Comparative Proteomic Analysis of Plant–Pathogen Interactions in Resistant and Susceptible Poplar Ecotypes Infected with *Botryosphaeria dothidea*

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ABSTRACT

Poplar are important forestry species in China, but the *Botryosphaeria dothidea* pathogen causes serious economic losses worldwide. To identify candidate *B. dothidea* resistance proteins and explore the molecular mechanisms involved in poplar–pathogen interactions, proteomic responses of stem samples from resistant and susceptible poplar ecotypes to *B. dothidea* were investigated using nanoflow liquid chromatography–tandem mass spectrometry with label-free quantitative analysis. We identified 588 proteins, divided into 21 biological process categories including 48 oxidoreductases, 72 hydrolytic enzymes, 80 metabolic enzymes, and 29 proteins of unknown function. Differential proteome analysis revealed large differences between resistant *Populus tomentosa* Carr and susceptible *Populus beijingensis* Hsu ecotypes before and after inoculation. Among 102 identified proteins, 22 were highly upregulated in the resistant genotype but downregulated in the susceptible genotype. Proteins induced in *P. tomentosa* Carr in response to *B. dothidea* are associated with plant defenses including oxidoreductase activity (catalase, isocitrate dehydrogenase, and superoxide dismutase), phenylpropanoid biosynthesis and phenylalanine metabolism (alcohol dehydrogenase),

photosynthesis (ATP synthase subunit alpha, ATP synthase gamma chain, photosystem I P700 chlorophyll a apoprotein A2, photosystem II CP47 chlorophyll apoprotein), carbon fixation (pyruvate kinase, triosephosphate isomerase, malic enzyme, phosphoglycerate kinase, ribulose-1,5-bisphosphate carboxylase, and ribulose bisphosphate carboxylase small chain), and glycolysis/gluconeogenesis (fructose-bisphosphate aldolase). Kyoto Encyclopedia of Genes and Genomes pathway analysis identified 168 proteins related to metabolic pathways, 41 proteins related to the biosynthesis of phenylpropanoids, and 36 proteins related to the biosynthesis of plant hormones, the biosynthesis of alkaloids derived from ornithine, lysine, and nicotinic acid, and photosynthesis in response to *B. dothidea*. Our findings provide insight into plant–pathogen interactions in resistant and susceptible poplar ecotypes infected with *B. dothidea* and could assist the development of novel strategies for fighting poplar canker disease.

Keywords: nanoflow LC-MS/MS, plant proteomics, poplar–*Botryosphaeria dothidea* interaction

Poplar are among the most important forestry species in China (Zhang and Luo 2003), but the yield and quality are seriously compromised by poplar canker caused by *Botryosphaeria dothidea*. Related anamorphs are the most widespread pathogens in China and have caused severe economic losses (Huang and Su 2003; Su et al. 2003). Plant responses to infection with pathogenic fungi depend on the genetic basis of plant–pathogen interactions. The ability of plants to defend themselves against disease is associated with various proteins that can be up- or downregulated following infection (Yang et al. 2011). After contact with the pathogen, the host initiates a series of physiological, biochemical, and secondary metabolic responses involving surface molecular interactions and signal transduction. Plant acclimation to stress is

associated with profound changes in proteome composition (Kosová et al. 2011).

Previous studies on the mechanisms of interaction between poplar and ulcerative diseases have mainly focused on the pathogenicity and resistance of poplar to *B. dothidea*. Poplar canker is a facultative parasite with a wide distribution, extensive genetic diversity, and broad host specificity (Chen 2006; Chen et al. 1999; Liu et al. 1997; Zhao 1998; Zhengyu and Chuandao 1987). The hazard degree of metabolites and toxin extracts of *B. dothidea* on the branches of disease-resistant varieties such as *Populus tomentosa* Carr was lower than that on susceptible *Populus beijingensis* Hsu; meanwhile, the cell membrane permeability and proline content of *P. beijingensis* Hsu was more significant than that of *P. tomentosa* Carr (Hu and Zhu 1997a; Wei et al. 2009a; Zhu et al. 1997). Analysis of the structural and physiological changes of host callus tissue in *P. tomentosa* Carr and *P. beijingensis* Hsu infected with *B. dothidea* revealed membrane lipid peroxidation in the susceptible species, and peroxidase was induced earlier in *P. tomentosa* Carr (Wang 2007). In addition, accumulation of *P. tomentosa* Carr was clearly higher than that of *P. beijingensis* Hsu, and as infection progressed, an obvious “oxidation outburst” was apparent that was less evident in *P. beijingensis* Hsu (Wang 2007). Physiological studies revealed greater changes in superoxide dismutase (SOD) activity and some secondary metabolites in the resistant *P. tomentosa* Carr compared with the susceptible *P. beijingensis* Hsu at 72 h postinoculation (hpi) (Li et al. 2011).

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SOD is a key scavenger of reactive oxygen species (ROS) in plants and can quickly deal with damaging superoxide ions. ROS may serve as signaling molecules for hydrogen peroxide-induced regulation of defense responses (Wang 2007). In addition, once a pathogen is recognized by its host, a cascade of signaling reactions, controlled by enzymes via several pathways, becomes activated, leading to defense responses and ultimately resistance (El-Bebany et al. 2010; Hammond-Kosack 1996).

The mechanisms underpinning plant disease resistance can be divided into constitutive and inducible resistance (Blodgett et al. 2007; Franceschi et al. 2005; Stout et al. 2006). Constitutive resistance includes inherent structural resistance (cuticle and wax) and inherent biochemical resistance (chemical compounds released by plants). Inductive resistance includes inductive resistance of tissue structure as well as physiological and biochemical resistance. The induction of pathogenesis-related proteins is one of many plant defense responses against pathogens (van Loon et al. 2006). Previous studies revealed that invasion of canker can induce the accumulation of hydroxyproline-rich glycoprotein (HRGP) and the deposition of lignin in the cell wall of poplar, with obvious synchronism over time (Hu and Zhu 1997a; Ye et al. 1992). Treatment of poplar callus with an oligosaccharide elicitor from mycelium of the fungus *B. dothidea* showed that the activities of phenylalanine ammonia-lyase, chitinase, and β 1,3-glucanase in poplar cells, as well as accumulation of lignin and HRGP in poplar callus, were induced significantly (Hu and Zhu 1997b; Hu et al. 1999).

The integration of gene expression and functional knowledge from genomic studies with proteomics-based data is a powerful combination for investigating biology, including plant disease resistance (Jordan et al. 2006). Unlike the genome, the proteome reflects protein and gene functions under different physiological conditions or pathological states. The identification and characterization of pathogen-responsive proteins and their corresponding genes has proven to be of immense practical value. Proteomics has been successfully used to study different pathosystems and symbiotic interactions (Birch and Kamoun 2000; Kav et al. 2007). Proteomic-based technologies have been successfully applied to the systematic study of the proteomic responses in many plant species to a wide range of abiotic stresses (Mandelc et al. 2013). For example, proteomics analyses based on two-dimensional gel electrophoresis of poplar inoculated with *B. dothidea* demonstrated that upregulated expression of proteins in disease-resistant *P. tomentosa* Carr compared with susceptible *P. beijingensis* Hsu may be related to the constitutive mechanism of resistance to canker disease, and these proteins mainly include ribulose biphosphate carboxylase small chain, predictive protein, SOD, and isopentene isomerase (Li et al. 2011). The response mechanisms of plants to pathogen infection are highly complex, and combining the results of functional genomics and proteomics studies can provide a systems-level understanding of poplar and the molecular basis of plant-pathogen interactions, including with *B. dothidea*. Limited knowledge of pathogen-responsive protein expression remains a major gap in our understanding of the biological functions of genes and the linkage between gene expression and physiological functions. Therefore, comprehensive profiling of pathogen-responsive proteins is important for elucidating the molecular mechanisms of plant tolerance to pathogen infection.

The main objective of this study was to conduct a comparative proteomic analysis between resistant and susceptible poplar cultivars and their interactions with *B. dothidea*. Since proteins are directly related to functions in biological systems, and because liquid chromatography (LC)-tandem mass spectrometry (MS/MS) can identify differences in protein sequences, the results could highlight defense- and resistance-related factors in poplar. Identifying these factors through proteomics may clarify resistance-specific targets and reveal resistance mechanisms that are activated in poplar in response to infection with *B. dothidea*, of possible relevance to disease control and resistance breeding. Therefore,

since the molecular mechanisms underpinning plant defenses that constitute the interactions between poplar and *B. dothidea* are unknown, we carried out a differential proteomic analysis of poplar stems from (i) resistant *P. tomentosa* Carr (control) inoculated with *B. dothidea* (and uninoculated controls), (ii) susceptible *P. beijingensis* Hsu inoculated with *B. dothidea* (and uninoculated controls), and (iii) poplar plants bearing resistant and susceptible traits similar to *P. tomentosa* originating from *P. tomentosa* and *P. beijingensis* Hsu.

MATERIALS AND METHODS

Plant materials and growth conditions. Two poplar cultivars, one resistant (*P. tomentosa* Carr) and one susceptible (*P. beijingensis* Hsu) to *B. dothidea*, were grown on a compost substrate in plastic pots in a greenhouse with a 10- to 14-h/10 to 14-h light/dark photoperiod, with photosynthetic active radiation from fluorescent illumination of <30,000 lx, a 22 to 30°C/20 to 25°C day/night cycle, and 50 to 75% relative humidity. After a 5-month incubation, healthy seedlings with 5-mm-diameter stems were selected for use in the following experiments. *P. tomentosa* Carr and *P. beijingensis* Hsu plants were divided into two groups, and one was sampled directly (BCK, *P. beijingensis* Hsu controls; MCK, *P. tomentosa* Carr controls), whereas the other was inoculated with *B. dothidea* (BJJ, inoculated *P. beijingensis* Hsu; MJJ, inoculated *P. tomentosa* Carr).

Pathogen isolation and cultivation. The *B. dothidea* CXY001 strain was obtained from the Culture Collection of the Chinese Academy of Forestry following isolation from canker lesions in Beijing Yang. The pathogenicity of the strain was investigated and confirmed through single cell culture by an inoculation test. Before inoculation, the fungal strain was cultured on a dish at 27°C in the dark for 7 days, and a 5-mm-diameter fungal sample obtained using a punch was inoculated onto seedlings using an inoculating needle under sterile conditions.

Inoculation and sampling. The inoculation method was similar to that described previously (Zhao et al. 1999) with some modifications. Stems from seedlings of *P. tomentosa* Carr and *P. beijingensis* Hsu were inoculated with an agar disk containing mycelium of *B. dothidea*, but before inoculation, young seedlings were disinfected with 75% alcohol and stems were washed with sterile distilled water. Two plum-like needle holes were punctured into the stem using a sterile dissecting needle. The distance between the two needle holes was ~5 mm. The *B. dothidea* mycelium plus potato dextrose agar (PDA) was inoculated onto the stem with the mycelium side toward the needle holes. Mycelium-free PDA was used for control plants. To ensure 100% humidity and avoid dehydration, holes were wrapped in sterilized wet cotton and covered with plastic paraffin film (Parafilm) after inoculation. *B. dothidea* and mock-inoculated plants were maintained in a greenhouse under the conditions described above. At 72 hpi, stem segments (phloem) of 5 mm in size were harvested by cutting around the inoculation point, freeze-dried continuously for 4 days, and stored at -80°C. The dried material was milled to a fine powder with a freezer mill (SPEX Certiprep, Metuchen, NJ) and stored at -80°C until protein extraction. Four stems were pooled for each time point, and the experiment was repeated to obtain three biological replicates.

Protein extraction. A 1.5-g sample of stem powder was homogenized with 20 ml of 10% (wt/vol) trichloromethane (tricarboxylic acid [TCA]) (Bio Basic Inc., Markham, ON, Canada) and 2% (vol/vol) β -mercaptoethanol in acetone at 4°C for 1 h, then centrifuged at 16,000 $\times$ g for 20 min at 4°C. The resulting pellet was washed twice with 15 ml of 2% (vol/vol) β -mercaptoethanol/acetone (Bio Basic Inc.) and homogenized in 10 ml of extraction buffer comprising 100 mM of Tris-HCl, pH 8.65, 30% sucrose, 2% sodium dodecyl sulfate (Bio Basic Inc.), 1 mM of polyvinylpyrrolidone (Sigma Chemicals, St. Louis, MO), and 1 mM of phenylmethanesulfonyl fluoride (PMSF) (Sangon Biotech,

Shanghai, China) at 4°C for 1 h. After centrifugation at 10,000 × g for 10 min at 4°C, an equal volume of phenol equilibrated with 10 mM of Tris-HCl (pH 8.0) was added to the supernatant and the mixture was stirred for 15 min. Proteins from the upper phenol phase were precipitated by the addition of four volumes of 0.1 M ammonium acetate to methanol and incubated for at least 2 h. Precipitated proteins were pelleted by centrifugation at 30,000 × g for 20 min at 4°C and washed twice with ice-cold acetone. Pellets were redissolved in L3 solution (Amresco) comprising 7 M of urea, 2 M of thiourea, 4% NP40, 40 mM of Tris base, pH 8.5, 5 µl of 2 mM EDTA (GibcoBRL/Life Technologies, Waltham, MA), 5 µl of PMSF, and 5 µl of dithiothreitol (DTT) (Promega, Madison, WI). The protein concentration of each stem extract was evaluated using the Bradford method with bovine serum albumin (YPHBI0 Inc., Beijing, China) as the calibration standard.

Sample preparation for LC-MS/MS. Samples containing 1 M of DTT were dissolved using ultrasonication with a VCX130 instrument (SONICS, Newtown, CT) at 56°C for 1 h, and 0.5 miodoacetamide was then added and samples were incubated at room temperature in the dark for 45 min. Cold acetone [1:4 (vol/vol)] was then added, and samples were incubated at –20°C for 2 h. The supernatant was discarded following centrifugation at 20,000 × g for 20 min. The pellet was allowed to stand for 2 to 3 min and was then suspended with a pipette in 150 µl of 8 M urea, shaken, allowed to stand for 5 min, and dissolved by ultrasonication. The total content of soluble protein was quantitated precisely using a GE kit (GE Healthcare, Chicago, IL). Protein solutions containing 100 µg of protein were hydrolyzed with 1 µg/µl of trypsin at 37°C for 16 h, then desalinated with a Strata reagent kit. The extracted peptide mixture was dried and stored at –80°C until LC-MS/MS analysis.

Nanoflow LC-MS/MS analysis. The four reconstituted sample fractions (in triplicate) were analyzed by nanoflow LC-MS/MS using an LTQ-XL linear ion trap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptide digests were resolved by ultra-performance liquid chromatography (Nano LC-Ultra; Eksigent, Framingham, MA), coupled online via electrospray ionization to a hybrid linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap Velos; Thermo Fisher Scientific, Waltham, MA). Dried peptide samples were dissolved in 20 µl of 0.1% formic acid by centrifugation at 20,000 × g for 15 min, the supernatant was extracted, and half of the volume (10 µl) was injected into a Shimadzu nano HPLC instrument (Shimadzu, Kyoto, Japan), using an autosampler. Samples eluted from the 2-cm C18 trap column (200 µm internal diameter) were separated on a 10-cm C18 analytical column (75 µm internal diameter). Samples were injected into the sample ring using a constant flow rate of 5 µl/min and into the trap column by the sample pump at a rate of 40 µl/min. This process lasted a total of 4 min. Samples were eluted using a gradient concentration from 2 to 40% buffer B over 44 min at a flow rate of 400 nl/min, followed by a linear gradient from 40 to 80% for 2 min, and held at 80% for 4 min. Following this, the concentration of buffer B was reduced from 80 to 2% over 1 min and held at 2% for 5 min. The whole process lasted 60 min. Buffer solutions used for chromatography were 0.1% formic acid in 5% acetonitrile (buffer A) and 0.1% formic acid in 95% acetonitrile (buffer B).

Mass spectrometry (MS) data acquisition was performed in positive ion mode with an electrospray voltage of 1.5 kV and a temperature in the ion transport capillary of 250°C, and the Orbitrap mass spectrometer was scanned at a resolution of 6,000, covering a mass-to-charge ratio (m/z) range of 350 to 20,000. The LTQ-Orbitrap LC-MS/MS detection was performed at conventional resolution (Niehl et al. 2013). Dynamic exclusion of parent ions was enabled with one count for 30 s after acquisition with a tolerance of 10 ppm (Niehl et al. 2013).

Proteomic data analysis. MS data were obtained, and liquid phase diagrams and primary/secondary mass spectrograms were viewed using Xcalibur LCQuan software (version 2.7; Thermo Finnigan Inc., San Jose, CA) and Xcalibur MS acquisition software

(Xcalibur 2.1; Thermo Fisher Scientific). Useful peptide signals were extracted from raw data via denoising and deisotoping, and corresponding secondary MS data were saved as a peak list of two-dimensional data sets comprising m/z ratios and signal intensities. Reference protein sequence databases were constructed, which included the entire sequence of eudicotyledons (383,330). Peptides and their signal intensities were identified and calculated by comparing the peak list with the reference protein sequence database using MaxQuant 1.1.1.25 software (Department of Proteomics and Signal Transduction, Max-Planck Institute of Biochemistry, Munich, Germany). Identification of peptides and proteins using the NCBI eudicotyledons database was performed with full enzymatic cleavage, a maximum of two missed cleavages sites, 20-ppm first search tolerance, 20-ppm MS/MS tolerance, and 0.5-Da fragment ion tolerance. Oxidation (M) and acetyl (protein N-term) were considered as variable modifications. Carbamidomethyl (C) was considered a fixed modification. The false discovery rate was below 0.01 for identified peptides and proteins, and repetitive signals from the same peptide in the same sample were normalized. Identified proteins were grouped based on common peptide segments. The signal intensity of a protein was calculated by normalizing the relative signal strength of a peptide segment. According to the relative signal strength of the protein, relative quantification was determined (i.e., the differential abundance of proteins between two different samples was calculated).

Differentially expressed proteins (DEPs) were grouped using Gene Ontology (GO) annotation and UniProt databases (Emily et al. 2012), and qualitative and quantitative results were subjected to further biological analysis. Proteins exhibiting a fold change (FC) of >2-fold ($\pm$) in response to *B. dothidea* were considered differentially expressed. Identified proteins were then subjected to metabolic pathway enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database as described previously (Jie et al. 2012). KEGG pathways (<https://www.genome.jp/kegg/>) with corrected P values < 0.05 were considered to be statistically enriched in signaling pathway including proteins for which expression was altered in response to *B. dothidea*. Protein sequences were used to search InterPro member databases using the InterProScan Sequence Search server (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>) to identify signature motifs. The compiled text outputs were subjected to GO categorization using the Web Gene Ontology Annotation Plot (WEGO). Finally, the three groups of data sets were subjected to online analysis (<http://wego.genomics.org.cn>) (Chu et al. 2015).

RESULTS

Overview of peptide identification and quantitative proteomics analysis. Peptide identification and quantitative proteomics analysis were summarized from three aspects: number of peptide sequence, protein quantity, and their molecular weight distribution. In total, 1,913 peptide sequences were identified from the four samples (Table 1), and 611 proteins were identified and separately matched from all four isolates (MCK, MJJ, BCK, and BJJ) following LTQ-Orbitrap LC-MS/MS analysis. The mass distribution of most proteins spanned a wide range of molecular masses from 7.0 to 117 kDa, and 142 and 121 proteins were concentrated in 20 to <30 and 30 to <40 kDa, respectively (Fig. 1). Among all of the proteins detected, 532 matched the BCK sample proteins, 432 matched the BJJ sample proteins, 552 matched the MCK sample proteins, and 567 matched MJJ sample proteins.

Based on the number of unique proteins identified in each functional category, function classification of proteins is shown in Figure 2. A total of 559 proteins were divided into 21 functions reflecting different biological processes. Categories in which proteins were most highly enriched were metabolic enzymes and hydrolases, which accounted for ~13.61 and 12.24%, respectively. Other enriched functional categories were oxidoreductases (8.59%),

synthetases (7.69%), dehydrogenases (7.16%), transport proteins (5.55%), and functionally unknown proteins (4.93%). Of the 559 proteins, 29 could not be functionally classified because known conserved domains were not identified in their sequences.

Functional classification of identified proteins based on GO annotation. GO annotation was performed to functionally classify identified proteins, which provides details of the hierarchical relationships of cellular components, biological processes, and molecular functions. Analysis of putative functions revealed nine GO terms related to cellular components, eight GO terms for molecular functions, and 13 GO terms associated with biological processes (Fig. 3). In terms of the cellular component category, the most represented categories were cell, cell part, macromolecular complex, organelle, and organelle part. In the molecular function category, proteins with binding and catalytic activity were highly represented. Regarding biological process, most proteins were involved in cellular processes and metabolic processes.

Differential proteins between MJJ and MCK samples were identified. Regarding cellular component proteins, those related to cell, extracellular region, and macromolecular complex subcategories were upregulated, whereas downregulated proteins were associated with extracellular regions and organelles. Among biological process-related proteins, those involved in metabolic and cellular processes, localization, localization establishment, cellular component biogenesis, cellular component organization, and anatomical structure formation were upregulated. In the molecular function category, proteins related to binding and catalysis were upregulated, as were structural molecules, transporters, and translation regulatory proteins. By contrast, electron carriers, enzyme regulatory proteins, and proteins involved in pigmentation and biological regulations were downregulated. The number of protein-coding genes associated with binding and catalyst functions in the MCK group was lower than in the MJJ group, whereas the number of protein-coding genes involved in cellular and metabolic processes in MJJ was higher than that in MCK (Fig. 3A).

The number of protein-coding genes connected with binding and catalyst functions in BCK was higher than in BJJ, whereas the number of protein-coding genes associated with cellular and metabolic process subcategories was higher in BCK than in BJJ (Fig. 3B).

GO annotation (Fig. 3C) of these proteins showed that there were no significant differences among each group between BCK and MCK samples, except for the macromolecular complex subcategory within the cellular component category. The percentage of genes related to biological processes, biological regulation, response to stimuli, and pigmentation in MCK was greater than in BCK, whereas the percentage of genes associated with cellular

processes and metabolic processes was less in MCK. The number of protein-encoding genes with molecular functions linked to antioxidant and enzyme regulator subcategories in MCK was higher than in BCK. However, more binding- and catalysis-related proteins in MCK were downregulated than in BCK (Fig. 3C).

GO annotation (Fig. 3D) of these proteins showed that the percentage of genes encoding proteins related to cell, extracellular region, macromolecular complex, and antioxidant cellular component subcategories in MJJ was higher than in BJJ. Only the number of genes encoding proteins related to organelles in MJJ was lower than in BJJ. Levels of proteins associated with biological process, response to stimulus, cellular process, metabolic process, and cellular component biogenesis subcategories were higher in MJJ than in BJJ. The number of proteins linked to binding, catalytic, antioxidant, and structural molecule molecular functions in MJJ was higher than in BJJ, but the number of proteins related to electron carrier and transporter subcategories was lower in MJJ than in BJJ (Fig. 3D).

Analysis of KEGG metabolic pathways associated with DEPs. The molecular mechanisms underpinning the responses of *P. tomentosa* Carr and *P. beijingensis* Hsu to *B. dothidea* were further investigated through enrichment analysis of the KEGG metabolic pathways associated with DEPs. A total of 317 (BCK versus BJJ), 303 (BCK versus MCK), 404 (BJJ versus MJJ), and 318 (MJJ versus MCK) DEPs were enriched in 87 (BCK versus BJJ), 86 (BCK versus MCK), 88 (BJJ versus MJJ), and 80 (MJJ versus MCK) KEGG metabolic pathways, respectively. The number of

Fig. 1. Molecular weight distribution of differentially expressed proteins obtained by LTQ-Orbitrap liquid chromatography-tandem mass spectrometry analysis of *Populus tomentosa* Carr and *Populus beijingensis* Hsu infected with *Botryosphaeria dothidea*.

TABLE 1. Identification of mass spectrometry (MS), tandem mass spectrometry (MS/MS), and peptide sequences

Sample ^a	Identifier	MS spots (n)	Total MS/MS spots (n)	Peptides		Sequences identified (n)
				MS/MS identified (n)	MS/MS identified relative to total identified (%)	
BCK	BCK1	4,946	8,025	2,555	31.82	982
	BCK2	5,023	8,143	2,574	31.61	981
	BCK3	4,859	7,905	2,516	31.83	984
BJJ	BJJ1	4,549	6,673	2,004	30.70	860
	BJJ2	4,460	6,566	2,031	29.15	878
	BJJ3	4,617	6,775	1,975	32.04	846
MCK	MCK1	4,567	7,593	1,925	25.45	825
	MCK2	4,437	7,292	1,911	26.21	815
	MCK3	4,677	7,888	1,932	24.49	829
MJJ	MJJ1	4,454	7,492	2,341	31.34	1,066
	MJJ2	4,370	7,319	2,345	32.04	1,075
	MJJ3	4,538	7,671	2,334	30.43	1,044
Total		55,497	89,342	26,443	29.58	1,913

^a BCK = *Populus beijingensis* Hsu control, BJJ = *P. beijingensis* Hsu inoculated with *Botryosphaeria dothidea*, MCK = *Populus tomentosa* Carr control, and MJJ = *P. tomentosa* Carr inoculated with *B. dothidea*.

DEPs and the top 20 pathways with the largest number of proteins in each comparable group are shown in Figure 4. In this study, the differentially expressed genes involved in metabolism pathways were the most abundant in group BJJ versus MJJ, and the least in the group BCK versus MCK. In these pathways, the following were dominant: biosynthesis of phenylpropanoids, biosynthesis of plant hormones, biosynthesis of alkaloids derived from ornithine, lysine, and nicotinic acid, photosynthesis, biosynthesis of terpenoids and steroids, biosynthesis of alkaloids derived from terpenoids and polyketides, carbon fixation in photosynthetic organisms, biosynthesis of alkaloids derived from the shikimate pathway, biosynthesis of alkaloids derived from histidine and purine, glycolysis/gluconeogenesis, methane metabolism, phenylpropanoid biosynthesis, photosynthesis-antenna proteins, phenylalanine metabolism, glyoxylate and dicarboxylate metabolism, starch and sucrose metabolism, cysteine and methionine metabolism, plant-pathogen interactions, oxidative phosphorylation, glutathione metabolism, pyruvate metabolism, and the citrate/TCA cycle.

Proteins up- and downregulated in different treatments.

Statistical analysis of the number of DEPs and their functions across all four groups revealed 18 proteins related to plant-pathogen interactions, 11 related to peroxisomes, 15 related to ribosomes, nine related to the proteasome, 14 related to metabolism of xenobiotics by cytochrome P450, 32 related to phenylpropanoid biosynthesis and phenylalanine metabolism, four related to the phosphatidylinositol signaling system, 13 related to ascorbate and aldarate metabolism, 49 related to photosynthesis, 47 related to carbon fixation in photosynthetic organisms, 21 related to photosynthesis-antenna proteins, 21 related to oxidative phosphorylation, 29 related to glycolysis/gluconeogenesis, 15 related to the citrate/TCA cycle, and 61 linked to the biosynthesis of plant hormones (Table 2).

Comparison of DEPs in healthy *P. beijingensis* Hsu (BCK) and the infected *P. beijingensis* Hsu (BJJ) sample identified 386 DEPs, of which 84 and 302 were up- and downregulated in *P. beijingensis* Hsu, respectively, in response to *B. dothidea* infection. Among these, 27 downregulated proteins were related to phenylpropanoid biosynthesis and phenylalanine metabolism, 22 were associated with carbon fixation in photosynthetic organisms, 19 were related to glycolysis/gluconeogenesis, and 36 were associated with the biosynthesis of plant hormones (Table 2).

Compared with the MCK sample, 391 DEPs were identified, including 205 and 186 up- and downregulated proteins (Table 2). Among these, 14 upregulated proteins were related to phenylpropanoid biosynthesis and phenylalanine metabolism, 28 were associated with carbon fixation in photosynthetic organisms, and 25 were related to glycolysis/gluconeogenesis in response to *B. dothidea*, whereas 39 upregulated proteins were associated with the biosynthesis of plant hormones.

A total of 382 DEPs were identified between BCK and MCK samples, including 190 and 192 up- and downregulated proteins in the MCK sample (Table 2). Among these, 18 downregulated proteins were related to photosynthesis, 28 were associated with carbon fixation in photosynthetic organisms, 13 were related to glycolysis/gluconeogenesis, and 24 were associated with the biosynthesis of plant hormones.

A total of 505 DEPs were identified between BJJ and MJJ samples, including 352 and 153 up- and downregulated in MJJ. Among these, 28 upregulated proteins were related to phenylpropanoid biosynthesis and phenylalanine metabolism, 29 were associated with carbon fixation in photosynthetic organisms, 23 were related to glycolysis/gluconeogenesis, and 47 were associated with the biosynthesis of plant hormones (Table 2).

Fig. 2. Functional category coverage of total and functional identified proteins. The numbers in the pie chart represent the number of proteins with the same function. The percentages in parentheses refer to the proportion of such functional proteins in the total identified proteins.

Therefore, most of the identified proteins were upregulated in *P. tomentosa* Carr following *B. dothidea* inoculation, whereas most were downregulated in *P. beijingensis* Hsu after *B. dothidea* infection. Most of these proteins are linked to phenylpropanoid biosynthesis and phenylalanine metabolism, photosynthesis, carbon fixation in photosynthetic organisms, glycolysis/gluconeogenesis, and the biosynthesis of plant hormones.

Expression levels of DEPs in different treatments. The expression levels of 102 differential proteins in different treatments are listed in Table 3. Expression levels are represented by label-free quantitation (LFQ) intensity. Based on thresholds of a ≥ 2 -fold increase (+) or a ≤ 0.5 -fold decrease (–) in abundance, a large number of proteins displayed altered expression levels compared with controls (MCK for *P. tomentosa* Carr and BCK for *P. beijingensis* Hsu) following inoculation with *B. dothidea* (Table 3).

When *P. tomentosa* Carr (MCK) plants were inoculated with *B. dothidea*, expression of most proteins was upregulated. Proteins displaying the greatest upregulation were alcohol dehydrogenase (Q9SEP7, FC = 48.86), dehydroascorbate reductase (DHAR) class glutathione transferase (D2WL74, FC = 11.19), ATP synthase gamma chain (A9PJJ6, FC = 11.68), malic enzyme (B9GP43, FC = 9.79; B9GPX3, FC = 17.89; B9ICA1, FC = 28.03; and B9ILT5,

FC = 7.44), ribulose 1,5-bisphosphate carboxylase large subunit (fragment; Q33388, FC = 55.17), and glyceraldehyde-3-phosphate dehydrogenase (B9RAL0, FC = 10.84). Proteins exhibiting the largest decrease in expression were SOD [Cu-Zn] (A8CF89, FC = –0.78; and B2CP37, FC = –0.89), peroxidase (Q081T5, FC = –0.74), caffeoyl coenzyme A 3-O-methyltransferase 2 (Q0N3X0, FC = –0.67), photosystem II subunit Q-2 (D7M1Z0, FC = –0.92), cytochrome b6-f complex iron-sulfur subunit (B9I6H7, FC = –0.92), and chlorophyll A/B-binding protein (COL2S1, FC = –0.80). Therefore, we speculate that *P. tomentosa* Carr may resist *B. dothidea* infection by upregulating protein expression.

Expression of most proteins was downregulated in *P. beijingensis* Hsu inoculated with *B. dothidea*, and among the most downregulated were SOD [Cu-Zn] (A8FC89, FC = –0.87; and B2CP37, FC = –0.93), alcohol dehydrogenase (Q9SEP7, FC = –1.00), hydroxycinnamoyl coenzyme A shikimate/quinate hydroxycinnamoyl transferase-like protein (B9IFG0, FC = –1.00), peroxidase (Q43049, FC = –1.00), aspartate aminotransferase (Q43781, FC = –1.00; A9PHH1, FC = –0.98; and B9HAW0, FC = –0.84), ribosomal protein S27 (RPS27; C6SWV6, FC = –1.00), photosystem I P700 chlorophyll a apoprotein A2 (B7T455, FC = –0.98), oxygen-evolving enhancer protein 2 (P16059, FC = –1.00),

Fig. 3. Functional classification and comparison of differentially expressed proteins based on Gene Ontology annotation. **A**, Red bars indicate proteins that are significantly upregulated in the MJJ group relative to MCK, whereas violet bars indicate proteins significantly upregulated in MCK relative to MJJ. **B**, Red bars indicate proteins that are significantly upregulated in the BCK group relative to BJJ, whereas violet bars indicate proteins significantly upregulated in BJJ relative to BCK. **C**, Red bars indicate proteins that are significantly upregulated in the BCK group relative to MCK, whereas violet bars indicate proteins significantly upregulated in MCK relative to BCK. **D**, Red bars indicate proteins that are significantly upregulated in the BJJ group relative to MJJ, whereas violet bars indicate proteins significantly upregulated in MJJ relative to BJJ. MJJ = *Populus tomentosa* Carr inoculated with *Botryosphaeria dothidea*, MCK = *P. tomentosa* Carr control, BCK = *P. beijingensis* Hsu control, and BJJ = *P. beijingensis* Hsu inoculated with *B. dothidea*.

pyruvate kinase (A9PFJ7, FC = -1.00), malic enzyme (B9GPX3, FC = -1.00), phosphoglycerate kinase (D7L0K4, FC = -1.00), ribulose biphosphate carboxylase large chain (D6P9T4, FC = -1.00), chlorophyll A/B-binding protein (C0L2S1, FC = -1.00), isopentenyl diphosphate delta isomerase (A9ZN04, FC = -1.00),

and malate dehydrogenase (D7TG04, FC = -1.00). Therefore, we speculated that *P. beijingensis* Hsu may resist *B. dothidea* infection by decreasing its protein expression.

Levels of catalase (Q9ZPK7), peroxidase (Q43049), aspartate aminotransferase (A9PHH1), photosystem I P700 chlorophyll a

Fig. 4. Kyoto Encyclopedia of Genes and Genomes pathway annotation of differentially expressed proteins in *Populus tomentosa* Carr and *P. beijingensis* Hsu following *Botryosphaeria dothidea* infection. The numbers in the pie chart represent the number of differentially expressed proteins in each pathway. MJJ = *P. tomentosa* Carr inoculated with *B. dothidea*, MCK = *P. tomentosa* Carr control, BCK = *P. beijingensis* Hsu control, and BJJ = *P. beijingensis* Hsu inoculated with *B. dothidea*.

TABLE 2. Number of up- and downregulated differential proteins in different treatments^a

Related metabolic pathway	Total DEPs (n)	Proteins (n)			
		MJJ-MCK	BJJ-BCK	MCK-BCK	MJJ-BJJ
		Up/down	Up/down	Up/down	Up/down
Total up- and downregulated proteins in two sample comparisons		205/186	84/302	190/192	352/153
Plant-pathogen interactions	18	5/7	4/8	6/7	10/6
Peroxisome	11	3/4	2/4	2/6	3/2
Ribosome	15	6/3	4/6	6/4	7/3
Proteasome	9	1/2	2/6	3/1	5/2
Metabolism of xenobiotics by cytochrome P450	14	6/2	1/6	4/6	9/3
Phenylpropanoid biosynthesis and phenylalanine metabolism	32	14/11	0/27	13/12	28/3
Phosphatidylinositol signaling system	4	0/3	0/3	1/1	2/0
Ascorbate and aldarate metabolism	13	6/4	2/8	3/3	9/3
Photosynthesis	49	12/23	10/19	11/18	22/16
Carbon fixation in photosynthetic organisms	47	28/5	2/22	7/28	29/9
Photosynthesis-antenna proteins	21	0/14	7/7	9/2	6/11
Oxidative phosphorylation	21	13/5	3/8	3/9	8/4
Glycolysis/gluconeogenesis	29	25/0	2/19	5/13	23/1
Citrate/TCA cycle	15	10/3	1/8	3/5	8/4
Biosynthesis of plant hormones	61	39/4	4/36	11/24	47/5

^a DEP = differentially expressed protein, MJJ = *Populus tomentosa* Carr inoculated with *Botryosphaeria dothidea*, MCK = *P. tomentosa* Carr control, BJJ = *P. beijingensis* Hsu inoculated with *B. dothidea*, BCK = *P. beijingensis* Hsu control, and TCA = tricarboxylic acid.

TABLE 3. Proteins differentially expressed in *Populus beijingensis* Hsu and *Populus tomentosa* Carr in response to *Botryosphaeria dothidea* infection identified by label-free quantitation (LFQ) analysis^a

Number	Identifier	Protein name	Gene name	Protein databank	MW	PEP of protein	Os	Fold change ^b				
								A	B	C	D	
Plant-pathogen interaction proteins (n = 2)												
1	P27164	Calmodulin-related protein	CAM53	sp	21.113	1.6E-160	<i>Petunia hybrida</i>	-0.67		7.78	2.17	
2	D2CFI9	Leucine-rich repeat protein	LRR1	tr	40.079	2.41E-06	<i>Nicotiana tabacum</i>		1.00		-1.00	
Poplar peroxisome proteins (n = 7)												
3	Q9ZPK7	Catalase	CAT1	tr	57.411	7.23E-12	<i>Brassica juncea</i>	8.31	-0.73	-0.62	11.92	
4	A0EJ87	Isocitrate dehydrogenase [NADP]		tr	47.242	2.02E-08	<i>Populus canescens</i>	5.01	-0.67	-0.91		
5	A8CF89	Superoxide dismutase [Cu-Zn]	sod-1	tr	21.978	8.59E-37	<i>Populus alba</i>	-0.78	-0.87	0.17		
6	B2CP37	Superoxide dismutase [Cu-Zn]	SOD	tr	15.198	9.35E-06	<i>Potentilla atrosanguinea</i>	-0.89	-0.93	-0.51		
7	B9I887	Superoxide dismutase	POPTRDRAFT_730452	tr	25.315	3.96E-07	<i>Populus trichocarpa</i>		1.07	0.74		
8	B9IPE5	Type IIF peroxiredoxin	PtrPrxIIF	tr	21.924	4.25E-12	<i>Populus trichocarpa</i>		-1.00	0.84		
9	P93260	Glycolate oxidase	GOX	tr	40.612	0.000492	<i>Mesembryanthemum crystallinum</i>	-0.50	*	*		
Phenylpropanoid biosynthesis and phenylalanine metabolism proteins (n = 11)												
10	A9PDU7	Cinnamyl alcohol dehydrogenase-like protein	CADL10	tr	39.31	1.48E-10	<i>Populus trichocarpa</i>	*	-1.00	-1.00	*	
11	A9PHZ1	Cinnamyl alcohol dehydrogenase-like protein	CADL2	tr	39.364	1.35E-15	<i>Populus trichocarpa</i>	2.55		*	*	
12	D2XQZ8	Alcohol dehydrogenase		tr	41.794	0.000258	<i>Mangifera indica</i>	2.03	-1.00	-0.76	*	
13	Q9SEP7	Alcohol dehydrogenase		tr	41.233	6.62E-05	<i>Arabis turrita</i>	48.86	-1.00	1.10	*	
14	B9IFG0	Hydroxycinnamoyl coenzyme A shikimate/quinate hydroxycinnamoyl transferase-like protein	HCQL3	tr	47.875	0.000258	<i>Populus trichocarpa</i>	-0.74	-1.00	-0.81	*	
15	Q08IT5	Peroxidase		tr	34.041	4.16E-20	<i>Populus trichocarpa</i>	-0.74	-0.94	0	7.15	
16	Q0N3X0	Caffeoyl coenzyme A 3-O-methyltransferase 2	CCoAOMT2	tr	27.55	1.43E-10	<i>Populus trichocarpa</i>	-0.67	-0.55	1.90	1.14	
17	Q43049	Peroxidase	prxA3a	tr	37.079	3.24E-31	<i>Populus trichocarpa</i>		-1.00	2,201	*	
18	Q43781	Aspartate aminotransferase	AAT	tr	45.457	1.00E-19	<i>Populus trichocarpa</i>		-1.00	-0.55	*	
19	A9PHH1	Aspartate aminotransferase	POPTRDRAFT_835881	tr	44.474	3.55E-37	<i>Populus trichocarpa</i>		-0.98	9.63	416	
20	B9HAW0	Aspartate aminotransferase	POPTRDRAFT_654206	tr	51.046	3.16E-21	<i>Populus trichocarpa</i>		-0.84		17.48	
Cytochrome P450 metabolism proteins (n = 4)												
21	D2WL74	DHAR class glutathione transferase		tr	23.651	2.91E-29	<i>Populus trichocarpa</i>	11.19		-0.98	-0.60	
22	A9PHH6	Phi class glutathione transferase GSTF1	POPTRDRAFT_55081	tr	24.334	1.77E-78	<i>Populus trichocarpa</i>	-0.54				
23	A9PBD6	Phi class glutathione transferase	POPTRDRAFT_665133	tr	23.212	7.20E-102	<i>Populus trichocarpa</i>				2.83	
24	B9GQ64	Phi class glutathione transferase GSTF2	POPTRDRAFT_550820	tr	24.619	5.19E-28	<i>Populus trichocarpa</i>				2.49	
Ribosome proteins (n = 2)												
25	C6SWV6	Ribosomal protein S27	POPTRDRAFT_800083	tr	10.134	3.76E-14	<i>Populus trichocarpa</i>	2.48	-1.00	1.04	*	
26	D7LEE5	60S acidic ribosomal protein P0	ARALYDRAFT_483041	tr	33.806	3.39E-22	<i>Arabidopsis lyrata</i> subsp. <i>lyrata</i>	2.10	1.15	1.25	2.24	
Photosynthesis proteins (n = 20)												
27	A4GYP3	ATP synthase subunit alpha, chloroplastic	atpA	sp	55.322	3.94E-115	<i>Populus trichocarpa</i>	1.47		-0.83		
28	Q5QCF9	ATP synthase subunit alpha (fragment)	atp1	tr	45.135	2.36E-06	<i>Rafflesia pricei</i>	3.27	-0.80		10.90	
29	Q5QCG2	ATP synthase subunit alpha (fragment)	atp1	tr	45.064	4.05E-28	<i>Berlinianche aethiopica</i>	3.60	-0.65	-0.53	5.10	
30	A9PJJ6	ATP synthase gamma chain		tr	41.098	8.35E-13	<i>Populus trichocarpa</i> × <i>Populus deltoides</i>	11.68	-0.66	-0.97		
31	Q14FF0	ATP synthase subunit beta, chloroplastic	atpB	tr	53.623	5.96E-155	<i>Populus tomentosa</i>	2.07		-0.83		
32	C3UNX9	ATP Synthase subunit beta, chloroplastic	atpB	tr	53.671	1.97E-120	<i>Megaleranthis sanciculifolia</i>	2.31		-0.79		
33	A0ZZ52	Cytochrome b559 subunit alpha	psbE	tr	9.3864	9.34E-101	<i>Gossypium barbadense</i>		-0.67	1.06	3.07	
34	B7T455	Photosystem I P700 chlorophyll a apoprotein A2	psaB	tr	83.139	3.19E-16	<i>Pelargonium cotyledonis</i>	2.75	-0.98	-0.72	63.46	
35	A4GYS4	Apocytochrome f	petA	sp	35.038	2.19E-49	<i>Populus trichocarpa</i>	-0.64			-0.77	
36	B9I6H7	Cytochrome b6-f complex iron-sulfur subunit	POPTRDRAFT_663556	tr	24.34	4.36E-27	<i>Populus trichocarpa</i>	-0.84	1.00		-0.94	
37	B9RJ42	Photosystem II 22-kDa protein, chloroplast, putative	RCOM_1030930	tr	29.344	3.66E-08	<i>Ricinus communis</i>	-0.62	*	*	*	
38	B9S408	Photosystem I reaction center subunit II, chloroplast, putative	RCOM_0557350	tr	23.089	1.34E-67	<i>Ricinus communis</i>	-0.54		1.53		
39	D7M1Z0	Photosystem II subunit Q-2	PSBQ		24.722	1.83E-22	<i>Arabidopsis lyrata</i> subsp. <i>lyrata</i>	-0.92	*	*	*	
40	P16059	Oxygen-evolving enhancer protein 2	PSBP	sp	28.047	2.15E-15	<i>Pisum sativum</i>	-0.63	-1.00	2.70	*	
41	A4GYT7	Photosystem II CP47 chlorophyll apoprotein	psbB	sp	56.116	4.54E-138	<i>Populus trichocarpa</i>	0.96	-0.50	-0.45	1.17	
42	A4GYU0	Photosystem II reaction center protein H 1	psbH	tr	7.7299	1.57E-08	<i>Populus trichocarpa</i>		1.21		-0.65	
43	A4QK31	Apocytochrome f	petA	sp	35.398	3.13E-12	<i>Arabis hirsuta</i>		*		-1.00	
44	B6DZC6	Photosystem Q(B) protein (Fragment)		tr	36.315	4.22E-36	<i>Capsella bursa-pastoris</i>				1.00	
45	B7T2Y6	Photosystem II CP43 chlorophyll apoprotein	psbC	tr	51.918	7.18E-79	<i>Erodium texanum</i>		-0.86	-0.53	3.89	
46	Q9SXU4	Cytochrome b6-f complex iron-sulfur subunit (Fragment)		tr	16.862	2.45E-11	<i>Cicer arietinum</i>		*		-1.00	

(Continued on next page)

^a PEP = posterior error probability, MW = molecular weight, and Os = organism species.

^b A = indicates fold change in expression in MJJ compared with MCK, B = indicates fold change in expression in BJJ compared with BCK, C = indicates fold change in expression in MCK compared with BCK, and D = indicates fold change in expression in MJJ compared with BJJ. Asterisks indicate that the expression of this protein in the latter is 0 when the two are compared (i.e., the asterisk in group A indicates that the protein expression in the MCK sample is 0).

TABLE 3. (Continued from previous page)

Number	Identifier	Protein name	Gene name	Protein databank	MW	PEP of protein	Os	Fold change ^b			
								A	B	C	D
Carbon fixation in photosynthetic organisms (<i>n</i> = 25)											
47	A9P7U5	Pyruvate kinase	POPTRDRAFT_589553	tr	57.465	6.62E-42	<i>Populus trichocarpa</i>	3.11	-0.65		5.87
48	A9PFJ7	Pyruvate kinase	POPTRDRAFT_563414	tr	55.724	2.15E-07	<i>Populus trichocarpa</i>	7.07	-1.00	-0.78	*
49	Q42806	Pyruvate kinase, cytosolic isozyme		tr	55.302	1.34E-27	<i>Glycine max</i>	*			*
50	A9PE68	Triosephosphate isomerase	POPTRDRAFT_656103	tr	27.259	4.30E-35	<i>Populus trichocarpa</i>	1.34	-0.53	-0.96	-0.80
51	Q9SKP6	Triosephosphate isomerase, chloroplastic	TIM	sp	33.345	9.75E-06	<i>Arabidopsis thaliana</i>	2.12	-1.00		*
52	B9GP43	Malic enzyme	POPTRDRAFT_816506	tr	69.741	3.56E-11	<i>Populus trichocarpa</i>	9.79	-0.85	-0.93	3.82
53	B9GPX3	Malic enzyme	POPTRDRAFT_552135	tr	67.267	1.91E-16	<i>Populus trichocarpa</i>	17.89	-1.00		*
54	B9ICA1	Malic enzyme	POPTRDRAFT_731028	tr	67.172	3.35E-18	<i>Populus trichocarpa</i>	28.03		-0.84	7.43
55	B9ILT5	Malic enzyme	POPTRDRAFT_737670	tr	65.146	1.74E-67	<i>Populus trichocarpa</i>	7.44		-0.63	2.92
56	B9HY29	Phosphoglycerate kinase	POPTRDRAFT_833719		50.211	6.25E-26	<i>Populus trichocarpa</i>	6.49		*	*
57	B9HY30	Phosphoglycerate kinase	POPTRDRAFT_659332	tr	42.679	3.89E-88	<i>Populus trichocarpa</i>	4.89	-0.77	-0.61	9.19
58	D7L0K4	Phosphoglycerate kinase	ARALYDRAFT_478641	tr	50.007	1.33E-36	<i>Arabidopsis lyrata</i> subsp. <i>lyrata</i>	5.37	-1.00	-0.82	*
59	O82158	Phosphoglycerate kinase	PnCytPGK1	tr	42.486	1.33E-76	<i>Populus nigra</i>	3.02	-0.65		11.61
60	A6XHZ3	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (Fragment)	rbcL	tr	51.438	6.43E-37	<i>Ximenia americana</i>	6.79		-0.67	
61	D6P9T4	Ribulose bisphosphate carboxylase large chain	rbcL	tr	17.631	1.85E-12	<i>Berberis asiatica</i>	*	-1.00	-1.00	*
62	A9PFS6	Ribulose bisphosphate carboxylase small chain	POPTRDRAFT_813777	tr	20.379	4.17E-06	<i>Populus trichocarpa</i>	*	-0.55	-1.00	-0.97
63	A9PHQ1	Ribulose bisphosphate carboxylase small chain	POPTRDRAFT_580797	tr	20.39	0.000493	<i>Populus trichocarpa</i>	-0.53		-1.00	-1.00
64	A9PJL4	Ribulose bisphosphate carboxylase small chain			20.404	0.00059	<i>Populus trichocarpa</i> x <i>Populus deltoides</i>	0.96		*	*
65	A9P9U1	Fructose-bisphosphate aldolase	POPTRDRAFT_836979	tr	38.579	3.43E-39	<i>Populus trichocarpa</i>				1.40
66	A9PCR0	Malate dehydrogenase		tr	35.588	8.42E-83	<i>Populus trichocarpa</i>	-0.81		1.01	-0.66
67	B9GGU9	Malate dehydrogenase	POPTRDRAFT_707785	tr	35.498	8.30E-122	<i>Populus trichocarpa</i>	-0.73	-0.56		
68	P09559	Phosphoribulokinase, chloroplastic		sp	45.007	1.42E-06	<i>Spinacia oleracea</i>		-0.90	-0.81	1.73
69	Q33388	Ribulose 1,5-bisphosphate carboxylase large subunit (Fragment)	rbcL		51.647	2.54E-37	<i>Clethra alnifolia</i>	55.17	1.76	-0.97	
70	A9PI67		POPTRDRAFT_578586	tr	19.202	0.033689	<i>Populus trichocarpa</i>	-0.58	0.63	-0.51	-0.87
71	Q645N1	Malate dehydrogenase	mMDH	tr	36.04	6.25E-32	<i>Solanum lycopersicum</i>			-1.00	-1.00
Photosynthesis-antenna proteins (<i>n</i> = 8)											
72	A9PGD4	Light-harvesting complex II protein Lhcb6	Lhcb6-2	tr	27.206	1.88E-61	<i>Populus trichocarpa</i>	-0.69	4.77	1.55	-0.87
73	B9GML1	Light-harvesting complex I protein Lhca2	Lhca2-	tr	29.458	1.28E-15	<i>Populus trichocarpa</i>	-0.57	*	*	-0.65
74	B9I107	Light-harvesting complex II protein Lhcb1	Lhcb1-1	tr	28.053	3.54E-27	<i>Populus trichocarpa</i>		-0.88		9.46
75	B9IAR5	Light-harvesting complex II protein Lhcb2	Lhcb2-1	tr	28.513	4.82E-15	<i>Populus trichocarpa</i>	-0.55	-0.72	24.77	40.50
76	B9N4C4	Light-harvesting complex I protein Lhca4	Lhca4	tr	27.696	1.42E-26	<i>Populus trichocarpa</i>	-0.66			-0.67
77	COL2S1	Chlorophyll A/B-binding protein	CAB1	tr	28.182	3.19E-18	<i>Gardenia jasminoides</i>	-0.80	-1.00		*
78	P12470	Chlorophyll A/B-binding protein E, chloroplastic	CABE	sp	28.277	3.62E-21	<i>N. plumbaginifolia</i>	-0.59	2.64	1.83	-0.68
79	Q39286	Chlorophyll A/B-binding protein CP26 in PS II	Lhcb5*Bj1	tr	30.478	8.62E-30	<i>Brassica juncea</i>		-1.00	-1.00	
Oxidative phosphorylation proteins (<i>n</i> = 1)											
80	B9HWA2	ATP synthase subunit beta	PtrAtpB_1	tr	59.883	1.40E-159	<i>Populus trichocarpa</i>	3.02	-0.51		4.08
Proteasome proteins (<i>n</i> = 5)											
81	A9PAG0	Proteasome subunit alpha type	POPTRDRAFT_733926	tr	25.625	1.16E-44	<i>Populus trichocarpa</i>	-0.64	-0.60		
82	A9PCR5	Proteasome subunit alpha type	POPTRDRAFT_833008	tr	27.008	1.86E-47	<i>Populus trichocarpa</i>		-0.94	1.28	34.84
83	B9HDL1	Proteasome subunit alpha type	POPTRDRAFT_717215	tr	27.307	4.14E-13	<i>Populus trichocarpa</i>		-0.66		2.10
84	B9HT94	Proteasome subunit beta type	POPTRDRAFT_1089650	tr	29.375	4.16E-22	<i>Populus trichocarpa</i>		-0.91	2.52	72.95
85	B9N7I3	Proteasome subunit alpha type	POPTRDRAFT_745884	tr	26.031	4.17E-59	<i>Populus trichocarpa</i>		-0.54		
Glycolysis/ gluconeogenesis (<i>n</i> = 8)											
86	A9PEU6	Fructose-bisphosphate aldolase	POPTRDRAFT_822107	tr	42.494	0.0000419	<i>Populus trichocarpa</i>	0.26		*	*
87	A9PGW0	Fructose-bisphosphate aldolase		tr	42.825	1.14E-15	<i>Populus trichocarpa</i>	0.63	-0.58	-0.76	-0.07
88	B3F8I0	Glyceraldehyde-3-phosphate dehydrogenase, GAPD	SAHH2	tr	37.021	6.07E-21	<i>N. langsdorffii</i> x <i>N. sanderae</i>	*	-1.00	-1.00	*
89	B9RAL0	Glyceraldehyde-3-phosphate dehydrogenase	RCOM_1507100		36.839	1.88E-28		10.84	31.4	3.33	0.58
90	D2D308	Glyceraldehyde-3-phosphate dehydrogenase		tr	36.951	2.94E-83	<i>Gossypium hirsutum</i>	5.28		*	*
91	P17878	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic	GAPC	sp	36.587	8.89E-22	<i>Mesembryanthemum crystallinum</i>	1.56	-1.00		*
92	P29409	Phosphoglycerate kinase	184.23		45.572	1.10E-29	<i>Spinacia oleracea</i>	2.34	2.76		
93	Q68HC8	Glucose-6-phosphate isomerase	POPTRDRAFT_1071816	tr	67.442	6.97E-08	<i>Populus trichocarpa</i>	1.47			6.54
Biosynthesis of plant hormones (<i>n</i> = 9)											
94	B9N6I9	Citrate synthase	POPTRDRAFT_837325	tr	53.506	0.000134	<i>Populus trichocarpa</i>	2.09	5.81		-0.72
95	B9RQ33	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	RCOM_0952830	tr	90.187	1.98E-94	<i>Ricinus communis</i>	*	3.82	-1.00	463.9
96	O22111	6-Phosphogluconate dehydrogenase, decarboxylating	gnd	tr	56.373	8.60E-15	<i>Glycine max</i>	3.14		-0.59	3.21
97	B9GL99	6-Phosphogluconate dehydrogenase, decarboxylating	OPTRDRAPFT_640402	tr	53.948	2.52E-18	<i>Populus trichocarpa</i>	2.31		-0.63	-0.18
98	Q40345	Isocitrate dehydrogenase [NADP], chloroplastic		sp	48.383	0.000121	<i>Medicago sativa</i>	*		-1.00	-0.64
99	Q711R1	Allene oxide cyclase	aoc	tr	26.524	1.33E-15	<i>N. tabacum</i>		-0.77		1.36
100	A9PIJ2	Enolase	POPTRDRAFT_836259	tr	47.641	1.50E-122	<i>Populus trichocarpa</i>		-0.51		2.24
101	A9ZN04	Isopentenyl diphosphate delta isomerase	HbIPI II	tr	34.363	1.04E-10	<i>Hevea brasiliensis</i>		-1.00	3.87	*
102	D7TG04	Malate dehydrogenase	VIT_00006474001	tr	37.642	3.64E-24	<i>Vitis vinifera</i>		-1.00	0.84	*

apoprotein A2 (B7T455), proteasome subunit alpha type (A9PCR5), proteasome subunit beta type (B9HT94), light-harvesting complex II protein Lhcb2 (B9IAR5), and 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (B9RQ33) in *P. tomentosa* Carr after inoculation were significantly higher than in *P. beijingensis* Hsu. Therefore, we speculated that the strong resistance of *P. tomentosa* Carr may be related to regulation of these proteins.

In addition, when *P. tomentosa* Carr and *P. beijingensis* Hsu were inoculated with *B. dothidea*, proteins displaying the greatest downregulation were SOD [Cu-Zn] (A8FC89 and B2CP37), peroxidase (Q081T5), caffeoyl coenzyme A 3-O-methyltransferase 2 (Q0N3X0), malate dehydrogenase (B9GGU9), light-harvesting complex II protein Lhcb2 (B9IAR5), chlorophyll a/b-binding protein (COL2S1), and proteasome subunit alpha type (A9PAG0). Expression of calmodulin-related protein (P27164, FC = -0.67) and photosystem II subunit Q-2 (D7M1Z0, FC = -0.92) was decreased in *P. tomentosa* Carr after inoculation, but expression of calmodulin-related protein (P27164) in MCK was higher than in BCK (FC:7.78), and expression of photosystem II subunit Q-2 was higher in MCK than in BCK (Table 3). Expression of photosystem I P700 chlorophyll a apoprotein A2 (B7T455) and ribulose biphosphate carboxylase large chain (D6P9T4) was higher in BCK than in MCK, and expression was increased in *P. tomentosa* Carr after inoculation but decreased in *P. beijingensis* Hsu (Table 3). Expression of catalase and photosystem I P700 chlorophyll a apoprotein A2 was lower in MCK than in BCK, and levels were higher in MJJ than in BJJ.

DISCUSSION

Proteomics is a powerful tool for researching plant diseases from the perspectives of both hosts and pathogens. The biochemical changes resulting from infection are of great interest because they offer insight into the molecular mechanisms underpinning plant disease resistance (Rampitsch et al. 2006). Previous biochemical studies revealed that resistance reactions in poplar lead to hypersensitivity at 72 hpi. Therefore, to gain a better understanding of the molecular basis of the plant-pathogen interactions in resistant and susceptible plants, we analyzed the proteomes of representative resistant and susceptible cultivars at 72 hpi with *B. dothidea*. The results showed that *B. dothidea* inoculation triggered significantly different responses in susceptible and resistant cultivars, including DEPs. Proteomic profiling identified 386 and 391 DEPs in *P. beijingensis* Hsu and *P. tomentosa* Carr, respectively, compared with controls. A total of 382 and 505 DEPs were identified between *P. beijingensis* Hsu and *P. tomentosa* Carr before and after 72 hpi, respectively. Understanding the roles and expression patterns of these proteins in resistant and susceptible cultivars is essential to elucidate the molecular mechanisms underpinning resistance against *B. dothidea*, the fungus causing canker in poplar. Further analysis unveiled 102 DEPs, of which those closely related to disease resistance were mainly associated with plant-pathogen interactions, antioxidants, signaling systems, photosynthetic systems, metabolism, and biosynthesis. Interestingly, 22 were upregulated in the highly resistant genotype but downregulated in the susceptible genotype.

Proteins involved in plant-pathogen interactions. Two differential proteins, leucine-rich repeat protein (LRR1) and calmodulin-related protein (CAM53), were found to be involved in plant-pathogen interactions. In particular, expression of calmodulin-related protein (P27164) in *P. tomentosa* Carr was decreased after inoculation, but expression in MCK and MJJ groups was higher than in BCK and BJJ. Signaling pathways show that calmodulin-related protein stimulates the production of signaling substance nitric oxide, thereby triggering hypersensitive response, reinforcing the cell wall, and making poplar resistant (Supplementary Fig. S1). One possible explanation is that CAM53 mediates control of numerous enzymes, ion channels, and other proteins using Ca^{2+} during plant defense responses. The enzymes stimulated

by the calmodulin- Ca^{2+} complex include various protein kinases and phosphatases.

Proteins involved in antioxidant systems. Plants possess a set of enzymes and redox metabolites that carry out ROS detoxification as contributors to antioxidant systems, which are important for protecting cells against oxidative stress (Urbanek Krajnc et al. 2011). ROS play multiple roles in defense pathways, including activation of signaling cascades, cell wall fortification, and toxic free radical production (Torres 2010). ROS are believed to be a key indicator of the cell's ability to survive pathogen attack, and they also help maintain oxidative balance in the cell (Huerta-Ocampo et al. 2009; Kunkel and Brooks 2002; Xiao et al. 2009). Plants employ antioxidants such as ascorbate and glutathione, as well as detoxifying enzymes such as SOD, CAT, and enzymes in the ascorbate/glutathione cycle, to combat oxidative stress. Guaiacol peroxidase and ascorbate peroxidase (APX) are involved in protecting plant tissues against oxidative damage and in the detoxification of cytotoxic products (Campo et al. 2004). Similarly, there is a clear correlation between host plant resistance and the elimination of active oxygen radicals (Girotti 2001; Huang et al. 2009; Kocsy et al. 2001; Wei et al. 2009b). APX is a key enzyme in the ascorbate-glutathione cycle, an important antioxidant system for detoxifying AOS in plant cells. The observed increase in spot abundance for APX following *B. cinerea* infection is not surprising as it is probably necessary for detoxifying AOS produced during infection.

In this study, seven peroxisome-related proteins were identified as catalase (CAT1, no. 3), isocitrate dehydrogenase [NADP] (no. 4), SOD [Cu-Zn] (SOD and SOD₁, nos. 5, 6, and 7), type IIF peroxidase (PtcPrxIIF, no. 8), and glycolate oxidase (no. 9). These proteins are the main antioxidant enzymes related to ROS (Shetty et al. 2008). At 72 hpi with *B. dothidea*, the expression level of CAT1 (no. 3) enhanced ROS levels in the resistant *P. tomentosa* Carr but decreased them in the susceptible *P. beijingensis* Hsu cultivar. SOD (nos. 4, 5, and 6) showed no significant differences, but the expression levels of sod-1 and PtcPrxIIF in *P. tomentosa* Carr at 72 hpi were higher than in *P. beijingensis* Hsu. Meanwhile, proteins 3, 4, and 6 were expressed at lower levels in *P. tomentosa* Carr than in *P. beijingensis* Hsu. This indicates that different antioxidant enzymes could be involved in antioxidant defenses against infection by *B. dothidea* in different cultivars. The above findings suggest that CAT1 and SOD might be important antioxidant enzymes in the poplar defense response to pathogen infection, possibly by coordinating an oxidative burst by decreasing the cellular levels of ROS-scavenging molecules. Our results showed that SOD1 expression levels were decreased in both the resistant *P. tomentosa* Carr and the susceptible *P. beijingensis* Hsu, and there was no significant difference between these two cultivars at 72 hpi. Thus, SOD1 may be regulated by many factors and cannot be used to estimate poplar resistance.

Proteins involved in photosynthetic systems. Photosynthesis, one of the most sensitive physiological processes, is subject to various stresses in green plants. Previous studies have shown that a range of stress conditions reduce photosynthesis, including pathogen infection (Amey et al. 2008; Mahmood et al. 2006). In this study, 11 photosynthesis-related proteins were identified, among which seven were upregulated in *P. tomentosa* Carr after inoculation with *B. dothidea*, including ATP synthase subunit alpha protein (nos. 27 and 28) and photosystem I P700 chlorophyll a apoprotein A2 (no. 34). Meanwhile, six proteins were downregulated, including apocytocrome f (no. 35), cytochrome b6-f complex iron-sulfur subunit (no. 36), photosystem II 22-kDa protein (chloroplast, no. 37), photosystem I reaction center subunit II (no. 38), photosystem II subunit Q-2 (no. 39), and oxygen-evolving enhancer protein 2 (no. 40). A total of eight proteins were downregulated in *P. beijingensis* Hsu at 72 hpi with *B. dothidea*, including ATP synthase subunit alpha protein (no. 29) and photosystem I P700 chlorophyll a apoprotein A2 (no. 34).

Photosynthesis is the main source of energy; hence, photosynthetic inhibition is one of the primary detrimental effects caused by pathogen infection (Kottapalli et al. 2009). Upregulation of proteins could be part of the rapid defense response to the fungus. It is probable that pathogens cause a gradual decline in the rate of photosynthesis in infected areas of plants as disease progresses (Fang et al. 2012). Downregulation of protein activity indicates that infection with *B. dothidea* markedly inhibits the rate and extent of photosynthesis (Fotopoulos et al. 2003; Swarbrick et al. 2006). Expression of photosystem I P700 chlorophyll a apoprotein (B7T455) was higher in BCK than in MCK, and expression was increased in *P. tomentosa* Carr after inoculation but decreased in *P. beijingensis* Hsu. Photosystem I P700 chlorophyll a apoprotein A2 is an integral membrane protein complex that uses light energy to mediate electron transfer from plastocyanin to ferredoxin (Golbeck 1987). The enhancement of host resistance may be associated with increased expression of these proteins.

Proteins involved in carbon fixation. In total, 25 proteins associated with carbon fixation in photosynthetic organisms exhibited altered expression levels, among which 18 were upregulated in *P. tomentosa* Carr, while 13 were decreased in *P. beijingensis* Hsu at 72 hpi with *B. dothidea*, including pyruvate kinase (nos. 47 and 48), triosephosphate isomerase (nos. 50 and 51), malic enzyme (nos. 52 and 53), phosphoglycerate kinase (PGK; nos. 57, 58, and 59), and ribulose biphosphate carboxylase large chain proteins (nos. 61 and 62). Pyruvate phosphate dikinase catalyzes the conversion of pyruvate to phosphoenolpyruvate and is involved in gluconeogenesis (Evans and Wood 1968; Kinoshita et al. 2000). Malic enzyme catalyzes the oxidative decarboxylation of malate to form pyruvate, an important reaction in a number of metabolic pathways. In *P. tomentosa* Carr cells subjected to *B. dothidea* infection, malic enzyme was upregulated ~28.03-fold (Table 3), in accordance with previous observations demonstrating modulation of this enzyme during responses to pathogen infection (Libik-Konieczny et al. 2012; Schaaf et al. 1995). This change in malic enzyme expression is also consistent with observations in *Vitis vinifera* subjected to *B. cinerea* infection (Dadakova et al. 2015). PGK is an enzyme that catalyzes the conversion of ATP to ADP and vice versa. This reaction is essential in most cells in aerobes for the generation of ATP and in plants for carbon fixation. Pyruvate kinase catalyzes the final step in glycolysis—namely, the conversion of phosphoenolpyruvate to pyruvate with concomitant phosphorylation of ADP to ATP. Ribulose biphosphate carboxylase (Karkehabadi et al. 2005; Taylor and Andersson 1997) catalyzes the initial step in the Calvin reductive pentose phosphate cycle in plants, and rubisco activase controls carbon fixation. Similar changes in expression of the rubisco subunit-binding protein have been reported in numerous plant species under various biotic and abiotic stresses (Demirevska et al. 2008; Fujiwara et al. 2006; Nakano et al. 2006; Sangha et al. 2013). Therefore, these proteins contribute to maintaining appropriate levels of photosynthesis and various metabolites in plants during defense responses to pathogen infection, and this may be one of the reasons why *P. tomentosa* Carr is more resistant than *P. beijingensis* Hsu. By contrast, malate dehydrogenase, an enzyme that reversibly catalyzes the oxidation of malate to oxaloacetate (Ingram-Smith et al. 2006), was downregulated in both *P. tomentosa* Carr and *P. beijingensis* Hsu after infection.

Proteins involved in the biosynthesis of hormone-related proteins. Biosynthesis is essential for cell function because it is responsible for the production of structural proteins, initiation factors, elongation factors, and aminoacyl tRNAs. Thus, it is closely related to plant growth and pathogen infection. Plant defenses against pathogens involve the synthesis of many compounds, which is an energy-demanding process (Heidel et al. 2004; Wang et al. 2012). Structural proteins (5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, no. 95) and ribosome-related proteins (RPS27, no. 25; and 60S acidic ribosomal protein P0, no.

26) that displayed altered expression were also found to be involved in hormone biosynthesis, phenylpropanoid biosynthesis, and phenylalanine metabolism, along with other ribosome- and biosynthesis-related proteins. 5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase is involved in the regulation of methionine biosynthesis (Zerillo et al. 2013).

Ribosomes are structures that catalyze mRNA-directed protein synthesis in all organisms. Kruger et al. (2002) reported that several ribosomal proteins and translation elongation factors were induced in wheat tissues after *Fusarium graminearum* infection, suggesting that RPS27 may be involved in resistance to canker in poplar (Kruger et al. 2002). These biosynthesis-related proteins were upregulated in the resistant cultivar after *B. dothidea* inoculation but diminished in the susceptible cultivar, suggesting that they must play an active role in biosynthesis and thereby affect plant growth and resistance.

Proteins involved in other metabolic pathways. Our proteomic analysis unveiled proteins that are potentially involved in other metabolic pathways, including phenylpropanoid biosynthesis and phenylalanine metabolism, cytochrome P450 metabolism, ribosomes, oxidative phosphorylation, and glycolysis/gluconeogenesis. Interestingly, an alcohol dehydrogenase (nos. 12 and 13) and a glycolytic enzyme were upregulated in the resistant *P. tomentosa* Carr after infection. This could indicate an attempt by the plant to facilitate the glycolytic/fermentative pathway, thought to be crucial for reducing the supply of hexoses required for fungal growth. Some of our findings are inconsistent with those of previous studies. For example, downregulation of alcohol dehydrogenase in strawberry leaves has been suggested to improve plant performance in response to *Colletotrichum fragariae* stress (Fang et al. 2012). The spot abundance of a DHAR class glutathione transferase (cytochrome P450-related protein) was increased in *P. tomentosa* Carr following pathogen exposure (Table 1). Expression of the GSTF2 gene in resistant *P. tomentosa* Carr infected with *B. dothidea* was higher than in *P. beijingensis* Hsu (Table 3).

Glutathione S-transferase (GSTs) catalyze the conjugation of glutathione to a wide range of electrophilic substrates, including phytotoxic compounds, to tag them for vacuolar import and subsequent detoxification. GSTs induced during stress may detoxify metabolites produced during oxidative damage or phytotoxins produced by pathogens (Gronwald and Plaisance 1998). In addition, polyphenol oxidase (nos. 9 and 15) can oxidize tyrosine to quinones, which are subsequently converted to 3-methoxy-4-hydroxyl mandelic acid (3-methoxy-4-hydroxymandelate) by catechol-O-methyltransferase (no. 16). Finally, 3-methoxy-4-hydroxyphenylethyleneglycol is transformed into more toxic substances that are used to resist canker bacteria under the influence of alcohol dehydrogenase (nos. 10, 11, 12, and 13). Thus, it can be inferred that these proteins may be involved in poplar resistance to *B. dothidea*.

This study provides the first proteomic analysis of the responses of resistant and susceptible poplar cultivars to infection by *B. dothidea*. It also elucidates the differential expression and composition of the major proteins responding to *B. dothidea* inoculation after 72 h. The results indicate that the differential expression of proteins is dependent on plant genotype and pathogen infection. Among 102 identified proteins, ~20 (catalase, isocitrate dehydrogenase, alcohol dehydrogenase, RPS27, ATP synthase subunit alpha, ATP synthase gamma chain, photosystem I P700 chlorophyll a apoprotein A2, photosystem II CP47 chlorophyll apoprotein, pyruvate kinase, triosephosphate isomerase, malic enzyme, phosphoglycerate kinase, ribulose biphosphate carboxylase large chain, ribulose biphosphate carboxylase small chain, and fructose-biphosphate aldolase) were upregulated in the highly resistant genotype but downregulated in the susceptible genotype (Table 3). Most of these proteins are linked to phenylpropanoid biosynthesis and phenylalanine metabolism, photosynthesis, carbon fixation in photosynthetic organisms, or glycolysis/gluconeogenesis. These proteins may play an important role in the regulation of host defense mechanisms. KEGG metabolic pathways identified >168

proteins related to metabolic pathways, >41 proteins related to the biosynthesis of phenylpropanoids, and >36 proteins related to the biosynthesis of plant hormones, the biosynthesis of alkaloids derived from ornithine, lysine, and nicotinic acid, and photosynthesis in poplar in response to *B. dothidea*. Further work will be directed at the functional characterization of candidate genes in response to *B. dothidea* infection. The observed changes in protein abundance reflect changes in gene expression and imply the biological roles of these proteins in the defense response against *B. dothidea*. Further exploration and quantification of these defense response proteins will improve our understanding of *B. dothidea*–poplar interactions and could facilitate the development of resistance gene-based biomarkers for molecular-assisted breeding and enhanced disease resistance through genetic modification and gene cloning technology.

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