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ORIGINAL ARTICLE

A comparative cell wall proteomic analysis of cucumber leaves under *Sphaerotheca fuliginea* stress

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Abstract Powdery mildew, caused by *Sphaerotheca fuliginea* (*S. fuliginea*), is the most devastating disease that hampers cucumber plants cultivation and productivity. Cell wall proteins (CWPs) play a crucial role in response to biotic stress as a frontline defense of plants. In this work, we present a comparative cell wall proteomic approach to explore differentially expressed proteins in both highly resistant and highly susceptible cucumber leaves after 24 h of exposure to *S. fuliginea*. After extraction conducted by a destructive procedure with salts, glucose-6-phosphate dehydrogenase (G6PDH) activity and SDS-PAGE assessments were performed to determine the cytosolic contamination. Label-free quantitative proteomics approach was used to gain a comprehensive understanding of differentially regulated CWPs between the two lines after *S. fuliginea* inoculation. Among more than 200 proteins identified, 71 were significantly altered between the two lines. Most of these identified proteins were predicted to be CWPs except some classical cytosolic proteins. These differentially expressed CWPs belonged to different functional categories including defense, metabolism, redox regulation and cell wall arrangement. The expression levels of seven proteins selected were determined using RT-PCR. We found that resistant cucumber line is believed to start a series of disease-resistant mechanisms against pathogen. This study provides useful information on cell wall proteomic changes between a resistant and a susceptible genotype under infected conditions.

Keywords Cell wall proteins (CWPs) · *Cucumis sativus* · Powdery mildew · Glucose-6-phosphate dehydrogenase (G6PDH) · Label-free quantitative proteomics approach · RT-PCR

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Introduction

Cucumber (*Cucumis sativus* L.) plants are often subjected to many biotic stress during their lifetime and correspondingly evolved defense mechanisms to protect themselves. Powdery mildew caused by *Sphaerotheca fuliginea* (*S. fuliginea*) is one of the most devastating cucumber diseases, which limit cucumber production worldwide (Fukino et al. 2013). Now control measures based on the use of resistant cultivars remain the most effective and environment friendly method. Previous investigation of the mechanism of cucumber resistance to *S. fuliginea* inoculation was focused on inheritance of resistance (Kooistra

1971; Zhou et al. 2013) and physiological mechanisms (Fan et al. 2009; Wei et al. 2004). Since proteomics technology has drastically expanded in the last few years, much attention has been given to plant proteins that response to biotic or abiotic stress (Quirino et al. 2010).

Cell wall is the first barrier in plant–pathogen interaction, which is a dynamic organization that comprises polysaccharides, proteins and lignins (Jamet et al. 2006). Recent evidences indicated that cell walls could: (1) regulate cell expansion and division (Pereyra et al. 2010), (2) be relevant in signaling pathway (Esquerré-Tugayé et al. 2000; Sattelmacher 2011), (3) be involved in biotic and abiotic stress responses (Dahal et al. 2010; Ellis et al. 2002; Kong et al. 2010; Vogel et al. 2004). It is CWPs that reflect this broad function diversity. CWPs play essential roles during plant development and in adaptation to environmental cues; extensive studies leading to their identification and characterization have been undertaken. In the last 10 years, plant cell wall proteomics has greatly contributed to a wider knowledge of CWPs. An effort was made to estimate CWPs in many plants, including Arabidopsis, rice, tomato, maize (Albenne et al. 2013), after some model plants genome was completely sequenced. In this paper, we consider cell wall proteins (CWPs) to be all proteins secreted into the extracellular space as well as proteins bound loosely to cell wall and non-protoplast proteins.

Although there is an increasing amount of researches on proteins using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) followed by mass spectrometry (MS) or tandem mass spectrometry (MS/MS) in the past years, proteins with extreme ranges in molecular weights and pI values, and low abundance proteins still remain limit detection (Fang et al. 2006). In particular, CWPs have not been as well studied as intracellular proteins. Recently, quantitative proteomics approach, including label-based quantitative proteomics approach [such as stable isotope labeling by amino acids in cell culture (SILAC) and isobaric tags for relative and absolute quantitation (iTRAQ)] and label-free quantitative proteomics approach, has been applied to identify and quantify differentially expressed proteins from complex biological samples (Wang et al. 2008). However, label-based quantitative proteomics approach has disadvantages such as relative high cost, procedural complexity, and the potential danger for artifacts (Shen et al. 2009). Therefore, there has been increased interest in label-free quantitative proteomics approach to address some of the issues of label-based approach and achieve faster, cleaner and simpler quantification results (Zhu et al. 2010).

The proteins secreted into the plant cell wall by the plants and pathogen during the plant–pathogen interactions can play important roles in establishing the outcome of plant–pathogen interactions; plant cell wall proteomics has become a new tool to identify candidate proteins involved in biotic stress responses. Over the past 10 years, several groups have used proteomics to identify proteins in response to biotic or abiotic stress in cucumber (Du et al. 2010; Fan et al. 2009, 2014; Segarra et al. 2007). However, CWPs associated with both susceptible and resistance cucumber lines in the interaction with *S. fuliginea* were not characterized. We sought to present a comparative analysis of differentially regulated CWPs between highly resistant and highly susceptible cucumber leaves after *S. fuliginea* inoculation for 24 h by label-free quantitative proteomics technology. Proteomics approach was, therefore, undertaken to analyze the broad spectrum of the CWP profiles that could provide further insight to understand the defense responses of the cucumber plants. The cell wall proteome analysis will also present additional information to our earlier results in leaf intracellular proteins analysis.

Materials and methods

Plant materials and treatment

Two cucumber sister lines, one highly resistant (B21-a-2-1-2) and the other highly susceptible (B21-a-2-2-2) to *S. fuliginea*, were obtained from Liaoning Academic of Agricultural Science. These lines were selected from a segregated population derived from four generation selfing of a cultivar which was from South Korea. The two lines are similar in the plant type, commodity characteristics, resistance to Fusarium wilt and downy mildew, and so on, but different in the traits of resistance to powdery mildew.

Cucumber seeds of both lines were washed in sterile water for 15 min at 58 °C and then soaked in water for 5 h at room temperature. The seeds of white tip were sown in pots with a nutrient compound of peat soil–vermiculite (1:2, v/v) under greenhouse conditions. At 3rd–4th leaf stage, the second leaf blades were inoculated with *S. fuliginea* and incubated for 24 h. Then, the second leaves collected from 200 plants of each line at this time were placed in liquid nitrogen and stored at −80 °C until extraction of proteins was performed.

Extraction of CWPs and total proteins

CWPs extraction was performed according to Feiz et al. (2006) with slight modifications. The plant tissue was transferred to a pre-chilled mortar, and grounded with a pestle in liquid nitrogen to a fine powder. The powder was suspended with acetate extraction buffer containing 5 mM sodium acetate, pH 4.6, 0.4 M sucrose, 1 mM phenylmethyl sulfonyl fluoride (PMSF), and 1 % (v/v) isopropyl alcohol. The mixture was stirred for 15 min, and then incubated at

4 °C for 30 min after adding polyvinylpolypyrrolidone (PVPP) while stirring. After centrifugation for 15 min at 13,000×*g* and 4 °C, the pellet was further extracted by two successive centrifugations in acetate extraction buffer containing 5 mM sodium acetate, pH 4.6, 1 mM PMSF, 1 % (v/v) isopropyl alcohol, respectively, and 0.6 and 1 M sucrose. The residue was washed by centrifugation in 5 mM sodium acetate, pH 4.6, while filtered on a layer of nylon net. After grinding, the resulting cell walls were lyophilized to dry powder. Proteins were extracted by $CaCl_2$ solution containing 5 mM sodium acetate, pH 4.6, 0.2 M $CaCl_2$, 1 mM PMSF, and 1 % (v/v) isopropyl alcohol. Cell walls were resuspended by vortexing for 10 min at room temperature, and then centrifuged for 15 min at 4000×*g* and 4 °C. The supernatant was then collected, quantified with a BCA (bicinchoninic acid) Protein Assay Kit (Bio-Rad, USA) and stored at −80 °C for subsequent experiments. Total proteins preparation was conducted as described in Fan et al. (2008).

Determination of G6PDH activity and SDS-PAGE

G6PDH, a cytosolic marker enzyme, is used to test cytosolic contamination (Kong et al. 2010). Assay kit (Solarbio company, China) was applied to determine the G6PDH activity. The ratio of CWPs G6PDH activity to total proteins G6PDH activity stands for cytosolic contamination. The SDS-PAGE was performed on SE260 (GE-Healthcare) with 20 µg protein solution each sample.

Protein digestion

Protein digestion (250 µg for each sample) was performed according to the FASP procedure described by Wisniewski et al. (2009). Briefly, the detergent, DTT and other low-molecular-weight components were removed using 200 µL UA buffer (8 M Urea, 150 mM Tris–HCl pH 8.0) by repeated ultrafiltration (Microcon units, 30 kD) facilitated by centrifugation. Then, 100 µL 50 mM iodoacetamide in UA buffer was added to block reduced cysteine residues and the samples were incubated for 30 min in darkness. The filter was washed with 100 µL UA buffer three times and then 100 mL 25 mM NH_4HCO_3 twice. Finally, the protein suspension was digested with 5 µg trypsin (Promega, USA) in 40 µL 25 mM NH_4HCO_3 overnight at 37 °C, and the resulting peptides were collected as a filtrate. The peptide content was estimated by UV light spectral density at 280 nm using an extinctions coefficient of 1.1 of 0.1 % (g/L) solution that was calculated on the basis of the frequency of tryptophan and tyrosine in vertebrate proteins.

Liquid chromatography (LC)–electrospray ionization (ESI) Tandem MS (MS/MS) analysis by Q exactive

The peptide of each sample was desalted on C18 Cartridges (Empore™ SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 mL, Sigma), then concentrated by vacuum centrifugation and reconstituted in 40 µL of 0.1 % (v/v) trifluoroacetic acid. MS experiments were performed on a Q Exactive mass spectrometer that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific). 5 µg peptide was loaded onto a the C18-reversed phase column (Thermo Scientific Easy Column, 10 cm long, 75 µm inner diameter, 3 µm resin) in buffer A (2 % acetonitrile and 0.1 % Formic acid) and separated with a linear gradient of buffer B (80 % acetonitrile and 0.1 % formic acid) at a flow rate of 250 nL/min controlled by IntelliFlow technology over 120 min. MS data were acquired using a data-dependent top 10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 *m*/*z*) for HCD fragmentation. Determination of the target value is based on predictive automatic gain control (pAGC). Dynamic exclusion duration was 25 s. Survey scans were acquired at a resolution of 70,000 at *m*/*z* 200 and resolution for HCD spectra was set to 17,500 at *m*/*z* 200. Normalized collision energy was 30 eV and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1 %. The instrument was run with the peptide recognition mode enabled. MS experiments were performed triply for each sample.

Sequence database searching and data analysis

The MS data were analyzed using MaxQuant software version 1.3.0.5. MS data were searched against the UniProtKB Cucurbitaceae database (4898 total entries, downloaded 12/02/13). An initial search was set at a precursor mass window of 6 ppm. The search followed an enzymatic cleavage rule of Trypsin/P and allowed maximal two missed cleavage sites and a mass tolerance of 20 ppm for fragment ions. Carbamidomethylation of cysteines was defined as fixed modification, while protein N-terminal acetylation and methionine oxidation were defined as variable modifications for database searching. The cutoff of global false discovery rate (FDR) for peptide and protein identification was set to 0.01. Label-free quantification was carried out in MaxQuant as previously described (Schwanhäusser et al. 2011). Intensity-based absolute quantification (iBAQ) in MaxQuant was performed on the identified peptides to quantify protein abundance.

RNA preparation and real-time quantitative PCR analysis

Total RNA was isolated from different cucumber phenotype (B21-a-2-1-2 and B21-a-2-2-2) at different treating time after incubating them with *S. fuliginea* (0, 12, 24, 48 h). Total RNA was isolated using RNAprep Pure Plant Kit and cDNA was generated using a FastQuant cDNA first strand synthesis kit (TianGen Biotech, China) according to the manufacturer's instructions. Quantitative real-time PCRs were conducted using SYBR Green I 96-I system (Roche fluorescence quantitative PCR instrument, Basle). Reaction mixtures consisted of 4.5 μL of 2× SuperReal PreMix Plus (TianGen Biotech, China), mix of primers (0.2 μL of forward and reverse primer for proper gene) and 4.3 μL of RNase-Free ddH_2O and 1 μL of cDNA. The PCR program was set up in seven stages: (1) 95 °C for 15 min (Preincuubantion), (2) 95 °C for 10 s, (3) 58 °C for 20 s, (4) 72 °C for 30 s, (3) repeated 40 times (Amplification), (5) 95 °C for 0.5 s, (6) 60 °C for 1 min and (Melt) (7) 50 °C for 30 s (Cooling). The primers were synthesized by BGI Tech (China) and the PCRs quality was estimated based on melting curves. The gene-specific primers employed are shown in Table 1.

Statistical and bioinformatics analysis

The primers were designed using Primer Premier 5.0 software. Data obtained from Maxquant were analyzed by Perseus software version 1.3.0.4. Differential expression analysis was evaluated with UniProt and Gene Ontology (GO) database. The presence of secretion signals in the identified proteins was predicted by SignalP (V.3.0, http://www.cbs.dtu.dk/services/SignalP) and SecretomP (V.2.0, http://www.cbs.dtu.dk/services/SecretomP).

Table 1 List of gene-specific primers employed for qRT-PCR

Genes	Primer
XTH13	F: 5′-AAGGCGACCGAGAACAGAGG-3′
	R: 5′-TGGCACATCGTCCACAAAGA-3′
UGPase	F: 5′-CGATGTTTCGTTTGGGTCTG-3′
	R: 5′-GGCCATTGATTTCCTTGTTTG-3′
MCO	F: 5′-CATACCCTCCAAACGACCTA-3′
	R: 5′-GAAGCGAGTGGAAACGACA-3′
stellacyanin	F: 5′-GACGCCTTGGGTTGGACTGT-3′
	R: 5′-TTGTTCGCCACGGAGATGGGATTG G-3′
alpha-expansin 8	F: 5′-GCTTCTGGCACAATGGGT-3′
	R: 5′-AGTGGCGGTGACGATGAT-3′
olp	F: 5′-GCGTTTTGCTGTAGAGGGC-3′
	R: 5′-GCATAAGCGAAGGAGTATCGT-3′
CuPi1	F: 5′-GCACCAAAACAACGAAAAGG-3′
	R: 5′- GGCTATAAGGACCGCTACCAT-3′
18SrRNA	F: 5′-ATGATAACTCGACGGATCGC-3′
	R: 5′-CTTGGATGTGGTAGCCGT-3′

Results and discussion

Disease symptoms development

Cucumber plants were grown in the greenhouse and then treated with *S. fuliginea* at the seedling stage. Leaves from cucumber sister lines were observed after *S. fuliginea* inoculation for symptoms assessment. Plants of the susceptible line shown in Fig. 1a (6 days post-*S. fuliginea* inoculation) and Fig. 1b (11 days post-*S. fuliginea* inoculation) exhibited the typical whitish appearance of powdery mildew disease on leaves, as well as wilting symptoms, and necrotic tissues. However, plants of the resistant line shown in Fig. 1c (6 days post-*S. fuliginea* inoculation) and Fig. 1d (11 days post-*S. fuliginea* inoculation) exhibited no significant change and wilt slightly.

CWPs purification and cytosolic contamination detection

In cell wall proteomics surveys, the selection of a suitable purification protocol is a crucial step (Witzel et al. 2011). CWPs are embedded in an insoluble polysaccharide matrix and interact with other components, bringing extraction challenging. Until now, the efficient CWPs extraction methods include non-destructive techniques (vacuum infiltration) and destructive techniques (purified with salts or chelating agents) (Feiz et al. 2006; Jamet et al. 2006; Negri et al. 2008; Zhou and Liu 2011). We compared different methods to extract CWPs of cucumber leaves and showed that destructive technique purified with salts to be applicable to our purpose, since this method can gain more proteins and lower cytosolic contamination (Meng et al. 2015). We removed most of the intracellular proteins from the cell walls using sucrose gradients and extensive washing with low ionic strength acidic buffer.

To detect cytosolic contamination in cucumber leaf cell wall proteomic studies, the rate of G6PDH activity from the CWP fraction and the total protein fraction was analyzed. As shown in Table 2, the cytosolic contamination rate from each sample CWP fraction was lower than 3 %. Ribulose bisphosphate carboxylase/oxygenase (RuBisCO) protein, a cytosolic marker protein, can be used as a positive control to test cytosolic contamination. As indicated in Fig. 2, the clear protein bands showed no obviously high abundant cytoplasm RuBisCO protein bands and mainly in 15–40 kDa. Based on these results, it is validated that the

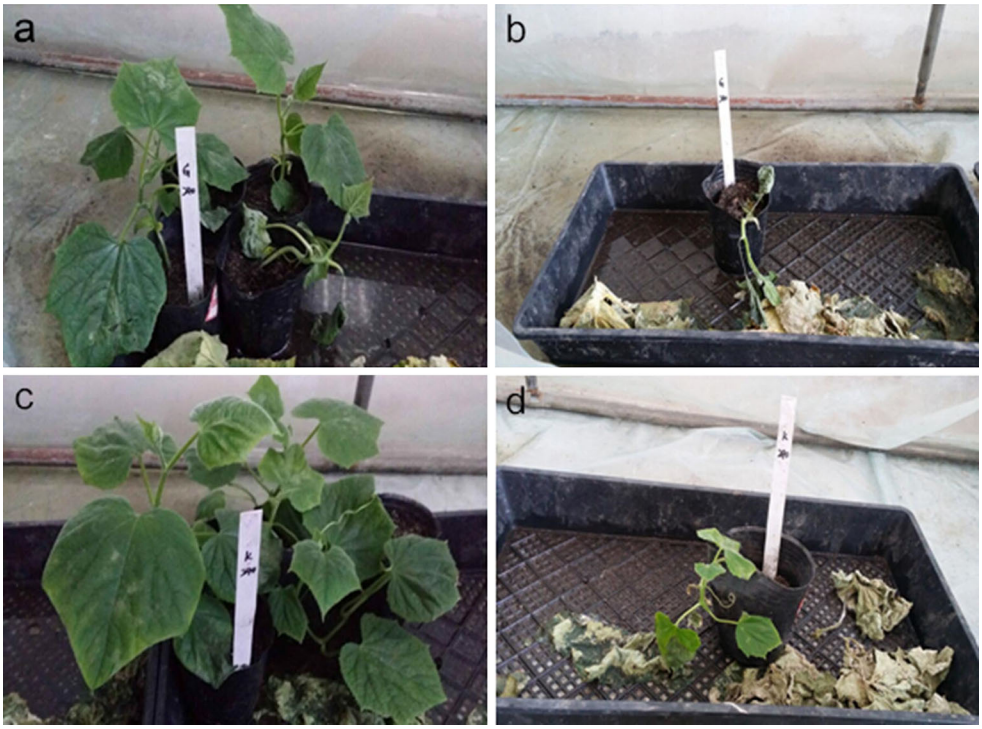

Fig. 1 Appearance of susceptible and resistant plants after pathogen inoculation. **a**, **b** Susceptible plants B21-a-2-2-2 treated with *S. fuliginea* for 6 and 11 days, respectively. **c**, **d** Resistant plants B21-a-2-1-2 treated with *S. fuliginea* for 6 and 11 days, respectively

Table 2 Protein content and contamination rate of cucumber leaves

Species	Protein content (μg/μL)	Contamination rate (%)[a]
Susceptible plants	3.6	0.58
Resistant plants	2.9	0.79

[a] Contamination rate shows the ratio of cell wall proteins glucose-6-phosphate dehydrogenase (G6PDH) activity and total proteins G6PDH activity

purification technique effectively enriched for cucumber leaf CWPs.

Differential CWPs analysis by label-free quantitative shotgun proteomics

We applied an LC–MS/MS-based label-free quantitative proteomics approach to compare the differential cell wall proteome of two cucumber accessions displaying different phenotypes to *S. fuliginea* infestation. The criterion defined for the reliable quantified proteins was that proteins had to be identified in at least three replicates with quantitative ratio. Protein variation was found comparing protein profiles between susceptible and resistant plants in response to *S. fuliginea* inoculation. From the cell wall proteome samples of B21-a-2-1-2 and B21-a-2-2-2, LC–MS/MS analysis revealed a total of 750 unique peptides. Of these,

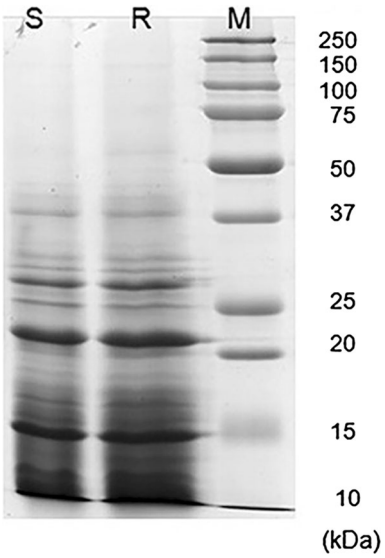

Fig. 2 SDS-PAGE of cucumber CWPs. *S* susceptible plants B21-a-2-2-2, *R* resistant plants B21-a-2-1-2, *M* marker

200 proteins were identified in B21-a-2-1-2 and B21-a-2-2-2 based on intensity-based absolute quantification method (iBAQ) (Supplemental data Table S1). Collectively, 71 proteins displayed more than twofold quantitative alterations ($p < 0.05$) in the cell wall proteome comparison between B21-a-2-1-2 and B21-a-2-2-2 (Table 3). There

Table 3 List of leaves cell walls proteins which are differential regulated in cucumber genotypes B21-a-2-1-2 (resistant) and B21-a-2-2-2 (susceptible) after *S. fuliginea* challenge for 24 h

Protein name	Accession[a]	Species[b]	Peptides[c]	Unique peptides[d]	Sequence coverage (%)[e]	Molecularmass (kDa)[f]	Significance analysis		SiP-SeP[i]
							iBAQ S/R[g]	*t* test *p* value[h]	
CWPs involved in metabolic activities									
Xyloglucan endotransglucosylase/hydrolase	N0DXB3	*Cucumis sativus*	2	2	7.5	34.007	0.5287299	0.004502	Y
Xyloglucan endotransglucosylase/hydrolase	N0DX86	*Cucumis sativus*	3	3	12.4	33.225	0.351001	0.0005112	0.8188
Acid alpha galactosidase 2	Q2HYY3	*Cucumis sativus*	3	3	6.9	46.635	2.7540887	0.0050218	Y
Polygalacturonase	Q9SLP3	*Cucumis sativus*	2	2	5.5	47.28	2.6760561	0.0011123	Y
Glyceraldehyde-3-phosphate dehydrogenase	E1B2J6	*Cucumis sativus*	7	3	24.4	36.361	1.2303494	0.0115513	0.6022
Chloroplast NADP-dependent glyceraldehyde 3-phosphate dehydrogenase B subunit	Q9FV16	*Cucurbita pepo*	4	3	27.5	18.176	0.2439328	0.0001834	0.7226
UDP-glucose pyrophosphorylase	Q19TV8	*Cucumis melo*	12	9	31.1	52.061	1.2974336	0.0122027	0.7257
Acid alpha galactosidase 1	Q2MK92	*Cucumis sativus*	9	9	27.6	45.697	3.5586479	5.23E-05	Y
Aconitate hydratase	P49608	*Cucurbita maxima*	3	3	3.5	98.004	1.0766842	0.0449661	0.7179
Cell wall apoplastic invertase	E5GCB6	*Cucumis melo* subsp. melo	3	3	8.4	45.225	5.0630671	0.0069585	0.9427
Photosystem I iron-sulfur center	G3ETV0	*Cucumis melo* subsp. melo	5	5	72.8	8.9804	1.2413054	0.0140321	0.588
Redox state regulated proteins									
Ferredoxin-NADP reductase	E5RDD5	*Cucumis melo* subsp. melo	14	14	41.1	40.481	1.7078858	0.002275	0.5019
Chloroplastic alkenal/one oxidoreductase	E2S0A5	*Cucumis sativus*	11	2	35.5	40.525	1.4879382	0.003132	0.9368
Cytosolic alkenal/one oxidoreductase	E2S0A6	*Cucumis sativus*	10	9	40.9	34.422	0.4447521	9.853E-05	0.8903
Ascorbate oxidase	E7BBM8	*Cucumis sativus*	12	12	28.1	65.848	1.4322034	0.0417263	0.7365
Multicopper oxidase	E5GCD6	*Cucumis melo* subsp. melo	3	3	7.4	60.505	1.9436187	1.103E-05	Y
Proteins related to cell wall structure									
Stellacyanin	Q96403	*Cucumis sativus*	4	4	41.2	19.313	1.735634	0.0269295	
Expansin S1	Q39625	*Cucumis sativus*	1	1	3.2	27.215	0.7606143	0.0199444	Y
Alpha-expansin 8	Q8W5A5	*Cucumis sativus*	1	1	7.9	13.575	1.2384719	0.0104306	0.8771
Defense related proteins									
Chloroplast HSP70	A2TJV6	*Cucumis sativus*	17	17	28.9	75.395	0.7509006	0.0008159	0.8958
HSP23.5	H6TB40	*Citrullus lanatus*	2	2	11.9	23.232	1.6231575	0.0071698	0.6129

Table 3 continued

Protein name	Accession[a]	Species[b]	Peptides[c]	Unique peptides[d]	Sequence coverage (%)[e]	Molecularmass (kDa)[f]	Significance analysis		SiP-SeP[i]
							iBAQ S/R[g]	*t* test *p* value[h]	
Osmotin-like protein	Q9SPH1	*Benincasa hispida*	2	2	10.9	26.689	0.8672533	0.0114347	Y
Superoxide dismutase [Cu–Zn]	A4UDF2	*Cucurbita ficifolia*	1	1	12.4	10.598	0.9417714	0.0047309	0.7401
Pathogen-induced protein CuPi1	O24512	*Cucumis sativus*	1	1	14.9	9.7604	5.7470835	0.0139533	Y
Truncated processed peroxidase	Q39650	*Cucumis sativus*	4	3	33.1	15.614	1.956156	0.0244419	0.6818
Netting associated peroxidase	Q6UBM4	*Cucumis melo*	4	2	16.5	37.215	1.9801724	0.022251	Y
Peroxidase 2	P19135	*Cucumis sativus*	2	2	9.2	31.856	3.9711427	0.0018252	0.7743
Peroxidase	Q39652	*Cucumis sativus*	8	8	28.9	35.746	2.6204811	0.0385137	Y
Others									
60S ribosomal protein L36/44	F8RHB5	*Cucurbita pepo*	2	2	23.4	7.1964	2.3237048	0.0109317	0.8637
Ribosomal protein	B0F825	*Cucumis sativus*	7	7	48.4	17.278	0.6005789	0.0083363	0.5863
30S ribosomal protein S12	G3ETW4	*Cucumis melo* subsp. melo	2	2	13	13.764	0.6746604	0.0225877	0.8356
Poly(A)-binding protein	Q9M549	*Cucumis sativus*	13	13	27.3	70.505	0.6705877	0.0028089	0.9519
Zinc finger-homeodomain protein 1	B0LK19	*Cucumis sativus*	1	1	3.3	32.812	1.6111206	0.039103	0.9039
Ubiquitin conjugating enzyme	E5GCG7	*Cucumis melo* subsp. melo	1	1	7.4	16.502	1.3751772	0.0111432	0.9056
Peptidyl-prolyl cis-trans isomerase	Q52UN0	*Cucumis sativus*	4	4	29.7	18.123	1.8715909	0.001898	0.8743
Putative uncharacterized protein 8C01	Q58A12	*Cucumis melo*	2	2	16	14.775	2.9807875	0.0426651	0.5264
BZIP2	F1DQG1	*Cucumis melo*	3	3	23.4	17.537	0.2963738	6.8E-05	0.8835
Patellin 1	Q2Q0V7	*Cucurbita pepo*	1	1	1.8	67.014	2.1837825	0.0094501	0.884
6,7-Dimethyl-8-ribityllumazine synthase	B7SIS4	*Cucumis sativus*	1	1	3.1	24.227	1.6372118	0.0150346	0.9292
High mobility group protein	Q4ZH67	*Cucumis sativus*	2	2	9.6	16.101	0.8117534	0.0234885	0.7279
CR9 protein	Q39636	*Cucumis sativus*	1	1	6.6	14.703	0.7201578	0.0159413	0.7087
Proteins were identified that did not have a signal peptide sequence nor were they predicted to be nonclassical secretory proteins could still be true CWPs									
Malate dehydrogenase	A1BQK6	*Cucumis sativus*	5	5	48.1	14.087	1.5626514	0.0334549	0.4358
Triosephosphate isomerase	A1BQP5	*Cucumis sativus*	2	2	29.2	7.9329	1.438014	0.014071	0.103
Ascorbate peroxidase	C3VQ49	*Cucumis sativus*	6	6	29.3	27.409	0.6396008	0.0077635	0.4044
Peroxidase	Q39653	*Cucumis sativus*	11	9	41.8	32.276	1.9140388	0.0011483	0.3089
Chloroplast chaperonin	E5GC96	*Cucumis melo* subsp. melo	3	3	23.7	16.501	0.1530418	0.0036725	0.3343

Table 3 continued

Protein name	Accession[a]	Species[b]	Peptides[c]	Unique peptides[d]	Sequence coverage (%)[e]	Molecularmass (kDa)[f]	Significance analysis		SiP-SeP[i]
							iBAQ S/R[g]	*t* test *p* value[h]	
Nucleoside diphosphate kinase	E5GBW3	*Cucumis melo* subsp. melo	3	2	17.6	25.109	2.1330225	0.0180958	0.1263
Nucleoside diphosphate kinase	L7S218	*Cucurbita maxima*	4	3	29.7	16.339	1.4445701	0.0015954	0.0416
Heat shock protein 70	Q9M4E7	*Cucumis sativus*	14	3	27.5	71.488	1.6225253	0.0074039	0.4364
ATP synthase epsilon chain	G3ETZ1	*Cucumis melo* subsp. melo	3	3	23.5	14.775	1.2171572	0.0217914	0.0586
Histone H4	Q14TA7	*Cucumis sativus*	6	6	41.7	11.184	1.3462255	0.0459516	0.3261
Histone H2A	Q58A21	*Cucumis melo*	1	1	8.9	8.3567	1.4123841	0.0128105	0.0835

The listed proteins are reproduced in three biological replications and are statistically significant (Student's *t* test, $p \leq 0.05$)

[a] Protein database accession number (UniProt)

[b] Plant species from which the protein was annotated

[c] Number of matched peptides of LC–MS/MS data with the corresponding protein in MSDB, SwissProt and NCBInr databases

[d] Number of matched unique peptides of LC–MS/MS data with the corresponding protein in MSDB, SwissProt and NCBInr databases

[e] Percentage of peptide sequences coverage for the identified protein by LC–MS/MS

[f] Molecular weight of protein

[g] Molecular weight of proteinThe ratio in the abundance of iBAQ % iBAQ between susceptible phenotype and resistant phenotype

[h] *t* test

[i] The results of SignalP (SiP) and SecretomP (SeP) analysis. Y presence of signal peptide evaluated from SignalP. The numbers are Secretom NN-score calculated from SecretomP, in case no signal peptides were identified, and the NN-score exceeding the normal threshold of 0.5 was considered as secretory protein

were 22 up-regulated and 49 down-regulated proteins in B21-a-2-1-2 as compared with B21-a-2-2-2. The 71 differential proteins were categorized into extracellular region (7 %), macromolecular complex (18 %), cell (34 %), organelle (26 %), membrane-enclosed lumen (4 %) and membrane (11 %) through (GO) database. And 52 of the 71 differential proteins were predicted to be CWPs. 11 of the identified CWPs had traditional signal peptides, 30 of the identified CWPs were predicted to be nonclassical secretory proteins, and 11 different cellular-localized proteins that did not have a signal peptide sequence nor were they predicted to be nonclassical secretory proteins were also identified in previous cell wall proteomics studies. These 52 differential CWPs were also classified according to their biological functions and signaling pathways, respectively. Most of the identified proteins corresponded to defense, metabolic pathways, redox activities and cell structural alters.

Defense-related proteins

Several defense-related proteins are differentially expressed in B21-a-2-2-2 (highly susceptible) and B21-a-2-1-2 (highly resistant) after *S. fuliginea* inoculation, such as osmotin-like protein (olp), heat-shock protein70 (HSP70), heat-shock protein 23.5 (HSP23.5), superoxide dismutase (SOD) [Cu–Zn], pathogen-induced protein CuPi1 and peroxidases (PODs). In our investigation, most defense-related proteins displayed the higher abundance in resistant cucumber line than those in susceptible cucumber line except HSP23.5 and pathogen-induced protein CuPi1.

Olp, a PR-5 family member, has been found in various plant species in response to pathogen infection (Abdin et al. 2011; Choi et al. 2013; Rather et al. 2015). HSPs are highly conserved in all organisms and constitute a large family of proteins, such as HSP10, HSP40, HSP60, HSP70, and HSP90. HSPs play a key role in protection of cells against stress (Calderwood et al. 2006; Garrido et al. 2001; Sun et al. 2002). Interestingly, in this study, HSP70 occurred in higher abundance in resistant cucumber line, while HSP23.5 presented higher abundance in susceptible cucumber line, proving evidence of the main role of these proteins in defense mechanisms. Lower regulation of HSP70 in the susceptible plants would probably cause impairments in protein structure and function (Timperio et al. 2008). SOD has been ascribed the biological function as a key enzyme in the cells protection against oxidant agents and, thus, plays a pivotal role in the protection of aerobic organisms against oxygen-mediated damages (Sfaxi et al. 2012). Pathogen-induced protein CuPi1 is a phloem lectin protein and expressed in systemic acquired resistance. However, there is far less information available on their disease resistance mechanism. PODs include large numbers of isoforms, which possess a broad range of biological functions including hydrogen peroxide detoxification, lignin biosynthesis, hormonal signaling and stress response (Gao et al. 2009). Class III plant peroxidases catalyze oxidoreduction between H_2O_2 and various reductants and belong to the PR-protein 9 subfamily (González et al. 2010). In many plant species, up-regulated peroxidases are in line with resistance (Passardi et al. 2005).

CWPs involved in metabolic activities

CWPs involved in metabolic activities such as xyloglucan endotransglucosylase/hydrolase (XTH), acid alpha galactosidase, polygalacturonase (PG), cell wall apoplastic invertase, UDP-glucose pyrophosphorylase (UGPase), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and aconitate hydratase (ACO) were differentially accumulated in the two sister lines. XTHs and Chloroplast NADP-dependent GAPDH B subunit in B21-a-2-1-2 occurred in higher abundance than that of B21-a-2-2-2. However, PG, cell wall apoplastic invertase, UGPase and ACO were higher in B21-a-2-2-2.

XTH, acid alpha galactosidase, PG and cell wall apoplastic invertase are carbohydrate-modifying proteins and belong to the glycoside hydrolases (GHs) family which is involved in biosynthesis, modification and degradation of cell wall components (Sharma et al. 2013). XTHs, cell wall-modifying enzymes, mainly involved in the modification of the cell wall during synthesis and also stress responsive fortification processes (Miedes et al. 2011; Saladié et al. 2006). The hot pepper XTH (*CaXTHs*) gene constitutive expression has been reported to enhance drought and salt tolerance in transgenic plants (Cho et al. 2006). Down-regulating alpha galactosidase which is involved in many aspects of plant metabolism can improve the freezing tolerance of plants (Pennycooke et al. 2003). It has been reported that over-expression of PG in rice decreased pectin content and cell adhesion and enhanced its susceptible to abiotic stress (Liu et al. 2014). An induced activity of cell wall invertase (Cw-Inv) can mediate the reduced export of sucrose or enhanced import of hexoses (Proels and Hückelhoven 2014). An increase in the apoplastic hexose-to-sucrose ratio has been suggested to strengthen plant defense (Sonnewald et al. 2012). Therefore, up-regulation of Cw-Inv can enhance plant defense. However, Roy et al. (2013) reported that tobacco (*Nicotiana tabacum*) Nin88, a presumed fully active Cw-Inv, fails to degrade sucrose. UGPase is an important regulatory enzyme for the development of plants and was suggested to play a role in sucrose/polysaccharides metabolism and cell wall biosynthesis (Wang et al. 2011) and also has a novel function as a cell death regulator (Chivasa et al. 2013). Over-expression of UGPase in *Larix gmelinii*

was reported that enhanced vegetative growth in transgenic *Arabidopsis thaliana* and increased the contents of soluble sugars and cellulose, and thickened parenchyma cell walls (Li et al. 2014). GAPDH is an abundant metabolic enzyme that plays important roles in a number of fundamental cell pathways (Sirover 2011) and is specially targeted to the cell wall during crop–pathogen interaction (Gokulakannan and Niehaus 2010). These changes might strengthen the cell wall through influencing polysaccharide degradation and synthesis and declining the cell wall polysaccharide metabolism during pathogen invasion.

The abundance of Photosystem I iron-sulfur center was lower in the leaves of resistant plants than that in susceptible plant. Photosystem I iron-sulfur center is believed to be important for photosynthesis. Therefore, the suppression of photosystem I iron-sulfur center is led by damage or inhibit PSI due to stresses. It is assumed that cucumber leaves inoculated with *S. fuliginea* could inhibit the leaf carbohydrate oxidation pathways leading to the decrease in the overall energy production.

Redox state regulation

Most CWPs related to redox state regulation showed lower abundance in resistant line B21-a-2-1-2 than those in susceptible line B21-a-2-2-2 in our investigation.

The intensity of ascorbate oxidase (AO) and multicopper oxidase (MCO) was now observed to be significantly lower in B21-a-2-1-2 compared to B21-a-2-2-2 after challenge with *S. fuliginea*. Ascorbate plays a key role in defense against potentially damaging external oxidants (Pignocchi et al. 2003). AO, an apoplastic enzyme, catalyzes the oxidation of ascorbate to yield water and, thus, controls the redox state of the apoplastic ascorbate pool (Pignocchi et al. 2006). Multicopper oxidases (MCOs) are a family of enzymes which belong to blue copper proteins. MCOs have the ability to catalyze the four-electron reduction of molecular oxygen to water coupled with one-electron oxidation of the substrate (Hoegger et al. 2006). However, relatively little information is available on the biological functions for AO and MCOs.

Stellacyanin is a blue copper-containing glycoprotein containing one copper atom in a blue or type I site isolated originally from *Rhus verniciferu* (Peisach et al. 1982). Reinhammar (1972) reported the oxidation–reduction potentials of the electron acceptors in stellacyanin, suggesting that it may have a function of a diffusible electron transfer protein. The presence of a cell wall structural protein domain suggests that stellacyanin may have possibility of associations with other structural proteins (Nersissian et al. 1996). It is reported that the Arabidopsis blue copper binding gene (*AtBCB*) has the function in aluminum (Al) stress resistance (Ezaki et al. 2005).

Ferredoxin-NADP reductase (FNR) is a flavoenzyme which catalyzes the last step of photosynthetic electron transport and plays a role in tolerance to oxidative stress (Rodriguez et al. 2007). FNR is used for carbon fixation, nitrogen metabolism, and lipid and chlorophyll biosynthesis, as well as for stromal redox regulation (Chaki et al. 2011).

NADPH-dependent alkenal/one oxidoreductase (AOR) is a major plant enzyme catalyzing reduction of an α,β-unsaturated bond in reactive carbonyls which is, therefore, essential for maintaining cellular homeostasis (Yamauchi et al. 2012). Two distinct AORs, chloroplastic AOR and cytosolic AOR, were found differentially expressed in the two species after *S. fuliginea* infection. Up- and down-regulated AOR suggests that different classes of NADPH-dependent reductases cooperatively contribute to the detoxification of reactive carbonyls (Yamauchi et al. 2011).

Protein related to cell wall structure

Expansins are highly conserved and non-hydrolytic cell wall-loosening proteins and comprise a large superfamily with at least two major branches (α-expansins and β-expansins) (Lee et al. 2001). Expansins were first cloned from cucumber and involved in a variety of plant developmental processes during which cell wall modification occurs (Cosgrove 1998; McQueen-Mason and Cosgrove 1995). Recent discoveries have uncovered that expansins not only regulate wall expansion during cell growth but also have key roles in plant–environment or plant–pathogen dialogue (Lee and Kim 2013). Plants may alter cell wall structure as indicators in resistance to pathogen attack. These changes might increase wall disassembly facilitating the fungus colonization of the progress of the infection.

Others

Plants will develop complex defense systems to cope with and combat against biotic stress. Apart from the four main groups of CWPs above, other proteins involved in disease resistance are also identified. Pathogen stress proteins may alter regulatory networks and induce genes with protective functions, such as ribosomal protein, poly (A)-binding protein and zinc finger-homeodomain protein 1. High mobility group protein is the most abundance non-histone that binds to and induces conformational changes in DNA and has also been isolated from a variety of plants (Grasser et al. 1994). Peptidyl-prolyl cis–trans isomerases (PPIases) possess chaperone activity and catalyze protein folding, which was also found changes during abiotic or biotic stress (Kromina et al. 2008). Ubiquitin-conjugating enzyme belongs to the ubiquitin pathway enzymes that are involved in protein degradation. Patellin1 (PATL1), a plant Sec14-related protein, is thought to involve in membrane trafficking

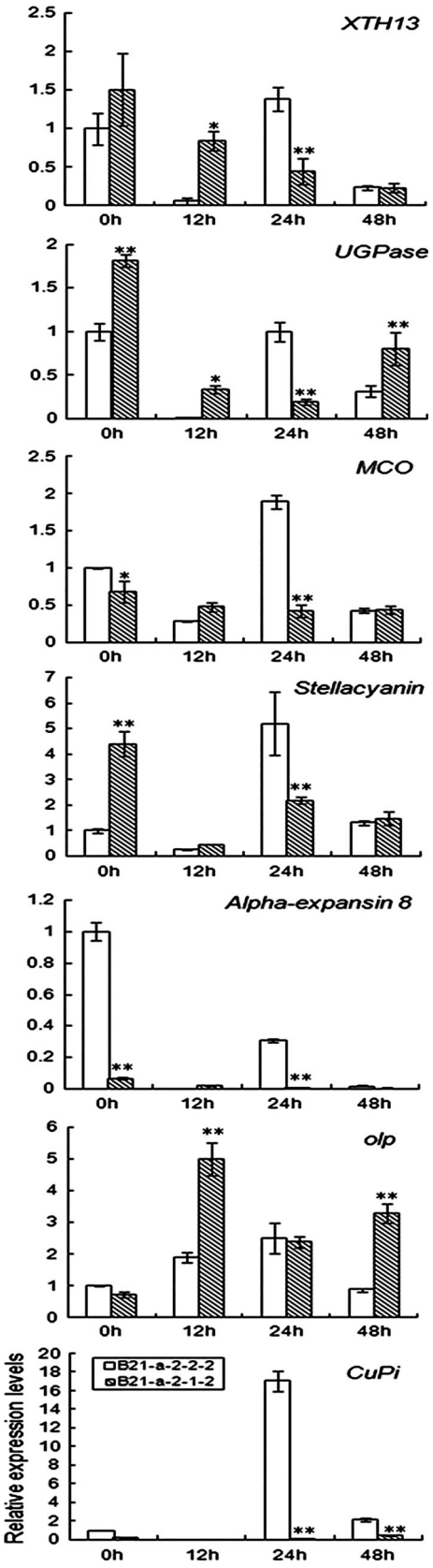

Fig. 3 The expression levels of *XTH13*, *UGPase*, *MCO*, *Stellacyanin*, *Alpha-expansin 8*, *olp* and *CuPi* in B21-a-2-1-2 and B21-a-2-2-2 lines after *S. fuliginea* infection for 0, 12, 24 and 48 h were determined by qRT-PCR. The expression level in B21-a-2-2-2 was normalized as 1. Data represent mean ± SE of three biological replicates. *Asterisk* or *asterisks* indicate significant difference at $P < 0.05$ or $P < 0.01$ compared with B21-a-2-2-2 by Student's *t* test, respectively

associated with cell plate maturation during the late stages of cytokinesis (Peterman et al. 2006). Down-regulation of CR9 could be closely related to wounding (Teramoto et al. 1995). Putative uncharacterized protein 8C01 and 6, 7-dimethyl-8-ribityllumazine synthase possess transferase activity. Nevertheless, the significance of these CWPs in biotic tolerance is incomplete without the knowledge of their function. Hence, further research is necessary to reveal their disease resistance.

Most CWPs belong to multiprotein families, and proteins in the same family can have different cellular localizations (Zhu et al. 2006). In this study, 11 different cellular-localized proteins that did not have a signal peptide sequence nor were they predicted to be nonclassical secretory proteins, such as malate dehydrogenase, triosephosphate isomerase, ascorbate peroxidase, peroxidase, heat-shock protein 70, histone H4, histone H2A, ATP synthase epsilon chain, chaperonin and 2 nucleoside diphosphate kinase, were also identified in previous cell wall proteomics studies. That is because current protein identification process still has limitations for distinguishing family members. And the conventional theory for protein trafficking still cannot explain why the proteins without any known secretory signal peptides could also secreted into extracellular space. For example, malate dehydrogenase, a cellular-localized protein, is proposed to be involved in response to H_2O_2 in cell walls (Zhou et al. 2011). Therefore, further experiments would be required to confirm their presence in cell wall. Based on the above finding, it can be argued that the differentially expressed proteins play important roles in determining the resistance or susceptibility of the plants.

Differential expression of regulated cell wall protein genes

To investigate the mRNA expression of the CWPs related to resistance response of cucumber under pathogen stress, seven proteins (olp, pathogen-induced protein CuPi1, XTH, UDPase, MCO, stellacyanin and alpha-expansin 8) were selected for mRNA expression analysis. As shown in Fig. 3, two genes, *XTH 13* and *olp*, exhibited no good correlation between mRNA and protein levels at 24 h indicated in Table 3. Transcription levels of *UGPase*, *MCO*, *stellacyanin*, *alpha-expansin 8* and *CuPi* showed

good correction at the same time points with protein levels (Fig. 3; Table 3). The variance might due to the posttranscriptional regulation of *XTH 13* and *olp*.

The synthesis of defense-related proteins can generally induce the general defense responses when plants are challenged with biotic stresses. We observed olp and CuPi which, related to defense, were of higher abundance in susceptible genotype at 24 h after pathogen invasion. CuPi is present in both phenotypes at low level; however, it is increased dramatically upon invasion with pathogen at 24 h in susceptible phenotype. With no pathogen treatment, the relative expression level of *olp* in susceptible cucumber line was sparsely higher than that in resistant cucumber line. Following treatment for 12 and 48 h, resistant cucumber line exhibited higher relative expression levels. Thus, resistant cucumber line is believed to start the disease-resistant mechanism earlier.

XTH13 and *UGPase* were correspondingly decreased in resistant cucumber plants, while *XTH13* was increased in susceptible cucumber line 24 h after infection. Reduction of these metabolism proteins in our study is in line with previous reports (Dahal et al. 2010). The decreased abundance of *XTH13* during pathogen invasion can be assumed as consequences of declined cell wall polysaccharide metabolism and mechanical stability in resistant cucumber line. On the other hand, suppression of *UGPase* in the resistant plants could be correlated to trigger a cell death, thus inhibiting pathogen growth.

The resistant line showed a lower expression of *MCO* and *stellacyanin* at mRNA level that was in accordance with its expression at protein level. Interestingly, both *MCO* and *stellacyanin* exhibited higher in resistant plants than those in susceptible plants 12 h after invasion. Thus, it is assumed that the expression of MCO could be helpful to maintaining metal homeostasis of resistant phenotype at early time against pathogen.

Expansins are implicated in the control of plant growth via loosening of the extracelluar matrix (Goh et al. 2012). The susceptible phenotype displayed the higher abundance of *alpha-expansin 8* than the resistant one without any treatment. That is why the resistant cucumber line grew better than the susceptible one. The reduction of *alpha-expansin 8* in both phenotypes due to pathogen invasion in our study may reflect tightening cell wall during disease expression.

Conclusion

CWPs play a crucial role in response to biotic stress as the first barrier of plants. In this study, a destructive procedure with salts, G6PDH activity and SDS-PAGE assessments and label-free quantitative proteomics approach were used to gain a comprehensive understanding of differentially regulated CWPs between highly resistant and highly susceptible cucumber leaves after *S fuliginea* infection for 24 h. The contamination rate presented in Table 2 and Fig. 2 based on the G6PDH activity and SDS-PAGE is low. However, MS technologies are much more sensitive than biochemical methods and easily detect minor proteins. It is possible that infected cells are damaged and that intracellular proteins may be released in cell walls and stick in an unspecific way to cell wall polysaccharides. As a consequence, the intracellular is unavoidable.

The differential expression of defense-related, cell wall structural related, redox state regulation and metabolic proteins in both lines triggered by the pathogen supports their crucial roles in the defense mechanism. According to these changes, conclusion can be made as follows: under *S. fuliginea* infestation, (a) resistant cucumber line is believed to start the disease-resistant mechanism and metal homeostasis maintaining mechanism earlier than susceptible one, (b) metabolism-related CWPs help decline cell wall polysaccharide and energy metabolism and mechanical stability, thus improving plant tolerance, and (c) tightening cell wall is carried out by expansins. Our results will contribute to the future plant defense research as well as to protection of crops against pathogen invasion.

Author contribution statement Haiyan Fan is responsible for experimental design, experimental data analysis and writing. The work of protein extraction, G6PDH activity analysis, SDS-PAGE assessment, RT-PCR and writing was done by Xiangnan Meng. The breeding and cultivation of two cucumber sister lines were done by Tiefeng Song and Juyong Zhao. Yang Yu is responsible for mass spectrometry analysis. The manuscript was revised and refined by Na Cui and Kexin Meng.

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Compliance with ethical standards

Conflict of interest The authors have declared that no conflict of interest exists.

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