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ORIGINAL ARTICLE

Comparative proteomic analysis of host responses to *Plasmodiophora brassicae* infection in susceptible and resistant *Brassica oleracea*

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Abstract

Clubroot disease, caused by *Plasmodiophora brassicae*, is one of the most devastating diseases affecting members of the Brassicaceae family. It is difficult to control by chemical or cultural means, and the molecular mechanisms underlying interactions with *Brassica oleracea* (cabbage) remain poorly understood. Herein, we used a proteomic approach to investigate *B. oleracea*–*P. brassicae* interactions during the early phases of infection in above-ground tissues. Proteins were isolated from the aerial parts of clubroot-susceptible (CT-18) and -resistant (YCR) cabbage cultivars at 5 days after inoculation with *P. brassicae* or buffer (mock) and resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis. A total of 24 differentially modulated proteins were identified in at least two biological replicates, and exhibited altered expression between mock and *P. brassicae* treatments and/or in the different cabbage cultivars. Most of the identified proteins are involved in oxidative stress, abscisic acid (ABA) metabolism, glucose-mediated signalling and responses to stimuli. Resistant YCR plants harboured an increased abundance of ABA-responsive protein, fructose-bisphosphate aldolase and glucose sensor interaction protein compared with CT-18 plants in both mock and *P. brassicae*-treated samples, suggesting that they may mediate basal defences against *P. brassicae* infection in YCR. Specifically, we observed that susceptible (CT-18) plants expressed higher levels of cobalamin-independent methionine synthase than YCR, which may enhance susceptibility of the host. Further investigation of the identified proteins will likely facilitate the identification of key molecular determinants, potentially improving clubroot disease resistance in future cabbage crop species.

Keywords *Brassica oleracea* · Clubroot disease · MALDI-TOF/TOF MS · *Plasmodiophora brassicae* · Proteomics

Introduction

Clubroot disease, caused by the soil-borne obligate biotrophic fungus *Plasmodiophora brassicae*, is one of most serious fungal diseases affecting crucifers (Diederichsen et al. 2009). The disease is characterised by gall development on the roots of infected plants that disrupts water and nutrient uptake, and causes a 10–15% reduction in crop yield worldwide (Dixon 2009). Once a field is infected, it is hard to control this pathogen by cultural practices due to the longevity of dormant spores in the soil (Wallenhammar and Arwidsson 2001), and chemical treatment is undesirable due to high cost, inefficient use and negative environmental impact. Developing genetically resistant cultivars is, therefore, an attractive alternative strategy for managing this disease.

Plants have evolved two levels of defence responses against invading pathogens: basal resistance and pathogen

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race-specific resistance (Kou and Wang 2010). Race-specific resistance is triggered by the recognition of pathogen effector molecules through host resistance (*R*) genes (Bent and Mackey 2007). With the exception of *Brassica juncea* and *B. carinata*, resistant accessions can be found in all major brassica crops, and most clubroot resistance (CR) is regarded as *P. brassicae* race-specific resistance. In *B. oleracea*, one broad-spectrum locus was identified, indicating race-independent CR in some resistant accessions of this species (Diederichsen et al. 2009). Manipulating the *CR* gene through plant breeding became an important research objective for brassica vegetables after discovering that some European turnips are resistant to the pathogen (Buczacki et al. 1975; Matsumoto et al. 1998). Resistance against race 2 *P. brassicae* was introduced from *B. napus* into *B. oleracea* (Chiang et al. 1980), and most turnips possess dominant monogenic genes together with other genes that have a minor effect on CR (Yoshikawa 1993). The discovered CR lines of European fodder turnips were used as sources of resistance genes for breeding a number of Chinese cabbage cultivars in Japan. However, decay of resistance in Chinese cabbage crops was shown only several years after the generation of these CR cultivars (Kuginuki et al. 1999). European field isolates of *P. brassicae* that can overcome commercial sources of resistance present wide genetic and pathogenic variability (Hwang et al. 2012), and European turnips still display resistance to pathogens isolated from areas where resistance in Chinese cabbage crops was severely diminished, indicating that one or more CR genes remained present but some resistance genes were lost during breeding of the CR cultivars (Kuginuki et al. 1999). Additionally, a number of the pathogenic races isolated caused multiple infections in the same field (Buczacki et al. 1975; Jones et al. 1982), indicating an intricate genetic basis for CR in cruciferous crops and, moreover, expression of resistance is often quantitative. A better understanding of the mechanisms of resistance to clubroot is, therefore, required for effective CR cultivar breeding programs.

P. brassicae is an obligate biotrophic pathogen that can only grow on its host and cannot be cultured in vitro, making it difficult to study plant–pathogen interactions. Proteomic approaches can determine the differential accumulation and modification of proteins. These methods have been extensively applied to study plant–pathogen interactions, the results of which have extended our knowledge on biotic stresses in host plants (Agrawal et al. 2005; Butt and Lo 2007; Mathesius 2009; Mehta et al. 2008; Quirino et al. 2010; Vincent et al. 2012). However, to date, only a few proteins associated with brassica crop plant–fungal pathogen interactions have been identified using proteomic analysis (Quirino et al. 2010; Yao et al. 2011).

In the present study, to gain an in-depth understanding of *B. oleracea–P. brassicae* interactions, proteomic experiments were carried out using the two-dimensional electrophoresis (2-DE) technique in combination with MALDI-TOF/TOF mass spectrometry (MS). In particular, we compared proteomic changes of above-ground tissues in *P. brassicae*-resistant (YCR) and -susceptible (CT-18) cabbage cultivars relatively soon after fungal infection of roots. Comparative analysis identified 24 proteins differentially accumulated in mock- and *P. brassicae*-inoculated leaves, including 14 specific to CT-18 and 6 unique to YCR plants. The identified proteins are involved in various metabolic pathways and processes, including responses to stimuli, biosynthesis of plant hormones, amino acid and carbohydrate metabolism, photosynthesis, and oxidative stress, while some were unclassified using genome annotation (Gene Ontology; GO). The functional implications of the identified proteins are discussed, with particular focus on their putative roles in susceptible or resistant responses to fungal pathogen infection.

Materials and methods

Plant material and *P. brassicae* inoculation

Four genotypes either susceptible or resistant to *P. brassicae* were employed in this study, namely YCR (resistant) and CT-18 (susceptible) cabbage (*B. oleracea*), and Ohjora (resistant) and CR Hagye (susceptible) oilseed rape (*B. napus*). Seeds of each genotype were provided by the Asia seed company (Korea), and all seedlings were grown in a growth room maintained at 23 °C with a 16-h light:8-h dark photoperiod. The *P. brassicae* inoculum was prepared from mature galls of Chinese cabbage roots showing typical clubroot symptoms collected from Gangneung in Korea (Kim et al. 2016). Gall root material was stored at − 70 °C until needed (Chemical Biotechnology Research Center, Korea Research Institute of Chemical Technology). Galls were homogenised in deionised water, and resting spores were obtained by filtration of the homogenates through a double-gauze filter. Each inoculum was standardised to 10^7 spores/ml using a haemocytometer (Neubauer Improved, Germany). Roots of 10-day-old seedlings were inoculated with a 4×10^8/ml suspension per pot by soil drenching, and controls were mock-inoculated with tap water. Inoculated plants were incubated in a growth chamber at 20 °C with a 12-h light:12-h dark photoperiod for 3 days and then transferred to the growth room as described above. Infected plant material excluding roots (~ 100 leaves) was harvested at 5, 15 and 28 days after inoculation (DAI), weighed, rinsed briefly in tap water to remove soil, dried with paper towels, powdered in liquid nitrogen and stored at − 70 °C.

Disease rating

A mean disease index (DI) was calculated for root symptoms of each treatment group up to 35 days based on a five-point scale (0–4) as described previously (Kobelt et al. 2000), where 0 indicates fully resistant and 4 indicates fully susceptible plants (Fig. 1). In more detail, grade 0 = no symptoms, 1 = a few small, separate globular clubs on lateral roots, 2 = medium, separate globular clubs on lateral roots, 3 = intermediate symptoms and 4 = severe clubs on main roots. The mean DI was classified into three categories: resistant, mean DI ≤ 1.0; intermediate resistant, 1.0 < mean DI ≥ 2.0; susceptible, mean DI > 2.

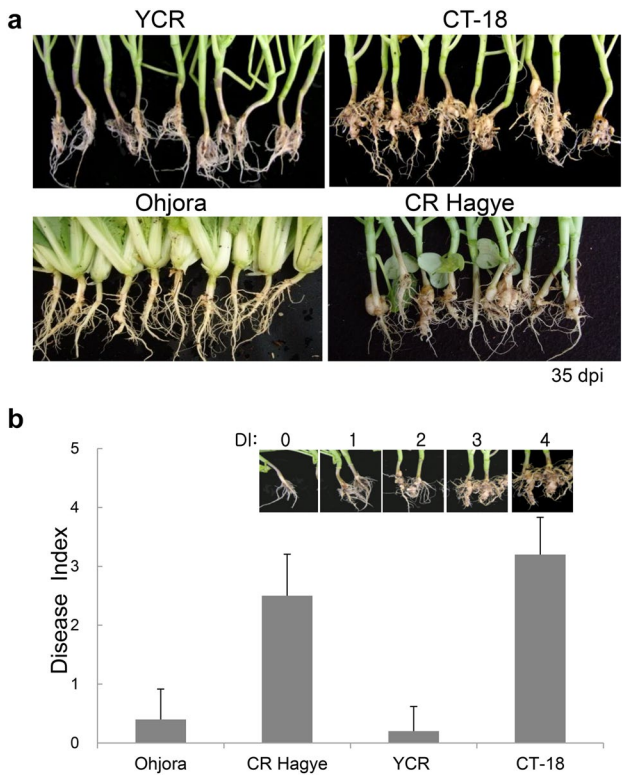


Fig. 1 Phenotypes of cabbage plants following root inoculation with *Plasmodiophora brassicae*. **a** Clubroot-infected roots of susceptible (CT-18) and resistant (YCR) cabbage plants. Pathogenicity was validated by comparison with *Brassica napus*-resistant (Ohjora) and -susceptible (CR Hagye) cultivars. Ten-day-old cabbage seedlings were inoculated by drenching with a *P. brassicae* spore suspension (inoculum density ~ 4.0×10^8 spores/pot). Images of phenotypes were photographed at 35 days after inoculation (DAI). **b** Measurement of disease index (DI) based on a five-point scale from 0 to 4 (0 = no symptoms; 1 = a few small, separate globular clubs on lateral roots; 2 = medium, separate globular clubs on lateral roots; 3 = intermediate symptoms; 4 = severe clubs on main roots). Root symptoms of each plant were recorded at 35 DAI. For disease indices, mean DI values ≤ 1.0 = resistant, 1.0 < DI ≥ 2.0 = intermediate resistant, and > 2 = susceptible

P. brassicae biomass estimation

For fungal biomass measurements, DNA was isolated from roots of mock- or *P. brassicae*-infected plant material at 5, 15 and 28 DAI using a DNeasy Plant Mini Kit (Qiagen, Germany). *P. brassicae*-specific primers were designed based on the internal transcribed spacer (ITS) region of *P. brassicae* (GenBank accession no. AF231027), and PCR using primers *ITS-F* (5′-CGAACTTCATTAAATTTGGGCTCTT-3′) and *ITS-R* (5′-ACTAGCATTCAAGCTGACTCG TTG-3′) was performed and analysed using a CFX Connect Real-Time System (BIO-RAD, USA) as described for previous gene expression experiments (Cao et al. 2007; Li et al. 2013).

Quantitative RT-PCR (qRT-PCR)

Total RNA from mock- or *P. brassicae*-infected leaf tissue was isolated using TRIzol reagent following the manufacturer's protocol (MRC, Canada), and quantified with a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). RNA samples were treated with RQ1 RNase-free DNase (Promega, USA) to remove genomic DNA, and first-strand cDNA was synthesised with Superscript II Reverse Transcriptase (Invitrogen, USA) according to the manufacturer's instructions. Primers (see Supplementary Table S1) were specifically designed to target amplicons (100–200 bp) using Primer Express 3.0 software (AB Applied Biosystems) and synthesised by Bioneer (Korea). Duplicate quantitative assays were conducted using iQ SYBR Green Supermix (BIO-RAD, USA) with a CFX Connect Real-Time System (BIO-RAD, USA) as previously described (Lee et al. 2012). Expression levels of each target gene were then quantified following normalisation against *actin* of *B. oleracea* (*BoActin*) as an endogenous reference.

Protein extraction

Arial tissues of non-infected and *P. brassicae*-infected plants were pooled, ground under liquid nitrogen and used for protein extraction as described previously (Kim et al. 2001). In brief, each sample was homogenised in Mg/NP-40 buffer (0.5 M Tris–HCl, pH 8.3, 2% v/v nonionic detergent NP-40, 20 mM $MgCl_2$ and 2% v/v β-mercaptoethanol), and centrifuged at 12,000 × *g* for 10 min at 4 °C. The resulting supernatant was subjected to phenol extraction for the recovery of total proteins by mixing with an equal volume of water-saturated phenol, vortexing vigorously and separating phases by centrifugation at 12,000 × *g* for 10 min at 4 °C. The phenol phase was recovered, and proteins were precipitated by adding 4 volumes of 0.1 M ammonium acetate in methanol, incubating at − 20 °C for 1 h, then centrifuging at 12,000 × *g* for 10 min at 4 °C. Pellets were washed once in 0.1 M ammonium acetate in methanol, and repeatedly

in ice-cold acetone until pellets were visible. Pellets were stored at − 20 °C in 80% (v/v) acetone until further analysis. Protein concentrations were evaluated using the 2D-Quant Kit (GE Healthcare, USA) according to the manufacturer's instructions.

Two-dimensional gel electrophoresis (2-DGE) analysis

Analysis by two-dimensional gel electrophoresis (2-DGE) was performed as previously described by Kim et al. (2012). For the first dimension, 500 µg of each protein sample was dissolved in rehydration buffer consisting of 7 M urea, 2 M thiourea, 4% v/v 3-[3-cholamidopropyl]dimethylammonio-1-propanesulfonate (CHAPS), 2 M dithioerythritol (DTT), and 0.5% v/v IPG buffer pH 4–7 (GE Healthcare) and loaded on a 24-cm IPG strip. First-dimensional separation of proteins was carried out on an IPGphore II platform (GE Healthcare) at 50 V for 4 h, 100 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 2000 V for 1 h, 4000 V for 2 h and 4000–8000 V for 5 h over a linear gradient, then at 120,000 Vh at 8000 V for 9 h. Before transfer to the second dimension, strips were first incubated in equilibration buffer (6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris–HCl, pH 6.8 and 0.01% w/v bromophenol blue) supplemented with 100 mM DTT, then replaced with equilibration buffer supplemented with 55 mM iodoacetamide in the dark, with gentle agitation for 30 min. After equilibration, proteins were resolved by 12% SDS-PAGE, and gels were stained with Coomassie Brilliant Blue R-250 (BIO-RAD, USA). 2-DGE images were acquired using an Umax Powerlook 1120 transmissive scanner (LaserSoft Imaging, Germany) and analysed by an ImageMaster 2D Platinum 6.0 (GE Healthcare), by which protein spots were detected, normalised and quantified using the mean intensity of all spots on the gel.

In-gel protein digestion

In-gel protein digestion was carried out as described previously (Kim et al. 2008). Protein spots of interest were excised, washed with 50% (v/v) acetonitrile in 0.1 M NH_4HCO_3 and dried in a SpeedVac evaporator (Hanil Science Industrial, Korea). Protein spots were then reduced with 10 mM DTT in 0.1 M NH_4HCO_3 for 45 min at 55 °C, and alkylated with 55 mM iodoacetamide in 0.1 M NH_4HCO_3 for 30 min at room temperature in the dark. Gel pieces were then washed with 50% (v/v) acetonitrile in 0.1 M NH_4HCO_3 and dried in a vacuum centrifuge. Protein spots were in-gel digested overnight at 37 °C in 10 µl of trypsin/Lys-C Mix (12.5 ng/ml in 25 mM NH_4HCO_3, sequencing grade; Promega, USA). The resulting peptides were extracted by adding 93:5:2 (v/v/v) distilled water:acetonitrile:trifluoroacetic acid (TFA), sonicating for 5 min, then centrifuging at 10,000 × *g* at 4 °C for 2 min. Matrix solution (α-cyano-4-hydroxycinnamic acid; Sigma-Aldrich, USA) dissolved in 100% acetone (40 mg/ml) and nitrocellulose in 100% acetone (20 mg/ml), nitrocellulose solution, and isopropanol were mixed at 100:50:50 (v/v/v). A two-point internal standard for calibration was used with a des-Arg^1-bradykinin peak ($m/z = 904.4681$) and an angiotensin 1 peak ($m/z = 1296.6853$).

MALDI-TOF/TOF MS

MALDI-TOF/TOF MS analysis was conducted as described previously (Kwon et al. 2010). Briefly, MS and MS/MS analyses were performed using an ABI 4800 Plus TOF-TOF Mass Spectrometer (Applied Biosystems, Framingham, MA, USA) fitted with a 200 Hz ND:YAG laser operating at 355 nm. The 10 most and 10 least intense ions per MALDI spot with a signal:noise ratio > 25 were selected for subsequent MS/MS analysis in the 1 kV mode with 800–1000 consecutive laser shots. During MS/MS analysis, air was used as the collision gas. All data were analysed using a Mass Standard Kit (Applied Biosystems, Framingham, MA, USA) for the 4700 Proteomics Analyser. MS/MS spectra were searched against the NCBI database using Protein Pilot version 3.0 (AB Sciex, Framingham, MA, USA) with MASCOT as the database search engine (ver. 2.3.0, Matrix Science, London, UK) using a peptide and fragment ion mass tolerance of 50 ppm. Carbamidomethylation of cysteines and oxidation of methionines were allowed during peptide searches, and one missed trypsin cleavage was allowed. The peptide mass tolerance and fragment mass tolerance of the selected proteins were set to 50 ppm. High-confidence identifications with statistically significant search scores (> 95% confidence, equivalent to the MASCOT expected values; $p < 0.05$) were consistent with the experimental isoelectric point (pI) and molecular weight (MW) of each protein, and accounted for the majority of ions present in the mass spectra.

Results and discussion

Comparison of disease phenotypes of cabbage cultivars to *P. brassicae*

To mimic natural *P. brassicae* infection of the crop cultivars (Diederichsen et al. 2009), roots of susceptible (CT-18) and resistant (YCR) cultivars of *B. oleracea* were inoculated with a *P. brassicae* spore suspension, and disease was rated based on typical clubroot symptoms to validate the pathogenicity of *P. brassicae*. Furthermore, pathogenicity assays were compared with susceptible (CR Hagye, Chinese cabbage) and resistant (Ohjora) cultivars of *B. napus* (Fig. 1a),

which were used as references. Infected plant roots were macroscopically observed at 5, 10, 15, 28 and 35 DAI where typical clubroot symptoms were shown only in susceptible cultivars (CT-18 and CR Hagye) at 35 DAI (Fig. 1a). Based on the phenotypic clubroot symptoms at 35 DAI, a DI score (0, 1, 2, 3 or 4) was assigned (Kobelt et al. 2000). CT-18 plants received a DI score of 3, similar to CR Hagye, and were thus classified as susceptible, while for YCR plants the DI score was 1, the same as that of the Ohjora line, and they were thus considered resistant to *P. brassicae* infection.

In planta fungal biomass was quantified using quantitative RT-PCR (qRT-PCR) by measuring the *ITS* gene of *P. brassicae* using total genomic DNA templates from the roots of mock- or *P. brassicae*-infected plants (YCR and CT-18) harvested at 5, 15 and 28 DAI (Fig. 2a, b, Supplementary Fig. S1B). A pair of primers was designed based on the conserved region between ribosomal DNA (rDNA) and *ITS* (Cao et al. 2007; Li et al. 2013). Consistent with the phenotype of large gall formation at 28 DAI, *ITS* transcripts were more than 3000-fold higher in CT-18 than in YCR, in which *ITS* expression was barely detectable (Supplementary Fig. S1). Moreover, fungal biomass of CT-18 was 1.8- and > 30-fold higher than that of YCR at 5 and 15 DAI, respectively (Fig. 2 and Supplementary Fig. S1); however, there were no symptoms on the infected CT-18 roots.

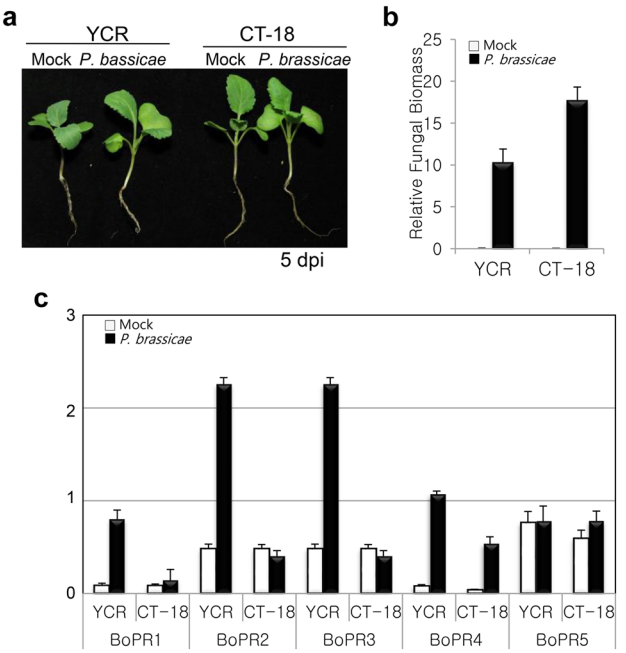


Fig. 2 Early regulation of the defence response in *B. oleracea* inbred line seedlings to *P. brassicae* infection. **a** Mock- or *P. brassicae*-infected seedlings of susceptible and resistant cabbage cultivars at 5 DAI. **b** Quantification of *P. brassicae* biomass in infected roots at 5 DAI. A region of the *P. brassicae*-specific *ITS* gene was examined using isolated DNA templates from roots of infected seedlings by qRT-PCR. Error bars indicate standard error of three technical repeats. **c** qRT-PCR analysis of the induction of *PR* genes in roots of cabbage cultivars following fungal infection. Total RNAs were extracted from root tissues of infected YCR and CT-18 seedlings at 5 DAI. The values were normalized to the level of *BoActin*. Error bars indicate standard error of three replicates

Expression of pathogenesis-related genes in the YCR or CT-18 cultivar to *P. brassicae* inoculation

To investigate whether the early defence responses to *P. brassicae* infection occur in the YCR-resistant cultivar, expression levels of *pathogenesis-related* (*PR*) genes were analysed in root tissues at 5 DAI (Supplementary Table S1) (Durrant and Dong 2004). The specificity of the amplified region of *B. oleracea* transcripts with all primers used was confirmed by sequencing and BLAST searches of the NCBI expressed sequence tag (EST) database. As shown in Fig. 2c, expression of *PR2* (β-*glucanase*) and *PR3* (*Endo-chitinase*) genes was highly up-regulated, and expression of *PR1* was also up-regulated relative to mock-infected YCR and susceptible (CT-18) cultivar plants infected with *P. brassicae* (Fig. 2c), although to a lesser extent. In addition, despite up-regulation of the *PR4* gene in *P. brassicae*-infected CT-18 relative to mock-treated CT-18, induction was even higher during the resistance responses in *P. brassicae*-inoculated YCR roots. However, there were no significant differences in the expression of *PR5* (*thaumatin-like*) between experimental groups. On the other hand, in the above-ground tissues of two cabbage cultivars at 5 DAI, no difference in pathogenic specific expression patterns of these genes was clearly observed (Supplementary Fig. S2). Taken together, these results suggest that an initial resistance response induced in YCR occurs in root tissues during *P. brassica* infection and the molecular mechanisms of the disease resistance response between underground and aboveground tissues are different.

Early proteomic changes in above-ground tissues of cabbage cultivars against *P. brassicae* infection

To identify differentially modulated proteins between resistant and susceptible cabbage cultivars in above-ground tissues to *P. brassicae* infection, proteomic profiles of seedling shoots were obtained using 2-DGE. For this, aerial shoot samples were collected and pooled from each cultivar either mock-infected or infected with *P. brassicae* at 5 DAI, before visible gall formation on infected roots since the growth of shoots was generally affected by clubroot disease development. Isolated proteins were resolved on high-resolution 2-D gels, which were then analysed using ImageMaster2DPlatinum software. The amount of loaded protein, number of spots resolved and proteins identified are summarised in Table 1. Analysis of 2-DGE maps for each sample yielded an average of 420 (± 30) spots per gel (Fig. 3 and Table 1). Comparative analysis of 2-DGE

Table 1 Protein spots and protein abundance resolved by two-dimensional gel electrophoresis

Feature	Details
Protein/gel	50 g (analytical)
	500 mg (preparative)
Staining	Coomassie blue
Total spots	420 (± 30)
Spots displaying significant differences	48 (5.18%)
Valid identified spots	44
Proteins identified in this study	24

gels using ImageMaster2DPlatinum software revealed 48 spots with significant differences ($p < 0.05$) in protein abundance between incompatible and compatible interactions, of which 44 were successfully identified by MALDI-TOF/TOF. Among the identified proteins, 24 were aligned to *B. oleracea* sequences and subsequently categorised according to biological process and/or subcellular localisation using GO annotation (Fig. 4). Based on the predicted biological functions of the identified proteins, they were divided into seven groups: response to stimulus (22%), amino acid metabolism (18%), carbohydrate metabolism (16%), photosynthesis (13%), biosynthesis of plant hormones (13%), oxidative stress (11%) and others (7%; Fig. 4a). The putative physiological functions of the proteins are closely linked to their subcellular localisation in plants (Anstead et al.

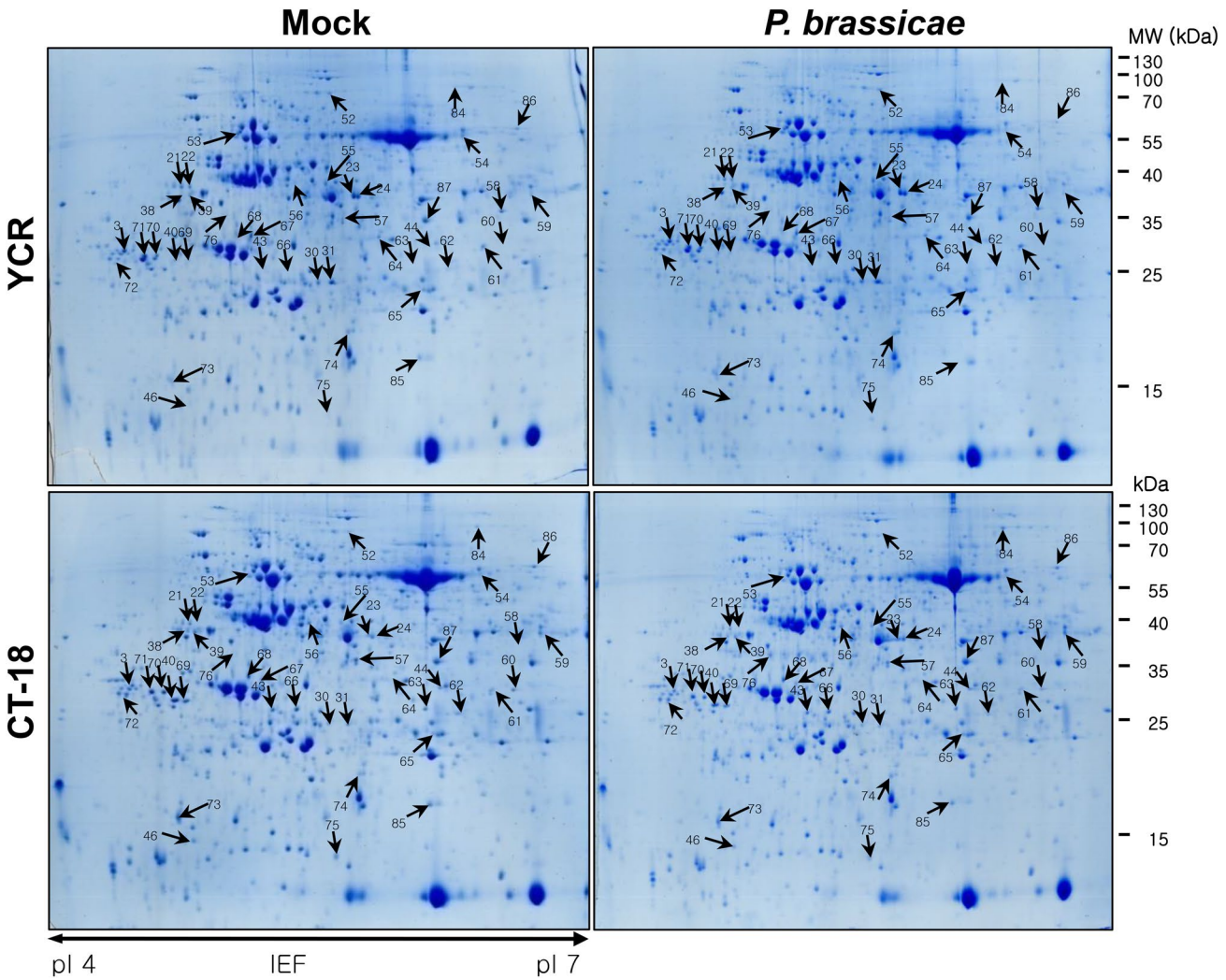


Fig. 3 Two-dimensional gel electrophoresis (2-DGE) protein profiles of mock- and *P. brassicae*-infected cabbage seedling shoots. Total isolated proteins from leaves of each *B. oleracea* inbred line were analysed by 2-DGE following mock treatment or fungal infection at 5 DAI. Proteins were first separated based on isoelectric point (pI) using isoelectric focusing (IEF), then by molecular weight via 12% SDS-PAGE. Gels were stained with colloidal Coomassie Brilliant Blue G-250. The molecular weights (MWs) of markers are shown in kDa

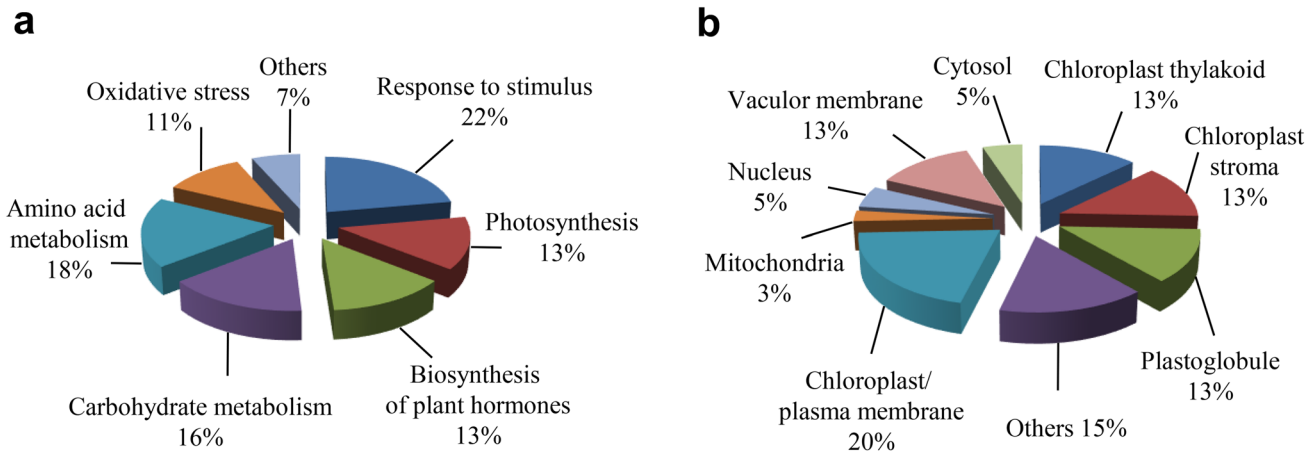


Fig. 4 Gene ontology (GO) analysis of identified protein spots in susceptible and resistant cabbage cultivars in response to *P. brassicae* infection. (**a** and **b**) Identified protein spots were assigned to biological process (**a**) and subcellular location (**b**) categories during incompatible (in YCR) and compatible (in CT-18) interactions with *P. brassicae* using GO annotation (Ashburner et al. 2000). Pie chart values are percentages of each group

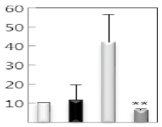
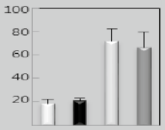
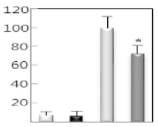
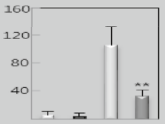
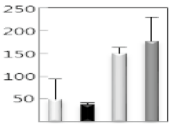
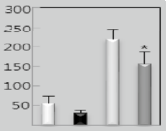
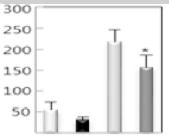
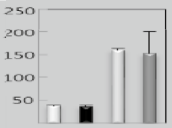
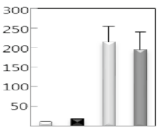
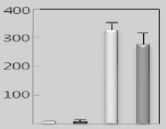
2013; Dalton et al. 1993; Lherminier et al. 2009; Li et al. 2006; Sun et al. 2014), and GO annotation has been broadly exploited to predict the locations of proteins (Ashburner et al. 2000; Lu and Hunter 2005). In this study, almost half (46%) of proteins identified were localised to the chloroplast, and specifically the thylakoid (13%), stroma (13%) and membrane (20%) of the organelle (Fig. 4b).

Differentially expressed proteins of *P. brassicae*-resistant or -susceptible cabbage cultivars

Comparative analysis using ImageMaster Program ver. 6 identified 24 protein spots displaying differential abundance in control (mock) or *P. brassicae*-infected cultivars (Student's *t* test, $^{**}p < 0.05$, $^{*}0.1 > p > 0.05$; Table 2). These 24 protein spots were classified into three groups: Group A contains 14 protein spots that were only present in the susceptible cultivar (CT-18); Group B includes six protein spots (22, 23, 24, 31, 53 and 67) that identified in the resistant cultivar (YCR); and Group C contains four protein spots (21, 54, 55 and 87) detected in both cultivars in response to *P. brassicae* infection (Table 2).

Of the 14 differentially enriched protein spots identified in susceptible CT-18 plants, only spot 84 showed increased abundance during *P. brassica* infection and 6 spots (38, 43, 46, 57, 58 and 72) were significantly downregulated compared to the mock-treated control. The other seven spots (39, 56, 59, 63, 66, 68, 73) did not changed their abundance under pathogen treatment. Spot 84 was identified as a cobalamin-independent methionine synthase isoform, which is involved in methionine regeneration from homocysteine to methionine (Eckermann et al. 2000; Ravanel et al. 1998). Cobalamin-independent MS (MetE) activity is found in higher plants, whereas cobalamin-dependent MS (MetH), which has a vitamin B_{12} (cobalamin) cofactor, is found in both animals and microorganisms (Rody and Oliveira 2018). Recently, it was reported that chloroplasts are autonomously involved in de novo biosynthesis of methionine (Ravanel et al. 2004). In addition, a previous study suggested a close metabolic link between plant defences against fungal pathogens and increased turnover of activated methyl groups in cultured cells and leaves of *Petroselinum crispum* (Kawalleck et al. 1992). On the other hand, *metE* mutations in *Ralstonia solanacearum* caused significantly reduced pathogenicity towards tomato plants (Plener et al. 2012). These studies indicate potent roles for MetE in compatible and incompatible interactions during pathogen invasion. Our results also suggest that *P. brassicae* infection enhances the susceptibility of plants by inducing MetE activity. Spots 31 (group B), 43 and 66 (group A) were all identified as RNA recognition motif proteins. Interestingly, only spot 43 of susceptible CT-18 plants reduced in protein abundance by pathogen infection. Spot 46 was identified as a thioredoxin (TRX) enzyme, and members of the TRX family are involved in responses to oxidative stress and pathogen defences (Laloi et al. 2004; Lee et al. 2005). Furthermore, plastidic 2-cysteine peroxiredoxin, a target for TRX in *Arabidopsis thaliana*, is involved in the protection of the photosynthetic apparatus against oxidative damage (Laloi et al. 2004). Spots 38 and 39 were close together with YCR specific spots 21 and 22 on the 2-D gel that were identified as peptidyl-prolyl *cis–trans* isomerase (PPIase). Cyclophilins, encoded by *CYP* genes, are a family of PPIase conserved throughout eukaryotes as well as bacteria. In plants, these proteins have been implicated in responses to biotic and abiotic stresses (Mokriakova et al.

Table 2 Proteins identified by MALDI-TOF/TOF MS

Spot no.	AC[¥]	Putative protein	Score	Expected	Mr[¶]/ pI[§]	SC[#]	Source	Relative protein content
Group A								
38	D7L9P5	Unknown	358	2.40E-29	48.30 /4.97	33%	*Arabidopsis lyrata* subsp. Lyrata	
39	D7L9P5	Unknown	315	4.70E-25	48.30 /4.97	42%	*Arabidopsis lyrata* subsp. Lyrata	
43	B9DG26	RNA recognition motif-containing protein	151	1.20E-08	27.70 /8.94	16%	*Arabidopsis thaliana*	
46	D7L429	Thioredoxin M4	75	1.00E-14	21.28 /9.00	20%	*Arabidopsis thaliana*	
56	D7KWJ1	Phosphoglycerae kinase	385	4.70E-32	42.15 /5.49	37%	*Arabidopsis lyrata* subsp. Lyrata	
57	D7KM98	Binding protein	355	4.70E-29	37.57 /8.50	40%	*Arabidopsis lyrata* subsp. Lyrata	
58	Q93X66	Enoyl-[acyl-carrier protein] reductase	344	5.90E-28	40.94 /9.30	53%	*Brassica napus*	
59	B6VCT5	Epithiospecifier protein	107	0.0003	37.89 /5.95	15%	*Brassica rapa* subsp. pekinensis	
63	E4MVU2	Ascorbate peroxidase 4	346	3.70E-28	38.10 /9.11	37%	*Eutrema halophilum*	
66	Q8VYM4	RNA recognition motif-containing protein	217	3.00E-15	37.73/ 8.94	32%	*Arabidopsis thaliana*	

2014; Sharma et al. 2008; Viaud et al. 2002). As these four spots were in close proximity to each other and were differentially modulated in different cultivars (spot 21 and 22 in YCR and spots 38 and 39 in CT-18), post-translational modifications such as phosphorylation, peptide cleavage or unfolding could be of relevance and result in significant differences in the responses against pathogen infection. Spot 58 was identified as enoyl-[acyl-carrier protein] reductase

Table 2 (continued)

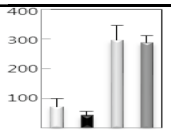
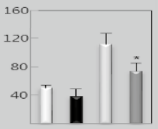
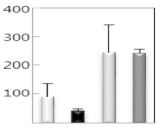
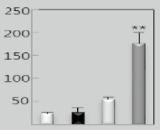
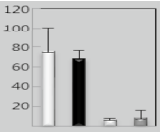
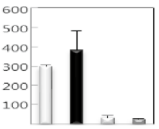
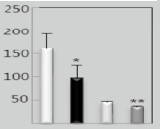
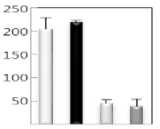
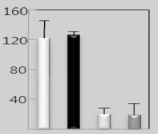
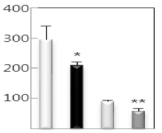
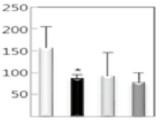
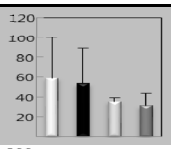
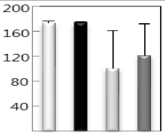
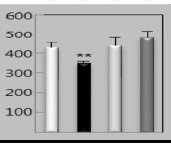
68	Q8W593-2	Isoform 2 of Probable lactoylglutathione lyase	387	3.00E-32	29.58 /5.14	64%	*Arabidopsis thaliana*	
72	Q2V614	Glycine-rich RNA-binding protein 5	151	1.20E-08	23.67/ 10.25	33%	*Brassica rapa*	
73	D7LPL3	Ribosomal protein L12-C	122	9.40E-06	19.58 /5.51	24%	*Arabidopsis lyrata* subsp. Lyrata	
84	D7LXP0	Cobalamin-independent methionine synthase	145	4.70E-08	84.64 /6.12	27%	*Arabidopsis lyrata* subsp. Lyrata	
Group B								
22	D7L9P5	Peptidyl-prolyl cis-trans isomerase	285	4.7E-22	48.30 /4.97	35%	*Arabidopsis lyrata* subsp. Lyrata	
23	Q944G9	Probable fructose-bisphosphate aldolase 2	288	2.40E-22	43.13 /6.78	34%	*Arabidopsis thaliana*	
24	E4MVZ7	Fructose-bisphosphate aldolase	89	0.021	43.12 /6.49	25%	*Eutrema halophilum*	
31	B9DG26	RNA recognition motif-containing protein	134	5.90E-07	27.70 /8.94	19%	*Arabidopsis thaliana*	
53	P11574	V-type proton ATPase subunit B1	525	5.90E-46	54.19 /4.98	55%	*Arabidopsis thaliana*	
67	D7KV14	Putative lactoylglutathione lyase	243	7.50E-18	39.66 /7.59	35%	*Arabidopsis lyrata* subsp. Lyrata	
Group C								
21	D7L9P5	Peptidyl-prolyl cis-trans isomerase	320	1.5E-25	48.30 /4.97	37%	Arabidopsis lyrata subsp. Lyrata	

Table 2 (continued)

54	A2VAK1	Ribulose bisphosphate carboxylase large subunit (Fragment)	149	1.90E-08	48.87 /6.13	35%	*Lepidium africanum*	
55	F4JUJ5	Fructose-bisphosphate aldolase	211	1.20E-14	41.55 /9.07	25%	*Arabidopsis thaliana*	
87	D7LDR2	Oxygenase activase	294	5.90E-23	52.21 /5.69	28%	*Arabidopsis lyrata* subsp. Lyrata	

The relative volumes of two replicates (± SD) are presented. Data analysis was performed using ImageMaster Program ver. 6. The 24 spots were classified as follows: Group A, present only in the susceptible cultivar (CT-18); Group B, present only in the resistant cultivar (YCR); Group C, present in both cultivars (in response to *P. brassicae* in all cases; see Fig. 3). $^{**}p < 0.05$; $^{*}0.1 > p > 0.05$ by Student's *t* test. Error bars represent the standard error of two replicates

[¥] *AC* accession number, [¶] *Mr* molecular weight, [§] *pI* isoelectric point, [#] *SC* sequence coverage, From left to right, □ = mock-inoculated YCR; ■ = *P. brassicae*-inoculated YCR; □ = mock-inoculated CT-18; ■ = *P. brassicae*-inoculated CT-18.

(ENR). ENR exhibits oxidoreductase activity and catalyses a reduction step in fatty-acid biosynthesis. ENRs have been widely used as natural antibacterial agents (Massengo-Tiasse and Cronan 2009). Moreover, a recent study reported that cyperin, a natural diphenyl ether phytotoxin produced in several fungal–plant pathogens, contributes to pathogenic virulence by targeting and inhibiting plant ENRs, suggesting that these enzymes may be good targets for the development of new herbicides derived from natural products (Dayan et al. 2008). Spot 72 was identified as a glycine-rich RNA-binding protein. This protein is known to be involved in RNA metabolism and has been reported to play a positive or negative role in plant defence responses, depending on the pathogen (Ciuzan et al. 2015).

Of the six protein spots isolated from resistant YCR plants, spots 24 and 67 showed a disease-specific decrease in expression, the other four (22, 23, 31, and 53) remained unchanged. Spots 23 and 24 and 55 were all identified as fructose-bisphosphate aldolase. Of these, spot 24 was only reduced in YCR plants during *P. brassicae* infection. Spots 21 and 87 (belong to group C) were identified as a PPIase and an oxygenase activase, respectively. The abundance of these spots was reduced in fungal-infected YCR relative to mock-treated controls, while there was no change in the CT-18 susceptible cultivar. Previous transcriptomic and proteomic studies reported up-regulation of TCA pathway-related genes in *P. brassicae*-infected roots during the early stages of infection (Devos et al. 2006; Siemens et al. 2006). Proteomics results revealed an increase in gene expression in galls, but this could also reflect the strong sink created by cytokinin (Devos et al. 2006). Spots 23, 24, 54, 55 and 87 were found to be implicated in the Calvin cycle, and spots 24 and 87 were specifically down-regulated in protein abundance in aerial shoots of the resistant YCR cultivar following *P. brassicae* infection. Spot 53 was identified as V-type proton ATPase subunit B1. In *A. thaliana*, vacuolar H+-ATPase B1 (VHA-B1) is one of the unconventional partners of nuclear-specific hexokinase 1 (glucose sensor AtHXK1) (Cho et al. 2006). These results suggest that the inhibition of energy metabolism of leaves during the infection by fungal pathogens in the roots can be an effective resistant reaction to inhibit the growth of pathogens.

Conclusion

To our knowledge, this is the first study to investigate club-root infection in susceptible and resistant cabbage cultivars using high-throughput proteomic analysis. We focused on the early stages of *P. brassicae* infection, preceding club-root development (5 DAI), to probe the early responses in the above-ground tissues of cabbage to this pathogen. To

understand plant–pathogen interactions, it is crucial to know how host proteins recognise and interact with pathogen-derived molecules, and elucidate how plant cells organise signalling pathways triggered upon recognition to coordinate appropriate resistance responses. Proteomic analysis revealed that spot 84 was increased in abundance only in susceptible cultivars in response to *P. brassicae* infection, implicating the corresponding protein as a biomarker for separating susceptible and resistant cultivars during the early stages of plant development. On the other hand, recently completed cabbage genomic information and gene editing techniques will help to characterize the function of proteins isolated in this study during *P. brassicae*–cabbage interactions.

GO analysis classified half of the identified differentially expressed proteins into response to stimulus, hormone synthesis and oxidative stress categories based on biological process. These proteins included some known to be involved in host plant responses to external environment stress, such as TRX (spot 44) and oxygenase activase (spot 87; Fig. 4). In addition, our proteomic data identified some protein spots in the same category that were present in susceptible and resistant cultivars for which expression patterns were not correlated with each other (Table 2). This apparent discrepancy indicates the possible contribution of post-translational regulation during compatible or incompatible interactions between cabbage and *P. brassicae*. The ultimate purpose of this investigation was to gain a deeper understanding of *B. oleracea*–*P. brassicae* interactions, and moreover, to identify new alleles for the development of new cultivars with broader resistance against *P. brassicae* infection.

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