly efficient machinery for detoxifying H_2O_2 and other ROS (Cai & Wong 1989). Up-regulation of these proteins implied that the antioxidative defense system in *P. massoniana* is provoked by PWN. Taken together, the fact that the expression of many proteins (spot 5, 6, 7, 8, 9, and 10) in GD_5 was higher than that of SX_1 after PWN invasion might imply that the hydrogen peroxide-scavenging capacity of GD_5 was higher than SX_1, which may be one of the important reasons why GD_5 is more resistant than SX_1.

Table 1. The 87 differentially expressed proteins identified in *P. massoniana* provenance GD_5 and SX_1 inoculated with *B. xylophilus* for two weeks.

[a]Spot no.	[b]Accession no.	[c]Protein name	[d]Protein score	[e]SC	[f]MP	[g]EpI	[h]Emw	GD_5		SX_1	
								CK	[i]TR	CK	TR
		Cell rescue/defense									
1.	gi|8272398	Abscisic stress ripening protein, *Pyrus pyrifolia*	160	35%	1	6.11	81.6	1.0	0.15*	1.0	0.18*
2.	gi|76782068	Pathogenesis-related protein 10-3.3, *Picea mariana*	279	21%	2	5.13	17.7	1.0	0.20*	1.0	0.32*
3.	gi|282767351	ABA and WDS induced 3 protein, *Pinus sylvestris*	70	12%	1	5.71	85.9	1.0	0.15*	1.0	0.11*
4.	gi|49616931	Betaine-aldehyde dehydrogenase, *Musa acuminata*	93	5%	1	6.29	29.4	1.0	0.35*	1.0	0.47*
		Redox homeostasis									
5.	gi|16798638	Cu-Zn-superoxide dismutase precursor, *Pinus pinaster*	83	5%	1	6.11	22.2	1.0	2.36*	1.0	0.11*
6.	gi|289187423	Glutathione S-transferase, *Pinus brutia*	136	13%	3	5.56	26.5	1.0	2.10*	1.0	0.69
7.	gi|289187423	Glutathione S-transferase, *Pinus brutia*	108	7%	2	5.56	26.5	1.0	3.59*	1.0	0.23*
8.	gi|350539113	Ascorbate peroxidase, *Solanum lycopersicum*	156	8%	2	8.65	42.4	1.0	3.77*	1.0	1.61
9.	gi|310587	Ascorbate peroxidase, *Spinacia oleracea*	93	7%	1	5.41	27.8	1.0	2.34*	1.0	0.34*
10.	gi|310587	Ascorbate peroxidase, *Spinacia oleracea*	133	7%	1	5.41	27.8	1.0	2.35*	1.0	1.16
11.	gi|332629597	Glyoxalase I homolog 2, *Allium cepa*	118	5%	1	5.74	40.6	1.0	1.21	1.0	3.06*
12.	gi|357520937	Lactoylglutathione lyase, *Medicago truncatula*	70	5%	1	6.56	39.0	1.0	0.83	1.0	2.24*
13.	gi|211906514	Lactoylglutathione lyase, *Gossypium hirsutum*	123	7%	2	5.69	32.6	1.0	0.77	1.0	2.53*
14.	gi|297849940	Oxidoreductase NAD-binding protein, *Arabidopsis*	65	3%	1	9.16	32.0	1.0	0.21*	1.0	0.24*
15.	gi|356526809	Quinone oxidoreductase,chloroplastic, *Glycine max*	150	5%	1	8.74	42.3	1.0	0.76	1.0	0.32*
		Photosynthesis									
16.	gi|132016	RuBisCO large subunit, *Pinus balfouriana*	507	9%	6	6.19	53.2	1.0	2.89*	1.0	2.24*
17.	gi|7524657	RuBisCO large subunit, *Pinus thunbergii*	359	8%	5	6.19	53.2	1.0	4.68*	1.0	6.38*
18.	gi|169930138	Chloroplast rubisco activase, *Capsicum annuum*	162	6%	2	8.09	48.5	1.0	0.34*	1.0	1.28*
19.	gi|169930138	Chloroplast rubisco activase, *Capsicum annuum*	172	6%	2	8.09	48.4	1.0	0.80	1.0	0.24*
20.	gi|169930138	Chloroplast rubisco activase, *Capsicum annuum*	162	6%	2	8.09	48.5	1.0	0.36*	1.0	0.63
21.	gi|169930138	Chloroplast rubisco activase, *Capsicum annuum*	175	6%	2	8.09	48.5	1.0	0.24*	1.0	0.03*
22.	gi|1006698	Rubisco binding protein, *Pseudotsuga menziesii*	143	34%	2	4.37	96.7	1.0	0.39*	1.0	0.12*
23.	gi|228016009	ATP synthase CF1, *Pinus resinosa*	924	30%	9	5.19	52.9	1.0	1.31	1.0	5.26*
24.	gi|357444609	ATP synthase, *Medicago truncatula*	399	4%	4	5.86	121.2	1.0	23.04*	1.0	0.19*
25.	gi|357444609	ATP synthase subunit, *Medicago truncatula*	425	4%	4	5.86	121.2	1.0	0.29*	1.0	0.42*
26.	gi|8132536	ATP synthase beta subunit, *Zamia furfuracea*	674	25%	7	5.06	52.2	1.0	0.29*	1.0	0.56
27.	gi|11769	Alpha subunit of ATPase, *Nicotiana tabacum*	119	3%	2	5.14	55.5	1.0	1.43	1.0	4.35*
28.	gi|132353340	Vacuolar ATP synthase, *Mesembryanthemum crystallinum*	247	4%	2	5.09	69.1	1.0	1.24	1.0	0.17*
29.	gi|55847719	Glyceraldehyde-3-phosphate dehydrogenase, *Pinus thunbergii*	172	5%	2	6.36	22.2	1.0	0.41*	1.0	4.93*

Table 1. (Cont'd.).

30.	gi|55847719	Glyceraldehyde-3-phosphate dehydrogenase, *Pinus thunbergii*	78	5%	1	6.36	22.2	1.0	0.45*	1.0	1.68
31.	gi|260903774	Glyceraldehyde 3-phosphate dehydrogenase, *Brevibacterium*	106	4%	1	4.97	35.9	1.0	0.02*	1.0	1.54
32.	gi|20788	Chlorophyll a/b-binding protein, *Pinus sylvestris*	147	7%	2	7.74	26.8	1.0	0.31*	1.0	0.40*
33.	gi|62733871	Chlorophyll a/b-binding protein CP26, *Oryza sativa*	66	4%	1	9.07	26.6	1.0	0.41*	1.0	1.36
34.	gi|131386	Oxygen-evolving enhancer protein 1, *Spinacia oleracea*	384	15%	4	5.58	35.4	1.0	0.83	1.0	0.34*
35.	gi|131385	Oxygen-evolving enhancer protein 1, *Solanum tuberosum*	207	8%	2	5.84	35.6	1.0	0.37*	1.0	0.96
36.	gi|22750	LHCP5 protein, *Pinus sylvestris*	138	5%	1	6.34	32.4	1.0	0.25*	1.0	0.82
37.	gi|217944	LHCP, *Lactuca sativa*	90	4%	1	5.29	28.3	1.0	0.45*	1.0	1.25
38.	gi|255568812	Carbonic anhydrase, *Ricinus communis*	74	5%	1	5.93	31.2	1.0	0.49*	1.0	0.99
39.	gi|2501354	Transketolase, *Craterostigma plantagineum*	116	1%	1	6.16	73.8	1.0	0.32*	1.0	0.76
40.	gi|2501356	Transketolase, chloroplastic, *Solanum tuberosum*	76	2%	1	5.94	80.3	1.0	0.44*	1.0	2.73*
41.	gi|22633	Fructose-bisphosphate aldolase, *Spinacia oleracea*	129	7%	2	7.57	42.7	1.0	0.33*	1.0	1.47
		Carbohydrate metabolism									
42.	gi|39939491	Malate dehydrogenase, *Pinus pinaster*	235	9%	2	6.18	35.8	1.0	0.49*	1.0	0.57
43.	gi|259414628	Malate dehydrogenase, *Larix kaempferi*	418	22%	6	7.63	43.4	1.0	0.47*	1.0	1.60
44.	gi|357132456	Malate dehydrogenase 1, *Brachypodium distachyon*	337	10%	2	8.54	35.6	1.0	0.43*	1.0	1.67
45.	gi|5420036	NADP-dependent malate dehydrogenase, *Selaginella martensii*	113	2%	1	6.68	48.1	1.0	0.95	1.0	5.53*
46.	gi|5420036	NADP-dependent malate dehydrogenase, *Selaginella martensii*	114	2%	1	6.68	48.1	1.0	0.30*	1.0	0.60
47.	gi|584872	Cinnamyl alcohol dehydrogenase, *Picea abies*	78	6%	1	5.69	39.3	1.0	0.63	1.0	4.24*
48.	gi|90110845	Enolase, *Oryza sativa*	419	12%	3	5.41	48.3	1.0	1.09	1.0	2.47*
49.	gi|22035897	Enolase, *Anisakis simplex*	66	3%	1	5.92	47.7	1.0	0.40*	1.0	1.85
50.	gi|211906460	Triosephosphate isomerase, *Gossypium hirsutum*	553	28%	6	6.00	27.5	1.0	2.04*	1.0	3.19*
51.	gi|46909437	Triosephosphate isomerase, *Patiria miniata*	66	5%	1	5.52	23.1	1.0	0.67	1.0	3.80*
52.	gi|11124572	Triosephosphat-isomerase, *Triticum aestivum*	100	9%	2	5.38	27.0	1.0	0.29*	1.0	1.12
53.	gi|1022805	Phosphoglycerate kinase, *Arabidopsis thaliana*	74	4%	1	4.93	41.9	1.0	0.34*	1.0	0.35*
54.	gi|332591479	Phosphoglycerate kinase 1, *Pinus pinaster*	387	8%	3	8.84	52.9	1.0	1.34	1.0	9.12*
55.	gi|148887793	UDP-glucose pyrophosphorylase, *Pinus taeda*	367	16%	5	5.21	53.2	1.0	0.45*	1.0	0.12*
56.	gi|13487787	ADP-glucose pyrophosphorylase subunit CagpS1, *Cicer arietinum*	416	10%	4	6.49	56.6	1.0	0.42*	1.0	0.43*
57.	gi|15240075	Succinate dehydrogenase, *Arabidopsis thaliana*	67	2%	1	5.86	70.2	1.0	0.27*	1.0	0.39*
58.	gi|904109	Adenosine triphosphatase, *Holboellia latifolia*	133	3%	1	5.19	53.8	1.0	1.30	1.0	2.12*
59.	gi|356533246	Adenosine kinase 2, *Glycine max*	87	4%	1	5.32	38.2	1.0	1.39	1.0	2.89*
60.	gi|21698922	Adenosine kinase, *Oryza sativa*	77	4%	1	5.29	32.6	1.0	1.29	1.0	2.96*
		Amino acid and nitrogen metabolism									
61.	gi|255542380	Cysteine synthase, *Ricinus communis*	155	8%	2	5.5	34.4	1.0	2.02*	1.0	2.35*
62.	gi|226508112	Cysteine synthase1, *Zea mays*	224	10%	3	6.97	41.8	1.0	2.65*	1.0	1.77

Table 1. (Cont'd.).

63. gi|255567778	Cysteine synthase, *Ricinus communis*	157	8%	2	7.60	43.4	1.0	1.23	1.0	2.18*
64. gi|351727959	OAS-TL4 cysteine synthase, *Glycine max*	120	3%	1	8.09	41.7	1.0	0.67	1.0	3.16*
65. gi|351727959	OAS-TL4 cysteine synthase, *Glycine max*	129	3%	1	8.09	41.7	1.0	4.96*	1.0	0.76
66. gi|121353	Glutamine synthetase, *Phaseolus vulgaris*	179	3%	2	6.77	47.5	1.0	3.97*	1.0	5.02*
67. gi|4138351	Glutamine synthetase, *Pinus sylvestris*	458	16%	5	5.88	39.4	1.0	3.09*	1.0	2.80*
68. gi|1709002	S-adenosylmethionine synthase, *Pinus banksiana*	313	10%	3	5.53	43.6	1.0	0.31*	1.0	0.41*
69. gi|357437449	Glycine dehydrogenase P, *Medicago truncatula*	105	3%	2	6.91	115.6	1.0	0.26*	1.0	0.85
	Protein biosynthesis									
70. gi|1706601	EF-Tu, *Chara connivens*	134	6%	2	5.78	45.5	1.0	0.44*	1.0	0.48*
	Protein folding and assembly									
71. gi|1620385	70kD heat shock protein, *Takifugu rubripes*	125	6%	2	6.40	40.5	1.0	0.16*	1.0	0.47*
72. gi|15242459	Mitochondrial Hsp70, *Arabidopsis thaliana*	169	5%	2	5.63	73.2	1.0	0.59	1.0	0.14*
73. gi|108707472	Hsp70, *Oryza sativa*	411	8%	6	5.30	71.9	1.0	0.29*	1.0	1.36
74. gi|20559	Hsp70, *Petunia x hybrida*	236	3%	5	5.07	71.1	1.0	0.39*	1.0	0.42*
75. gi|397482	Heat shock protein 70, *Arabidopsis thaliana*	215	3%	5	5.03	71.7	1.0	0.48*	1.0	1.06
76. gi|762844	Hsp70, *Solanum lycopersicum*	117	3%	4	5.18	71.8	1.0	0.39*	1.0	1.07
77. gi|226499860	Stromal 70 kDa Hsp, *Zea mays*	93	3%	2	5.08	74.8	1.0	0.96	1.0	3.93*
78. gi|16221	Chaperonin hsp60, *Arabidopsis thaliana*	187	3%	1	5.66	61.7	1.0	0.62	1.0	0.11*
79. gi|183675450	Cpn60, *Uncultured soil bacterium*	97	15%	2	4.86	20.2	1.0	1.51	1.0	0.20*
80. gi|461753	Chaperone protein ClpC, *Pisum sativum*	719	12%	8	6.55	102.8	1.0	0.81	1.0	3.08*
81. gi|1213116	Low molecular weight hsp, *Pseudotsuga menziesii*	63	5%	1	5.82	18.2	1.0	1.40	1.0	2.91*
	Protein degradation									
82. gi|15225839	Proteasome subunit alpha type-3, *Arabidopsis thaliana*	87	6%	1	5.93	27.6	1.0	0.70	1.0	3.20*
	Unclassified									
83. gi|145341034	AAA-metalloprotease FtsH, chloroplast, *Ostreococcus lucimarinus*	239	3%	2	5.23	67.7	1.0	0.98	1.0	0.06*
84. gi|340028721	2-octaprenyl-6-methoxyphenyl hydroxylase, *Paracoccus sp.*	56	3%	1	10.40	42.3	1.0	0.59	1.0	3.77*
85. gi|3415126	Phenylcoumaran benzylic ether reductase, *Pinus taeda*	106	3%	1	5.76	33.6	1.0	1.33	1.0	4.76*
86. gi|3415126	Phenylcoumaran benzylic ether reductase, *Pinus taeda*	236	15%	3	5.76	33.6	1.0	0.40*	1.0	5.60*
87. gi|161527594	Cobalamin biosynthesis CbiG protein, *Nitrosopumilus maritimus*	66	3%	1	5.37	38.6	1.0	6.97*	1.0	1.85

[a] Numbering corresponds to the 2-DE in Fig. 1., [b] Accession number from the NCBInr database; [c] Names and species of the proteins obtained via the MASCOT software from the NCBInr database; [d] MOWSE score probability for the entire protein; [e] The sequence coverage of identified proteins; [f] The total number of identified peptide; [g] EpI is experimental isoelectric point; [h] Emw is experimental molecular mass; [i] The protein abundance ratio (Treatment/Control) at each particular time point;

* Indicates significant (more than 2.0-fold or less than 0.5-fold) difference between control and treatment at 0.05 level

Differentially expressed proteins were associated with photosynthesis in *P. massoniana*: Photosynthesis is using the energy from sunlight to split water to liberate O_2 and converts carbon dioxide into organic compounds, especially sugars (Pollard & Cooper, 2009). In the present study, a total of 26 differentially expressed identities were found to be associated with the photosynthesis processes (Table 1). These proteins are implicated in four functional subgroups: 1) chlorophyll-binding proteins, 2) proteins participating in the Calvin cycle, 3) rubisco activases or –binding proteins, and 4) oxygen-evolving complex proteins of PS . In the first subgroup, two Chlorophyll a/b-binding proteins (spot 32 and spot 33, CP26 in PS), LHCP5 protein (spot 36) , LHCP protein (spot 37) and carbonic anhydrase protein (spot 38) were identified. CP26 and LHCP are components of the light-harvesting complex of PS in plants and facilitates light absorption and transfer of the excitation energy to reaction centers for change separation (Sancar & Hearst, 1993). Carbonic anhydrase is also involved in PS I (Grossman & Thiagalingam, 1993). These five proteins were down-regulated in GD_5 after inoculated with PWN and their expression changes were not obvious in SX_1 except spot 32. In the second subgroup, there were nine identities participating in CO_2 assimilation, including two RuBisCO large subunit (spots 16 and 17), one RuBisCO binding protein (spot 22), three glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (spots 29, 30, and 31), two transketolases (spots 39 and 40), and one fructose-bisphosphate aldolase (spot 41). Rubisco combines carbon dioxide dissolved in the cytosol with ribulose-1,5-bisphosphate to produce 3-phosphoglycerate (Wiebe *et al.,* 2007). G3PDH removes hydrogen from NADPH and adds it to the 1, 3-bisphosphoglycerate to make glyceraldehyde-3-phosphate in Calvin cycle (Askari *et al.,* 2006). Transketolase combines sedoheptulose-7- mitochondrial phosphate with another glyceraldehyde-3-phosphate to assemble ten carbons total in Calvin cycle (Wang *et al.,* 2003). Fructose-bisphosphate aldolase is also involved in Calvin cycle (Wan & Liu, 2008). Of these nine identities, seven proteins (spots 22, 29, 30, 31, 39, 40, and 41) were significantly ($p<0.05$) down-regulated in GD_5 after inoculated with PWN, but in SX_1 besides spot 22 also down-regulated, the remaining six spots expression changes were not obvious, and even expression quantity increased (spots 29, 40). And two proteins (spots 16 and 17) were found to show increased abundance in both GD_5 and SX_1. The third subgroup consisted of four proteins (spots 18,19,20 and 21) which were identified as Chloroplast rubisco activase, engaged in the activation of Rubisco. Except that spot 18 was up-regulated in SX_1, all other spots were down-regulated in both provenances after inoculated with PWN. The fourth subgroup was composed of two oxygen-evolving proteins (spots 34 and 35) which were down-regulated GD_5 and SX_1 after inoculated with PWN. In addition, six proteins (spots 23, 24, 25, 26, 27, and 28) were identified as ATP synthase or their submits, which participated in photosynthesis. Of these six proteins, two proteins (sports 23 and 27) were up-regulated and two proteins (sports 25 and 26) were down-regulated in both GD_5 and SX_1, one protein (sports 28) was down-regulated in SX_1 but its expression change was not obvious in GD_5. Only one protein (sports 24) was up-regulated significantly ($p<0.5$) in GD_5 and down-regulated remarkably ($p<0.05$)in SX_1.

It is generally believed that the proteins above mentioned in lower expression could decrease light absorption and electron transfer of PS I, CO_2 assimilation, and rubisco activation, which could impair the photosynthesis of plants, thus affect the normal growth of the plant. But in this experiment, the needles of GD_5 were not changed and the plants did not show any symptoms of disease in two weeks after inoculated with PWN. Therefore, our results suggested that the lower expression of these proteins in GD_5 does not play a major role in the resistance reaction. And ATP synthase protein (sport 24) which was up-regulated significantly in GD_5 may be balancing out the adverse factors and playing a protective role in the photosynthesis of plants**.**

Identified proteins were associated with carbohydrate metabolism in *Pinus massoniana*:After abiotic and biotic stress, plants decrease energy metabolism rates to conserve energy and limit further generation of ROS (Van *et al.,* 1994). There were 19 identities (spots 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, and 60) associated with energy metabolism pathway in this study. Of them, six proteins (42, malate dehydrogenase; 43, malate dehydrogenase; 44, malate dehydrogenase; 46, NADP-dependent malate dehydrogenase; 49, enolase; and 52, triosephosphate isomerase) were down-regulated only in GD_5 and didn't change their expression in SX_1 inoculated with PWN, and four proteins (53, PGK; 55, UDP-glucose pyrophosphorylase; 56, CagpS1; 57, succinate dehydrogenase) were down-regulated in both provenances. These ten proteins are all key proteins involved in producing energy for plants needed to fortify the resistance mechanisms, such as ion transport (Moller, 2001), reactive oxygen species scavenging (Jiang *et al.,* 2007) and osmolyte synthesis (Espartero *et al.,* 1994). Down-regulation of above proteins suggested that energy depletion was enhanced in both GD_5 and SX_1 after PWN invasion, but did not affect the GD_5 resistance. On the contrary, eight proteins (spot 45, NADP-dependent malate dehydrogenase; 47, cinnamyl alcohol dehydrogenase; 48, enolase; 51, triosephosphate isomerase; 54, phosphoglycerate kinase 1; 58, adenosine triphosphatase; 59, adenosine kinase; and 60, adenosine kinase) were found to be significantly ($p<0.05$) up-regulated only in SX_1 and not to change obviously in GD_5 and one protein (spot 50, triosephosphate isomerase) was found to be significantly ($p<0.05$) up-regulated in both provenances inoculated with PWN. NADP-dependent malate dehydrogenase and cinnamyl alcohol dehydrogenase are abundant enzyme that plays an important role in energy supply and in response to stress in plants (Wan & Liu, 2008). Enolase and triosephosphate isomerase may be essential for activation of the entire energy producing pathway (Bradford, 1976). It can be postulated that *P. massoniana* can provide more glucose for glycolysis pathway, in which key enzyme such

as enolase and triosephosphate isomerase is up-regulated. Phosphoglycerate kinase (PGK) is an enzyme that catalyzes the reversible transfer of a phosphate group from 1,3-bisphosphoglycerate (1,3-BPG) to ADP producing 3-phosphoglycerate (3-PG) and ATP. Like all kinases it is a transferase. PGK is a major enzyme used in glycolysis, in the first ATP-generating step of the glycolytic pathway (He *et al.,* 2007). Adenosine triphosphatase and adenosine kinase are both associated with energy metabolism (Csiszár *et al.,* 2011). The above data showed that SX_1 increased energy metabolism after PWN invasion, which may make SX_1 consume more energy in the body and reduce the resistance of the plants.

Identified proteins were associated with amino acid and nitrogen metabolism in *Pinus massoniana*: In the present study, 9 identified proteins (spots 61, 62, 63, 64, 65, 66, 67, 68, and 69) associated with amino acid and nitrogen metabolism were found to be differentially expressed in abundance in response to PWN in *P. massoniana* (Table 1). Of them, seven proteins (spots 61, cysteine synthase; 62, cysteine synthase1; 63, cysteine synthase; 64, OAS-TL4 cysteine synthase; 65, OAS-TL4 cysteine synthase; 66, glutamine synthetase; and 67, glutamine synthetase) were up-regulated in one provenance or in both provenances inoculated with PWN. Cysteine synthase is responsible for the final step in biosynthesis of cysteines (He *et al.,* 2007). Cysteine synthase is the key enzyme involved in assimilation of inorganic nitrogen into organic forms (Dixon *et al.,* 2002). It plays an essential role in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine (Wan & Liu 2008) and has been found to play an important role in enhancing rice tolerance to salt and chilling stresses (Ma *et al.,* 2012). Glutamine synthetase is a key protein for biosynthesis of glutamine which is involved in nitrogen metabolism (Pi *et al.,* 2010). Only two proteins (spots 68, S-adenosylmethionine synthase and 69, glycine dehydrogenase P) were down-regulated in both provenances after inoculated with PWN. These two proteins were both involved in amino acid and nitrogen metabolism (Parker *et al.,* 2006; Zhu *et al.,* 2007). Our results indicated that the amino acid and nitrogen metabolism were increased in both GD_5 and SX_1 after PWN invasion. So we deduced that the regulation of amino acid and the nitrogen metabolism does not seem to be directly involved in plant disease resistance mechanism.

Differentially expressed proteins were related to protein biosynthesis, protein folding and assembly and protein degradation *P. massoniana*: In the present study, one identified protein (spot 70, EF-Tu) was related to protein biosynthesis, eleven proteins related to protein folding and assembly (71, 70kD Hsp; 72, mitochondrial Hsp70; 73, Hsp70; 74, Hsp70; 75, Hsp70; 76, Hsp70; 77, stromal Hsp70; 78, chaperonin Hsp60; 79, Cpn60; 80, ClpC; and 81, low molecular weight hsp) and one protein related to protein degradation (spot 82, proteasome subunit alpha type-3) (Table 1). Firstly, EF-Tu (spot 70) which was functions in elongation of the growing peptide chains in chloroplast (Laloi *et al.,* 2004), was significantly ($p<0.05$) down-regulated in both GD_5 and SX_1 after inoculated with PWN. Secondly, there were eleven identified proteins related to protein folding and assembly. They were all heat shock protein family proteins which have been well studied and were known to be responsible for protein folding and assembly (Espartero *et al.,* 1995; Mittler *et al.,* 2004). Of them, eight proteins (spots 71, 72, 73, 74, 75, 76, 78, and 79) were down-regulated significantly or not changing their expression obviously in both *P. massoniana* provenances and three proteins (spots 77, 80, and 81) were up-regulated significantly in SX_1 and not changing their expression obviously in GD_5 inoculated with PWN. Lastly, only proteasome subunit alpha type-3 (spot 82) which selectively degrades various cellular proteins with specific degradation signals such as a multiubiquitin (Tchórzewski 2002), related to protein degradation, was up-regulated significantly ($p<0.5$) in SX_1 but its expression change was not obvious in GD_5 inoculated with PWN.

Taken together, the regulated expression response patterns of all the proteins in the above proteins indicated that the protein biosynthesis was inhibited as well as the protein folding and assembly. In addition, protein degradation pathway plays a dynamic and vital role in the regulation of different metabolic processes and in the cell's response to environmental stimuli. It removes irreversibly damaged polypeptides that may interfere with these pathways (Małgorzata & Barbara, 2004). Thus, the up-regulation of this protein in SX_1 indicated that protein degradation was enhanced in SX_1 by PWN invasion, which may be one important reason why SX_1 is more sensitive to PWN than GD_5.

Conclusions

In this study, to investigate changes of global proteins in *P. massoniana* response to PWN invasion, we performed a comparative proteome analysis of two provenances (*B. xylophilus* resistant, GD_5 and *B. xylophilus* sensitive, SX_1) inoculated with PWN. At last, 87 differentially expressed proteins were successfully identified by MALDI-TOF-TOF. The majority of the identified proteins in this experiment were related to cell rescue/defense (4.6%), redox homeostasis (12.6%), photosynthesis (29.9%), carbohydrate metabolism (21.8%), amino acid and nitrogen metabolism (10.3%), protein biosynthesis (1.2%), protein folding and assembly (12.6%), protein degradation (1.2%), and unclassified (5.8%). The conclusions of the present study were that cell rescue/defense and photosynthesis ability of GD_5 and SX_1 were both restrained, amino acids metabolism up-regulation and nitrogen metabolism enhanced and protein biosynthesis were down-regulation as well as the protein folding and assembly in the both experimental material. Difference was that Cu-Zn-superoxide dismutase (spot 5), glutathione S-transferase

(spot 6 and 7), and ascorbate peroxidase(spot 8, 9, 10) in the GD_5 expressed a large number of rising, and these six proteins expression levels were downgraded in SX_1. These six proteins involved in hydrogen peroxide scavenging capacity and protecting the redox homeostasis system from being damaged. Their up-regulation may be the main cause of GD_5 resistant to PWN. Moreover, the ATP synthase protein (spot 24) involved in photosynthesis was significantly up-regulated in GD_5 and significantly down-regulated in SX_1, which may have a certain positive role on resistance mechanism of GD_5. Also, proteins expression involved in the metabolism of carbohydrates showed that SX_1 energy consumption was higher than GD_5 and expression level of protein degradation up-regulation in SX_1 decreased the resistance of plants, which may also be one of the reasons for GD_5 resistant to Pine Wilt disease. Such a mechanism allows us to further understand and describe the possible cellular activities occurring in the provenance to handle PWN and provides new insights into the responses to PWN in resistant provenance.

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