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ORIGINAL ARTICLE

Comparative iTRAQ proteomic profiling of susceptible and resistant apple cultivars infected by *Alternaria alternata* apple pathotype

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Abstract Alternaria blotch, caused by the *Alternaria alternata* apple pathotype (*A. alternata* AP), is one of serious pathogen of apples. In order to better understand the molecular mechanisms that underlie the defense responses of apple resistance to Alternaria blotch disease, a comparative proteomic approach was applied to analyze of susceptible and resistant apple cultivars response to *A. alternata* AP infection using iTRAQ (isobaric tags for relative and absolute quantitation) technique. A total of 4225 proteins were identified, and 1226 proteins were quantified. Of the quantified proteins, 280 and 34 expressed differentially (fold change >1.5) at 72 h post-infection (HPI) in the susceptible ("Starking Delicious") and the resistant ("Jonathan") apple cultivars, respectively, compared with mock-inoculated controls. Most of the differentially expressed proteins (DEPs) were associated with host plant resistance to pathogens, including signal transduction, stress and defense, and photosynthesis metabolism. Among these proteins, beta-1,3-glucanase(PR2), thaumatin-like protein (PR5), and lipoxygenase were found in both susceptible and resistant hosts. However, endochitinase and (+)-neomenthol dehydrogenase were only detected in the resistant cultivar and increased in abundance in response to the pathogen attack. To study the role of pathogenesis-related (PR) proteins in the early infection process, their expressions at 6, 18, 36, and 72 HPI were analyzed by western blot. It showed that PR5 were accumulated to a high level at 6 HPI in "Jonathan," while cannot be detected in "Starking Delicious" until 18 HPI. The above results suggested that endochitinase and (+)-neomenthol dehydrogenase, as well as PR5 which exerts function at early stage, play important roles in apple plant against *A. alternata* AP infestation.

Keywords Apple · Alternaria blotch · *Alternaria alternata* Apple pathotype · Host resistance · Quantitative proteomics · Western blot

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Introduction

Apple (*Malus* × *domestica* Borkh.) is one of the most widely cultivated tree fruits in the world and is ubiquitous temperate fruit cultivated in Europe and Asia from antiquity (Sofi et al. 2013). In East Asia, where over 50% of the world's apples are produced, the majority of apple cultivars currently grown in the major production areas are susceptible to Alternaria blotch diseases (Li et al. 2013; Harteveld et al. 2014). Alternaria blotch caused by *Alternaria alternata* apple pathotype (Roberts 1924) has been a destructive apple disease in China and other East Asian countries (Jung 2007). *A. alternata* apple pathotype (AP) can cause symptoms on apple leaves, young shoots, and fruits, and symptoms appear as small, blackish spots with chlorotic margins that later extend into patches with a brownish-purple border (Lee et al. 2011). In epidemic years, this disease can cause serious defoliation and reduce tree growth, fruit quality, and yield (Lee et al. 2011; Li et al. 2012).

Several reports showed that some of the main cultivars are resistant to *A. alternata* AP, such as "Jonathan," "Gala," and "Jiguan," while "Starking Delicious," "Indo," and "Orin" are highly susceptible to infection (Abe et al. 2010; Zhang et al. 2014). Management of the Alternaria blotch occurs mainly through traditional chemical control agents instead of resistant cultivars. Therefore, studies designed to characterize host-pathogen interactions are not only essential for understanding host resistance in apple but also for the development of novel, safe, and more effective control strategies (Buron-Moles et al. 2015).

Recently, several studies have been conducted to understand how apple plants response to pathogen infections by transcriptomics methods (Harimoto et al. 2007; Xu et al. 2015). However, the changes in gene expression at transcript level do not often correspond with the changes at protein level (Gygi et al. 1999; Kaur et al. 2011; Fu et al. 2016). Therefore, investigation of changes in plant proteome is highly important since proteins, unlike transcripts, are direct effectors of plant stress response (Kosová et al. 2011). Proteomics is a useful technique and is now being widely used to study functional and regulatory aspects of proteins, for example by comparative proteomics, protein-protein interactions, and protein modifications (Chen and Harmon 2006). By the proteomic approach, we can obtain an understanding and identification of the functions of proteins expressed in a given condition (Mehta et al. 2008). Over the years, the most frequently used proteomic technique is the two-dimensional gel technique, by which differentially expressed spots are excised and analyzed by mass spectrometry (MS). However, some co-migrating proteins can compromise the accuracy of the quantification, and interfere with protein identification (Zieske 2006). In recent years, a new technique termed iTRAQ (isobaric tags for relative and absolute quantitation) has been applied for proteomic quantitation. iTRAQ labeling not only overcomes some of the limitations of 2D gel-based techniques but also improves the through put of proteomic studies. This technique has a high sensitivity, and the amine specific isobaric reagents of iTRAQ allow the identification and quantitation of up to eight different samples simultaneously (Aggarwal et al. 2006; Zieske 2006).

In this study, iTRAQ-based quantitative proteomic profiling in compatible ("Starking Delicious"-*A. alternata* AP) and incompatible ("Jonathan"-*A. alternata* AP) interactions was performed to gain molecular insights into defense mechanism of apple combat against *A. alternata* AP infection. Meanwhile, the iTRAQ-quantified differentially expressed pathogenesis-related (PR) proteins were further examined by western blot analysis. Our results showed that endochitinase and (+)-neomenthol dehydrogenase, as well as PR5 which exerts function at early stage, play important roles in apple against *A. alternata* AP infestation.

Materials and methods

Plant materials

Plant materials were taken from three-year-old apple plants (*Malus* × *domestica* Borkh.) grafted on *Malus robusta* Rehd. stocks. Two cultivars "Starking Delicious" and "Jonathan", which are susceptible and resistant to *A. alternata* AP, respectively (Abe et al. 2010), were used in this study. The plants were grown in the greenhouse at Nanjing Agricultural University, located in Nanjing, Jiangsu Province, China. The inoculation method was carried out according to the protocol of Abe et al. (Abe et al. 2010). *A. alternata* AP was cultured on potato dextrose agar (PDA; 3 g potato extract, 20 g dextrose, 15 g agar, 1 L water) medium for 7 days at 26 °C under dark conditions. For each cultivar, the fourth and the fifth youngest opened leaves from the shoot tips were picked from three apple plants separately. Six positions of each leaf were inoculated by mycelia biomasses of *A. alternata* AP. Leaves sampled at 0 and 72 HPI were used to extract total proteins for iTRAQ analysis, and leaves sampled at 6, 18, 36, and 72 HPI were used for western blotting.

Protein extraction

Apple leaves were ground in liquid nitrogen, then the cell powder was transferred to 50 mL centrifuge tube and sonicated three times on ice using a high-intensity ultrasonic processor (Scientz China) in lysis buffer (8 M urea, 1% Triton-100, 65 mM DTT, and 0.1% Protease Inhibitor Cocktail Set III). The remaining debris was removed by centrifugation (Thermo, GTR21-1) at 20,000×*g* at 4 °C for 10 min. Finally, the proteins were precipitated with 15% cold TCA for 2 h at −20 °C. After centrifugation at 4 °C for 10 min, the supernatant was discarded. The remaining precipitate was washed with cold acetone for three times and redissolved in the buffer (8 M urea, 100 mM TEAB, pH 8.0). Protein concentration was determined with 2D Quant kit (GE Health) according to the manufacturer's instructions.

Trypsin digestion

The protein solution was reduced with 10 mM DTT for 1 h at 37 °C and alkylated with 20 mM iodoacetamide for 45 min at room temperature in darkness. For trypsin digestion, protein samples were diluted by adding 100 mM TEAB (tetraethylammonium bromide) to urea concentration less than 2 M. Approximately 100 μg protein for each sample was digested with trypsin (Promega, V5111 Fitchburg, WI, USA). Trypsin was added to protein solution at the ratio of protein: trypsin =50:1 for the first digestion overnight and added at 100:1 for a second 4-h digestion.

TMT labeling and HPLC fractionation

After trypsin digestion, peptide was desalted by Strata X C18 SPE column (Phenomenex) and vacuum-dried. Peptide was reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for 8-plex iTRAQ kit (iTRAQ 8-plex kit, ABSciex). Briefly, one unit of iTRAQ reagent (defined as the amount of reagent required to label 100 μg of protein) was thawed and reconstituted in 24 μL ACN (acetonitrile). The peptide mixtures were then incubated for 2 h at room temperature and pooled, desalted, and dried by vacuum centrifugation. For the quantification of 12 samples, two labeling examples (8-plex and 5-plex) were carried out.

Samples were then fractionated into fractions by high pH reverse-phase HPLC using Agilent 300Extend C18 column (5 μm particles, 4.6 mm ID, 250 mm length). Briefly, peptides were first separated with a gradient of 2–60% acetonitrile in 10 mM pH 8.0 ammonium bicarbonate over 80 min into 80 fractions. Then, the peptides were combined into 18 fractions and dried by vacuum centrifuging.

LC-MS/MS analysis

Peptides were dissolved in 0.1% FA (formic acid), directly loaded onto a reversed-phase pre-column (Acclaim PepMap 100, Thermo Scientific). Peptide separation was performed using a reversed-phase analytical column (Acclaim PepMap RSLC, Thermo Scientific). The gradient was comprised of an increase from 6 to 20% solvent B (0.1% FA in 98% ACN) over 25 min, 20 to 35% in 8 min, and increasing to 80% in 3 min then holding at 80% for the last 4 min, all at a constant flow rate of 300 nl/min on an EASY-nLC 1000 UPLC system. The resulting peptides were analyzed by Q ExactiveTM hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific).

The peptides were subjected to nanospray ionization source followed by tandem mass spectrometry (MS/MS) in Q ExactiveTM Plus (Thermo Scientific)-coupled online to the UPLC. The intact peptides were detected in the Orbitrap at a resolution of 70,000. The peptides were selected for MS/MS using stepped normalized collision energy (NCE) setting as 30; ion fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans was applied for the top 20 precursor ions above a threshold ion count of 3E4 in the MS survey scan with 30.0 s dynamic exclusion. The electrospray voltage applied was 2.0 kV. Automatic gain control (AGC) was used to prevent overfilling of the ion trap; 5E4 ions were accumulated for generation of MS/MS spectra. For MS scans, the m/z scan range was 350–1800 Da. Fixed first mass was set as 100 m/z.

Database search

The resulting MS/MS data were processed using Mascot search engine (v.2.3.0). Tandem mass spectra were searched against *Malus* × *domestica* database (NCBI) concatenated with reverse decoy database. Trypsin/P was specified as cleavage enzyme allowing up to two missing cleavages. Mass error was set to 10 ppm for precursor ions and 0.02 Da for fragment ions. Carbamidomethyl on Cys, iTRAQ 8plex (N-term), and iTRAQ 8-plex (K) were specified as fixed modification and oxidation on Met was specified as variable modifications. False discovery rate (FDR) was adjusted to $<1\%$, and peptide ion score was set >20.

Functional classification, enrichment analysis, and subcellular localization

Gene ontology (GO) annotation proteome was derived from the Gene Ontology Consortium (http://www.geneontology.org/). Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to annotate protein pathway (http://www.genome.jp/kegg/). Subcellular localizations of proteins were determined using wolfpsort (http://genomics.cicbiogune.es/SECRETOOL/wolfpsort.php).

Western blot analysis

Apple cDNA encoding PR2 (gi:657953557) was amplified from apple leaves by RT-PCR and subcloned into prokaryotic expression vector pCzn1-His. The recombinant proteins were expressed in ArcticExpress (DE3) cells and purified using Ni-NTA spin column. Based on PR5 (gi:657978684) amino acid sequence, two peptides (PR5-1: CPNTVWPGTLTGDQKPQLS; PR5-2:TEYSEIFEKQCPQAYSYAYDDK) were synthesized as antigens. The purified proteins or the synthesized peptides were inoculated into New Zealand white rabbits to develop polyclonal antibodies. The sensitivity and specificity of antibodies were evaluated by enzyme-linked immunosorbent assay (ELISA). The protein conjugations, immunizations, and antiserum purifications were carried out by BPI (Beijing Protein Innovation Co., Ltd., Beijing, China). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 50 μg of proteins from "Jonathan" and "Starking Delicious" were separated by 5% stacking gel (5% Acr-Bis, 0.125 M Tris-HCl pH 6.8, 0.01% SDS, 0.01% Ammonium persulfate, 0.01% tetramethylethylenediamine) with 80 V for 30 min and 12% resolving gel (12% Acr-Bis, 0.375 M Tris HCl pH 8.8, 0.01% SDS, 0.01% ammonium persulfate, 0.04% TEMED) with the 120 V for 90 min, respectively. The separated proteins were transferred to a nitrocellulose membrane (Pall Life Sciences) with a constant electricity of 80 mA for 120 min. The membrane was immersed in blocking solution (5% non-fat milk-TBST) at room temperature for 120 min. The proteins were incubated with the polyclonal antibodies in

blocking solution. After washing, the membrane was incubated with HRP-conjugated secondary antibodies in blocking solution at room temperature for 90 min. The membrane was developed with a EnoGene™ ECL Plus Detection kit (Enogene Biotech. Co.,Ltd., Nanjing, China), and the signal was obtained by Tocan-3900 (Tanon Science & Technology Co.,Ltd. Shanghai, China).

Results

Phenotypic symptoms of the resistant and susceptible cultivars in response to pathogen infection

Phenotypic symptoms showed the existence of differences between resistant and susceptible apple cultivars when their leaves were inoculated with *A. alternata* AP. At 6 HPI, leaves of the susceptible cultivar appeared water soaked at the inoculated sites, while those in the resistant cultivar did not change obviously. Disease symptoms started to be visible at 18 HPI in the two cultivars. Brown blotches constituted of damaged tissues were more obvious in the susceptible leaves than in the resistant leaves. The blotches became larger and their color turned browner at 36 HPI and brown to a black-brown mix at 72 HPI on the susceptible cultivar leaves. The blotch size did not expand further after 18 HPI; however, in resistant cultivar, the blotch color was deeper (Fig. 1).

Identification of DEPs following pathogen infection

Proteome of apple leaves infected by *A. alternata* AP was analyzed by the iTRAQ technique. Three independent biological replicates were set; thus, 12 samples were included in the iTRAQ experiment: "Starking Delicious" 0 HPI (S0-1, S0-2, and S0-3), "Starking Delicious" 72 HPI (S72-1, S72-2, and S72-3), "Jonathan" 0 HPI (J0–1, J0–2, and J0–3), and "Jonathan" 72 HPI (J72-1, J72-2, and J72-3). After trypsin digestion, the proteins were labeled with isobaric tags. In the eight-plex experiment, eight samples cover two replicates, labeled with 113–119 and 121 iTRAQ tags. In the five-plex experiment, four samples cover one replicate, labeled with 113–116 iTRAQ tags; one sample (J72–1) which is common with the previous experiment was labeled with 117 iTRAQ tags and co-analyzed in a second iTRAQ experiment for calibration. The analytical separation and identification of the mixture were performed by LC-MS/MS. The proteins detected at least two of the three biological replicates were counted, and a total of 4225 proteins were identified, and 1226 proteins were quantified in the 12 samples (supporting information Figure S1; supporting information Excel S1). Compared with the controls, 280 and 34 proteins expressed differentially (fold change >1.5 or <0.67, $p < 0.05$) in the susceptible and resistant apple cultivars, respectively (Fig. 2a). In the susceptible cultivar, 90 proteins were upregulated and 190 were downregulated, while in the resistant cultivar, 18 proteins were upregulated and 16 were downregulated. Nineteen proteins were detected

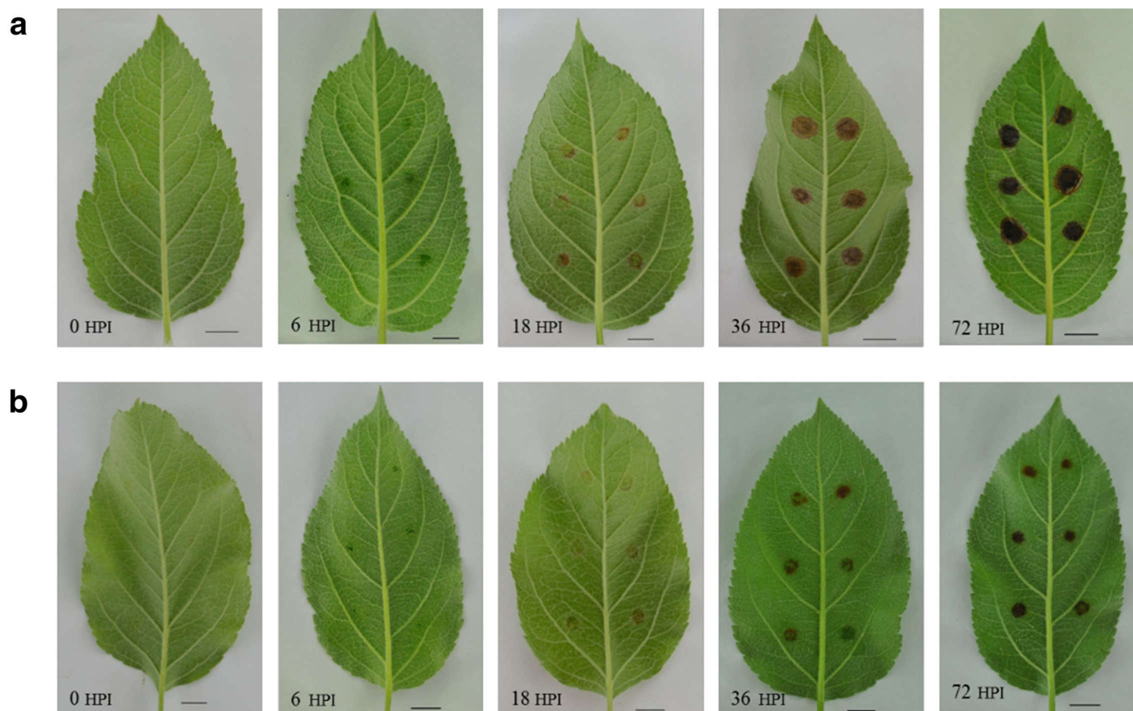

Fig. 1 Disease symptom on susceptible ("Starking Delicious") and resistant ("Jonathan") apple leaves after infection with *A. alternate* AP. Disease symptom on leaves of susceptible (**a**) and resistant (**b**) apple leaves at 0, 6, 18, 36, and 72 HPI. *Bar* = 1 cm

both in the susceptible and resistant cultivars, among which eight proteins were upregulated both in the susceptible and the resistant cultivars while eleven proteins showed downregulated in the two cultivars (Fig. 2b).

Functional analysis of DEPs responsive to *A. alternata* AP infection in apple leaves

According to GO annotation information of identified protein, the DEPs were classified into three GO categories including 17 functional categories as shown in Fig. 3. The main biological process category included "metabolic process" and "cellular process" in both cultivars. In the cellular component category, "cell," "macromolecular complex," "organelle," and "membrane" were mainly represented in "Starking Delicious," except "membrane," those were also represented mainly in "Jonathan." In the molecular functional category, "catalytic activity" and "binding" were mainly represented in "Starking Delicious," but in addition to the two functional categories, "structural molecule activity" also represented in "Jonathan" (Fig. 3).

To identify the biological pathways activated in the susceptible and the resistant apple cultivars, we further investigated these DEPs by using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The KEGG pathways of the great number of proteins mapped include metabolic pathways, biosynthesis of secondary metabolites, ribosome, photosynthesis, and photosynthesis-antenna proteins (supporting information Excel S2). The numbers of DEPs in the five most

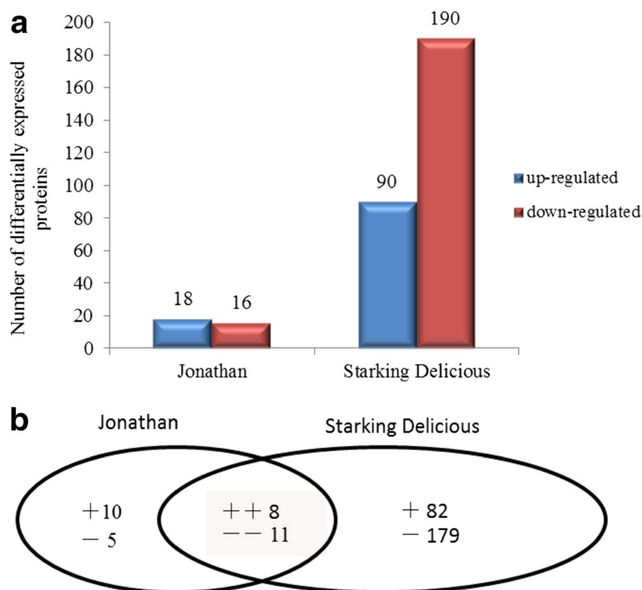

Fig. 2 Distribution of differentially expressed proteins between susceptible ("Starking Delicious") and resistant ("Jonathan") apple leaves after inoculated with *A. alternata* AP. **a** Number of upregulated and downregulated proteins. **b** Venn diagram analysis the differentially expressed proteins that were up- or downregulated in susceptible or resistant cultivars. The + and − indicate up- and downregulated proteins, respectively

abundantly represented KEGG pathways are presented in Table 1.

According to subcellular location annotation information of identified protein (supporting information Excel S3), 49 and 47% DEPs in the susceptible cultivar "Starking Delicious" and the resistant cultivar "Jonathan" were respectively located in chloroplast, 24 and 29% DEPs were assigned to the cytoplasm, 10 and 9% DEPs belonged to nuclear, and 5 and 6% DEPs were classified as mitochondria (Fig. 4).

DEPs detected specifically differential expression in the resistant cultivar

A total of 15 DEPs showed a specific differential expression in the resistant cultivar response to *A. alternata* AP, with 10 upregulated proteins, and 5 downregulated proteins (Table 2). Among of 10 upregulated proteins, four of them were related to defense, including two acidic endochitinase-like proteins, a small heat shock protein (17.9 kDa), and (+)-neomenthol dehydrogenase. Other six upregulated proteins were 30S, 40S ribosomal proteins, chlorophyll a/b binding protein, basic 7S globulin-like, lipoxygenase, and an uncharacterized protein. These proteins were upregulated 1.5-fold to 1.8-fold. Five proteins were downregulated including probable aquaporin PIP2-2, 2-methylene-furan-3-one reductase-like, 30S ribosomal protein S5, protein proton gradient regulation 5, and peptidyl-prolyl cis-trans isomerase FKBP16-4.

DEPs detected specifically differential expression in the susceptible cultivar

In total, 261 DEPs were found specifically differential expression in the susceptible cultivar after pathogen infection, with 82 upregulated and 179 downregulated proteins (supporting information Excel S4). Among the 82 upregulated proteins, 40 located in the chloroplast and seven proteins located in the mitochondrion, including chlorophyll a-b binding protein 8, 3-oxoacyl-[acyl-carrier-protein] synthase I, photosystem I reaction center subunit II, aconitate hydratase, probable enoyl-CoA hydratase, and 2-oxoglutarate dehydrogenase. The oxidation-reduction-related proteins, such as thioredoxin H-type-like, superoxide dismutase [Cu-Zn], glutathione S-transferase F9-like were upregulated. In addition, some proteins involved in secondary and amino acid metabolism, for instance, lipoxygenase, aspartate aminotransferase, and glutamate–glyoxylate aminotransferase 2 were also upregulated. However, 179 proteins were downregulated more than twice as many as upregulation proteins. Among them, a great number of proteins were energy metabolism-related, for example, pyruvate decarboxylase 2, malate dehydrogenase, NADP-dependent malic enzyme, and adenylosuccinate synthetase 2, and some proteins were identified as defense and calcium signal-related including leucine-rich repeat receptor-like protein, peroxiredoxin-2E-2, high

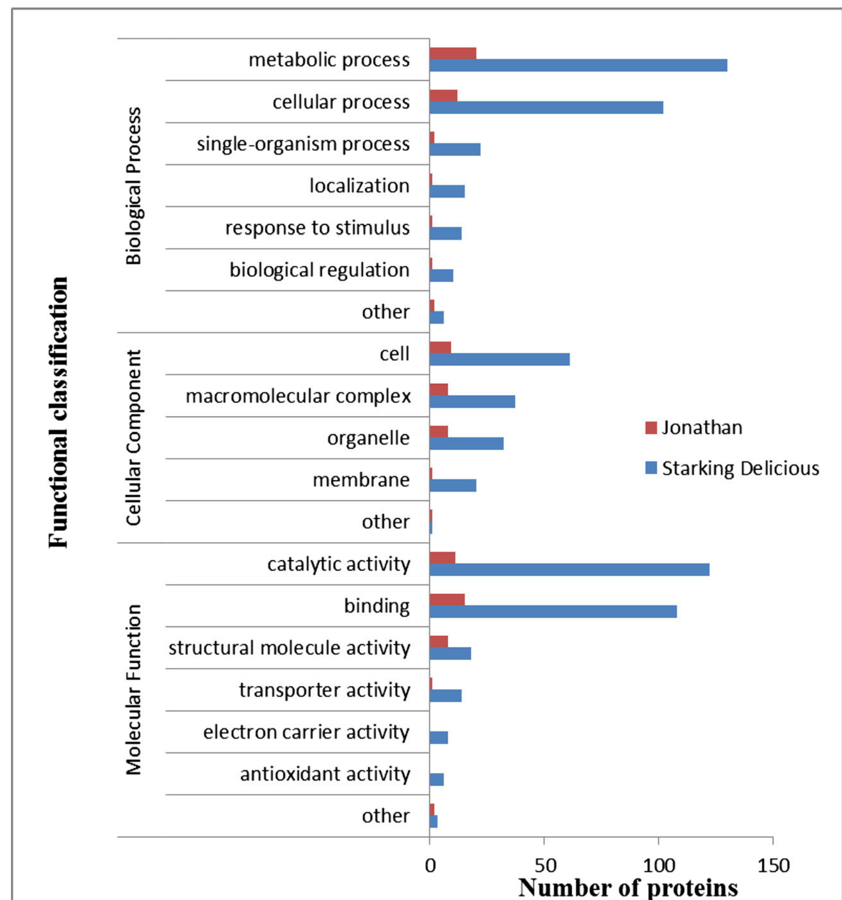

Fig. 3 Functional characterization of differentially expressed proteins in susceptible and resistant cultivars after inoculated with *A. alternata* AP

molecular weight heat shock protein, calcium-binding protein CML27, and 24 uncharacterized proteins.

DEPs detected both in the resistant and susceptible cultivars

There were 19 DEPs identified in both the resistant and susceptible cultivars (supporting information Excel S5). Eight of these proteins were upregulated in both the resistant and susceptible cultivar including a long chain acyl-CoA synthetase 4, a chlorophyll a-b binding protein CP29.3, a ATP-dependent zinc metalloprotease FTSH 2, a beta-amyrin 28-oxidase, two pathogenesis-related (PR) proteins (glucan endo 1,3-beta-glucosidase-like, and thaumatin-like protein 1a) and two ribosomal proteins (60S ribosomal protein L36-3-like and 50S ribosomal protein L29). Interestingly, a glucan endo 1,3-

Table 1 The top of six pathways of differentially expressed proteins in susceptible and resistant variety after inoculated with *A. alternata* AP

Pathway	Pathway ID	Starking Delicious			Jonathan		
		NO. of proteins with pathway annotation	Downregulated	Upregulated	NO. of proteins with pathway annotation	Downregulated	Upregulated
Photosynthesis	ko00195	11	10	1	1	1	
Biosynthesis of secondary metabolites	ko01110	35	21	14	4	2	2
Metabolic pathways	ko01100	76	50	26	7	3	4
Photosynthesis—antenna proteins	ko00196	4	1	3	2		2
Amino sugar and nucleotide sugar metabolism	ko00520	3	3		2	1	1
Ribosome	ko03010	18	9	9	7	2	5

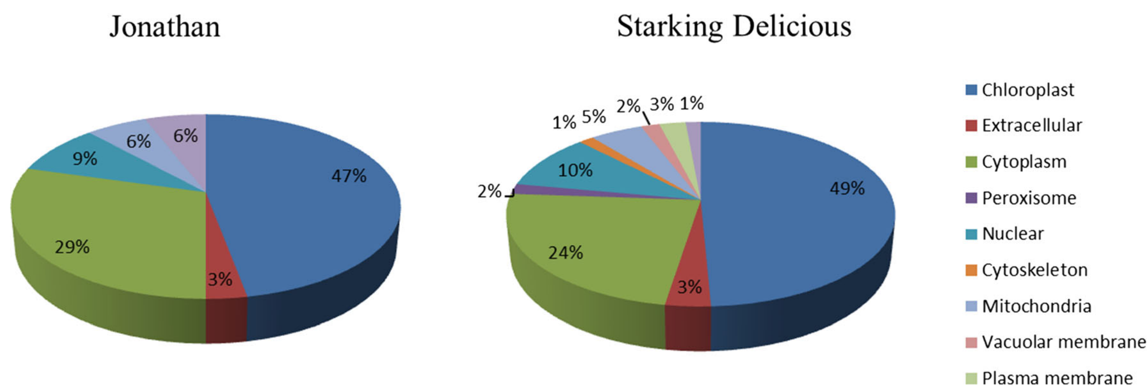

Fig. 4 Subcellular localization of differentially expressed proteins in susceptible and resistant cultivars after inoculated with *A. alternata* AP

beta-glucosidase-like (classified as PR2) and a thaumatin-like protein 1a (classified as PR5) were upregulated more significantly in the susceptible cultivar "Starking Delicious" (6.61-fold and 5.45-fold, respectively) than in the resistant cultivar "Jonathan" (2.88-fold and 2.22-fold, respectively). Eleven proteins were downregulated in both the resistant and susceptible cultivars, including four proteins (two ribosomal protein L1, one photosystem II 5 kDa protein, and 4-hydroxy-tetrahydrodipicolinate synthase), which were located in chloroplast, and two auxin-binding protein (ABP19a and ABP19b).

The PR proteins expressed in the early infection process

In order to study the paradox of PR protein expressions and resistance phenotype of the two apple cultivars, meanwhile to validate the proteomics data, we studied the expression of PR2 and PR5 by western blot analysis in the susceptible and resistant apple leaves in the early infection process. It showed that PR2 cannot be detected until 72 HPI in the resistant cultivar "Jonathan," while it expressed in the susceptible cultivar "Starking Delicious" since 18 HPI. On the contrary, PR5 was accumulated to a relatively high level at 6 HPI in "Jonathan," while cannot be detected in "Starking Delicious" until 18 HPI (Fig. 5). The expression of PR2 and PR5 reached a maximum at 72 HPI both in susceptible and resistant hosts, which was agreed with the proteomics analysis by iTRAQ method. Interestingly, we observed a higher molecular size band shift for PR5 at 72 HPI by immunoreactive analysis.

Discussion

The investigation of the pathogenic defense processes represents an important research goal for the development of resistant cultivars (Mazzeo et al. 2014). In the present study, we reported leaf proteome changes of two apple genotypes, "Jonathan" and "Starking Delicious," response to *A.alternata* AP. Using the iTRAQ-based quantitative proteomic approach, we aimed to better understand the molecular mechanism of the plant-pathogen interaction and the molecular mechanism of apple resistance against the pathogen.

Our experiment showed that the pathogen expanded rapidly on leaves of the susceptible cultivar after inoculation. By contrast, the development of the pathogen was blocked in the resistant plant (Fig. 1). Based on our previous transcriptomic analysis at 12, 18, 36, and 72 HPI that the differentially expressed genes at 72 HPI accounted for 80% of total DEGs, suggesting 72 HPI is an important time point for the apple-*A.alternata* AP interaction (data unpublished), we chose 72 HPI as sampling time for proteome analysis.

Several reports revealed that *A. alternata* AP causes Alternaria blotch in the apple host by producing a host-specific AM toxin (Sutton 1991; Johnson et al. 2000; Harimoto et al. 2008). To effectively combat invasion by pathogens, plants usually use various defense mechanisms to protect themselves. Chloroplasts are known to be responsible for the light-powered reactions of photosynthesis, upon which essentially all life depends (Waters and Langdale 2009; Jarvis and López-Juez 2013). Previous studies reported that chloroplasts can also serve as the primary site for the AM toxins (Otani 2000). In our study, a total of 159 DEPs were identified in the chloroplasts of the susceptible leaves. Among these proteins, about 67% DEPs were downregulated. By contrast, only 17 DEPs were located in the chloroplasts of the resistant leaves and about 47% DEPs were downregulated (supporting information Excel S3). Based on the acting site of the AM-toxins and known pathogenesis of the *A. alternata* pathogen (Harimoto et al. 2008), we speculated that *A. alternata* may act on cells of the susceptible apple leaves, causing chloroplasts' tissue damage. In addition, chloroplasts are the main sites of photosynthesis. In plants, interactions with pathogens have been shown to affect photosynthetic gene expression and activity (Herbers et al. 2000; Berger et al. 2004, 2007). In our study, about 2/3 DEPs in relation of photosynthesis were downregulated in susceptible leaves; by contrast, it was not observed in the resistant plant (Table 1). These

Table 2 DEPs detected only in the resistant cultivar upon pathogen infection

Accession no.	Protein description	Score	AASC (%)	MW(kDa)/pI	MP	Fold change	*P* value	Biological Process
gi| 657986370	Probable aquaporin PIP2–2	92	13.9	34.3/7.67	3	0.56	0.0030	Transport; localization; establishment of localization;
gi| 658025249	2-methylene-furan-3-one reductase-like	323	39.3	44.1/6.23	11	0.57	0.0012	Metabolic process; oxidation-reduction process
gi| 657983295	30S ribosomal protein S5	251	23.3	40.4/9.06	7	0.58	0.0048	Cellular protein metabolic process; biosynthetic
gi| 658010826	Protein proton gradient regulation 5	23	13.1	16.7/10.84	3	0.57	0.0272	–
gi| 658047826	Peptidyl-prolyl cis-trans isomerase	138	13.4	30.9/9.49	2	0.65	0.0020	Protein folding; metabolic process; cellular metabolic process
gi| 658039957	Basic 7S globulin-like	245	21.6	11.9/5.69	7	1.51	0.0005	–
gi| 657964825	Uncharacterized protein	43	3.1	108.8/4.97	2	1.53	0.0081	–
gi| 657968434	(+)-neomenthol dehydrogenas	121	24.7	38.5/5.20	5	1.62	0.0262	Metabolic process; biological_process
gi| 658064407	Chlorophyll a-b binding protein CP24 10A	292	28.3	36.7/6.85	5	1.62	0.0045	–
gi| 657972021	17.9 kDa class II heat shock protein-like	147	16	22.3/5.94	3	1.66	0.00007	–
gi| 485451127	Lipoxygenase	733	22.8	121.1/5.87	15	1.70	0.0001	Metabolic process; oxidation-reduction process; biological_process; single-organism metabolic process
gi| 657954307	30S ribosomal protein S20	34	11.1	26.3/10.51	2	1.72	0.0136	Cellular protein metabolic process; biosynthetic process; primary metabolic process; cellular metabolic process; organic substance
gi| 658016842	Acidic endochitinase-like	211	15.2	38.6/5.46	3	1.76	0.0018	Carbohydrate metabolic process; biological_process; organic substance metabolic process
gi| 658016846	Acidic endochitinase SE2-like	331	18.8	31.2/4.66	3	1.79	0.0023	Carbohydrate metabolic process; biological_process; organic substance metabolic process
gi| 657992836	40S ribosomal protein S15–4	190	11.2	22.6/10.44	1	1.80	0.0017	Cellular protein metabolic process; biosynthetic process; primary metabolic process; organic substance

AASC amino acid sequence coverage, *MP* matched unique peptide

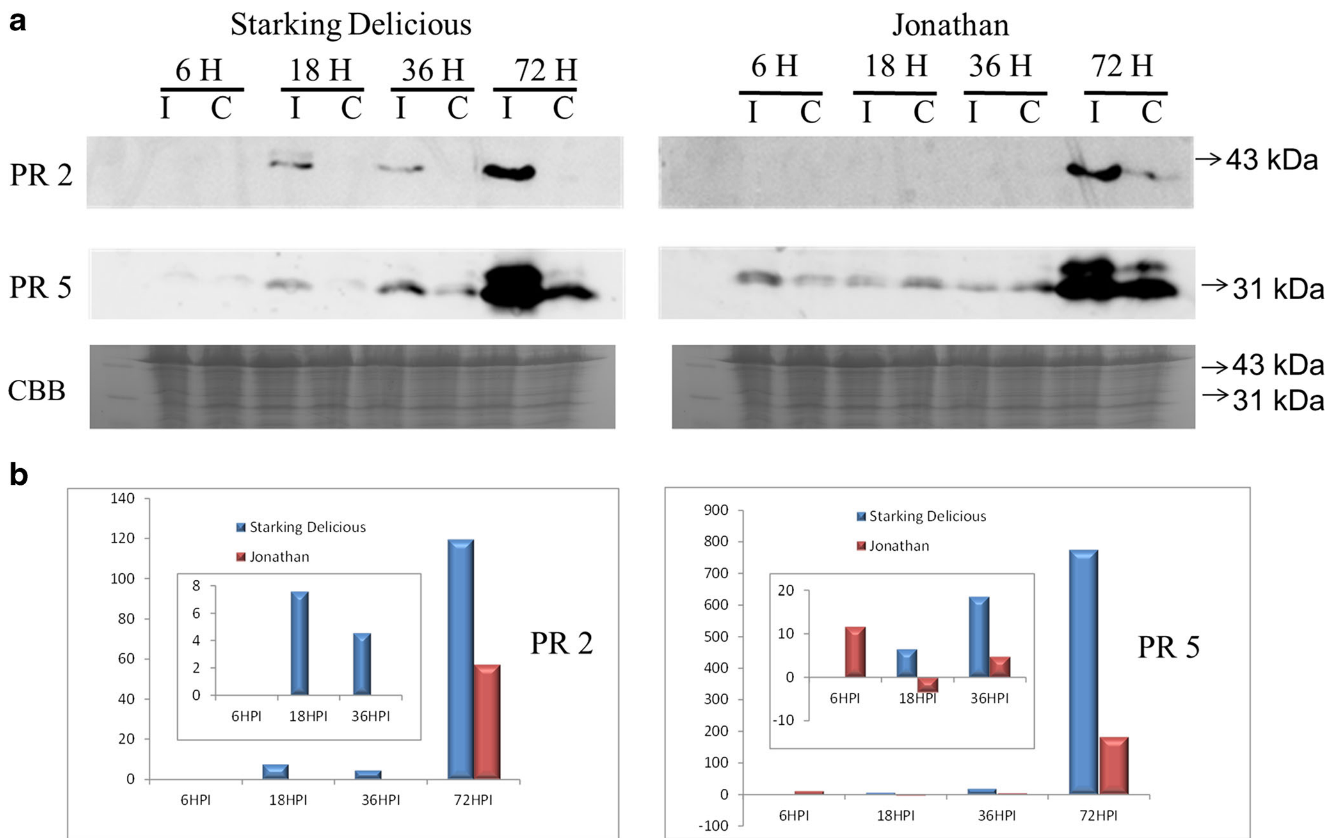

Fig. 5 Time course of expression accumulation of pathogenesis-related (*PR*) proteins after inoculated with *A. alternata* AP. Protein expressions were analyzed by western blot. Proteins were extracted from three biological replicates of control and *A. alternata* AP inoculated susceptible ("Starking Delicious") and resistant ("Jonathan") cultivars at 6, 18, 36, and 72 HPI. **a** Western blot (*WB*) detection of the expression of beta-1,3-glucanase(PR2), thaumatin-like protein (PR5) at different time points. *CBB*: Coomassie brilliant blue staining. **b** Quantification of signals on the membranes was performed by using Gel-pro analyzer

results indicated that expression of many chloroplast proteins is influenced by *A. alternata* AP infection, and chloroplasts and photosynthesis might be impaired by *A. alternata* AP in the susceptible cultivar.

It is well known that chitinase proteins are widely distributed across diverse biological systems (Grover 2012). Chitinases localize to vacuoles, and are involved in plant defense (Carter et al. 2004). Chitinolytic enzymes have the notable ability to degrade fungal hyphae (Joo 2005). In addition, oligomeric products of chitin could also act as signal molecules to stimulate further defense responses (Mazzeo et al. 2014). These enzymes have attracted much attention and become very important resources in the genetic engineering of crop plants for disease resistance (Abdallah et al. 2010). In the present study, two chitinases (acidic endochitinase-like and acidic endochitinase SE2-like) were detected, and both of them were upregulated in the resistance variety but not found in the susceptible cultivar, the expression pattern of which was similar to that in *Actinidia deliciosa* leaf apoplast infected with *Pseudomonas syringae* pv. *actinidiae* (Petriccione et al. 2014). Chitinases were accumulated in the resistant apple cultivar during pathogen attack, suggesting a significant contribution of them in triggering defense response to *A. alternata* AP pathogen infection. Additionally, the enzyme (+)-neomenthol dehydrogenase was identified only in the resistance cultivar and upregulated upon *A. alternata* AP infection. The (+)-neomenthol dehydrogenases belong to a large family of enzymes and characterized as classical short-chain dehydrogenases/reductases (Kallberg et al. 2002). The (+)-neomenthol dehydrogenases gene-silenced chili pepper plants became susceptible to *Colletotrichum coccodes*, suggesting the (+)-neomenthol dehydrogenase positively regulates plant defenses against pathogens (Choi et al. 2008). Some studies also underlined the contribution of (+)-neomenthol dehydrogenase in plant-fungi interactions, such as strawberry-*Fusarium oxysporum* f. sp. *Fragariae* (Fang et al. 2013). In our study, the (+)-neomenthol dehydrogenases only induced in the resistant cultivar implied they were important to apple fight against *A. alternata* AP.

Peroxiredoxins (Prxs) constitute a family of antioxidant enzymes. Prxs are ubiquitous thioredoxin- or glutaredoxin-dependent peroxidases to destroy peroxides (Jones et al. 2004; Rouhier et al. 2004; Borges et al. 2013). Plant Prxs can be organized into four distinct subgroups: 1Cys-Prx,

2Cys-Prx, type II Prx, and PrxQ (Rouhier and Jacquot 2002; Dietz 2003). Roles of Prxs in redox-signaling, desiccation tolerance, detoxification of reactive oxygen/nitrogen species, protection from pathogen attack, and other abiotic stresses have been confirmed in plant system (Finkemeier et al. 2005; Rouhier et al. 2004; Vidigal et al. 2013). The protein levels of Prx increased in leaves of common bean in response to infection with fungus *Pseudocercospora griseola* (Borges et al. 2013), and overexpression of this protein in *N. tabacum* increased tolerance to fungal diseases (Dietz et al. 2006). Peroxides, especially H_2O_2, are implicated in regulating disease resistance at various levels, and they are substrates for oxidative cross-linking of cell wall material and diffusible signals that induce the transcription of various resistance genes (Mellersh et al. 2002). In our study, five Prxs were detected only in the susceptible plant, one 2-Cys peroxiredoxin belonging to Prx IV and four proteins belonging to type II Prx, and all of them were downregulated after *A. alternata* AP infection. It is well known that the incompatible reaction is marked by an oxidative burst with the production of massive amounts of H_2O_2 and nitric oxide in infected tissues (Delledonne et al. 2001). In the experiment, the downregulation of Prx proteins in the susceptible cultivar suggested that the peroxide concentrations may be lower and the need for detoxification is presumably not as essential. Thus, it is likely that the decreased expression of Prx in "Starking Delicious" is part of reasons for its susceptibility to *A. alternata* AP.

Generally, host plants respond to pathogen attacks by producing a wide range of PR proteins (Van Loon et al. 2006; Li et al. 2011). Glucan endo-1,3-β-glucosidase, the members of the PR2 gene family, has been considered to act as antifungal proteins by hydrolyzing β-1,3-glucan, a major component of fungal cell walls (Sakamoto et al. 2011; Oide et al. 2013; Havanapan et al. 2016). Thaumatin-like protein, a class-5 PR protein (PR5), accumulates in the plants when they are infected by pathogens. Overexpression of PR5 in potato and tobacco leads to increased resistance to pathogenic fungi (Liu et al. 2012; Safavi et al. 2012; Acharya et al. 2013). In our study, a protein belonging to PR2 gene family and a thaumatin-like protein 1a (PR5) were all upregulated in both the susceptible and resistant leaves upon *A. alternata* AP infection, suggesting that the expression of PR2 and PR5 proteins play roles in both resistance and susceptible responses. It is interesting to note that PR2 and PR5 proteins are more abundant in the susceptible interaction than in the resistant reaction. This phenomenon was also found in the interaction of rice and *Xanthomonas oryzae* pv. *Oryzae* (Hou et al. 2012). There are two explanations proposed for this phenomenon. One is that PR proteins are associated with resistance even in the susceptible response. In the leaves of susceptible plants, there were greater quantities of pathogens, and correspondingly, more proteins were recruited to participate in the host-pathogen interaction process. In the resistance response, the number of the pathogens and the quantity of PR proteins were both lower, but the quantity of proteins per unit of fungus number was higher than that of the susceptible response. The other is that an unknown factor from the compatible pathogen detoxifies the PR proteins via protein modification or partial degradation (Hou et al. 2012). However, the interesting phenomena need further investigation and interpretation. In addition, we also found that PR2 is not expressed in mock-inoculated leaves, except for a low expression in the resistant leaves at 72 HPI (Fig. 5), suggesting that PR2 does not usually accumulate in healthy apple plants but is induced by pathogen infection. Moreover, in the experiment, PR5 responded to the pathogen infection earlier in the resistant leaves than in the susceptible ones. The similar results were also reported in mustard infected with white rust (Kaur et al. 2011). A higher molecular size band shift of PR5 at 72 HPI suggested post translation modification was occurred by phosphorylation, esterification, or glycosylation. Therefore, we supposed that PR5 might exert important function at early stage in resistant cultivar and play important roles in apple against *A. alternata* AP infestation.

Phytohormones play a key role in plant response to biotic and abiotic stresses (Jiang et al. 2015). Jasmonates (JA) is one of the most important signal molecules with key functions on the regulation of immune responses against necrotrophic pathogens (Thaler et al. 2012; Santino et al. 2013; Wasternack and Hause 2013). In the study, we obtained two upregulated lipoxygenases both in the resistant and susceptible apple leaves (gi:485451110 in the susceptible and gi:485451127 in the resistant cultivar). It is known that lipoxygenase is an important enzyme in the JA biosynthesis pathway (Schaller 2001; Vidhyasekaran 2007). Moreover, lipoxygenases biosynthesis products have several diversified functions, for instance, involving in the synthesis of a number of different compounds with antimicrobial activity and production of the hypersensitive response (HR) required for plant defense (Bannenberg et al. 2009; Zoeller et al. 2012; Munhoz et al. 2015). The upregulation of lipoxygenase in both the resistant and susceptible apple leaves suggested that JA signaling systems modulate apple immune responses against *A.alternata* AP.

Conclusion

The difference in protein expression patterns elicited by *A. alternata* AP in the susceptible and the resistant apple cultivars has resulted in the likely identification of key molecular components of this host-pathogen interaction. In this study, we identified proteins in the susceptible and resistant apple leaves induced by the *A. alternata* AP. The differentially expressed proteins were involved in metabolic pathways, biological processes, signal transduction, stress, and defense. Several key proteins including endochitinase, (+)-neomenthol

dehydrogenase and PR5 may play important roles in apple against *A. alternata* AP infestation. These findings provided valuable knowledge of the compatible and incompatible interactions between apple and *A. alternata* AP, as well as provided a valuable foundation for the further mechanism research of apple against the pathogen.

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Data Archiving Statement The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al. 2016) partner repository with the dataset identifier PXD005406 and 10.6019/PXD005406."

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