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Shotgun proteomics provides an insight into pathogenesis-related proteins using anamorphic stage of the biotroph, *Erysiphe pisi* pathogen of garden pea

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ABSTRACT

E. pisi is an ascomycete member causing powdery mildew disease of garden pea. It is a biotrophic pathogen, requiring a living host for its survival. Our understanding of molecular mechanisms underlying its pathogenesis is limited. The identification of proteins expressed in the pathogen is required to gain an insight into the functional mechanisms of an obligate biotrophic fungal pathogen. In this study, the proteome of the anamorphic stage of *E. pisi* pathogen has been elucidated through the nano LC–MS/MS approach. A total of 328 distinct proteins were detected from *Erysiphe* isolates infecting the susceptible pea cultivar, Arkel. The proteome is available via ProteomeXchange with identifier PXD010238. The functional classification of protein accessions based on Gene Ontology revealed proteins related to signal transduction, secondary metabolite formation and stress which might be involved in virulence and pathogenesis. The functional validation carried through differential expression of genes encoding G-protein beta subunit, a Cyclophilin (Peptidyl prolyl cis-transisomerase) and ABC transporter in a time course study confirmed their putative role in pathogenesis between resistant and susceptible genotypes, JI2480 and Arkel. The garden pea-powdery mildew pathosystem is largely unexplored, therefore, the identified proteome provides a first-hand information and will form a basis to analyze mechanisms involving pathogen survival, pathogenesis and virulence.

1. Introduction

Garden pea (*Pisum sativum* L.) is an important edible legume crop cultivated worldwide. The crop is consumed as vegetable and pulse due to its high nutritive value and is also used as fodder for cattle. *Erysiphe pisi* is an obligate biotrophic fungal pathogen which causes the destructive powdery mildew disease in garden pea worldwide. The disease appears as white specks on infected pea leaves which gradually infect the whole plant resulting in loss of nutrients to the fungus, disruption of photosynthetic process and premature death of leaves and other infected plant tissues. This devastative disease affects pea yield and its productivity potential leading to 25–50% yield losses globally (Bhardwaj and Sharma, 1984; Munjal et al., 1963; Warkentin et al., 1996). In India, a loss of 20–30% in pod number and 25% reduction in pod weight has been reported as a result of *E. pisi* attack (Smith et al., 1988). Nisar et al. (2006) have reported crop losses ranging from 25% to 86%.

Although powdery mildews are most commonly occurring Ascomycete plant pathogenic fungi, studies in these pathogens are limited due to their obligate biotrophic nature rendering difficulty in pursuing *ex-situ* or *in-vitro* studies. Studies have been carried to unravel the genetic and molecular nature of interactions between powdery mildews and their hosts, mostly on powdery mildew fungi infecting barley, wheat and *Arabidopsis thaliana*, the model plant (Bindschedler et al., 2011, 2009; Hacquard et al., 2013; Spanu, 2014; Spanu et al., 2010; Wicker et al., 2013). Though proteome based studies have been reported in the fungus, *Blumeria graminis*, another powdery mildew causing member of the Poaceae family, the pathogen is phylogenetically distinct from *E. pisi*, as it forms a separate clade from other powdery mildew fungi (Braun, 1987, 1981; Cook et al., 1997; Saenz and Taylor, 1999; Sperr, 1973; Zeller, 1995).

The whole genome sequencing studies of three of the powdery mildew fungi, *B. graminis* f. sp. *hordei* (*Bgh*), *Erysiphe pisi* and *Golovinomyces orontii* deciphered sequence sizes of ~120 Mb, ~151 and ~160 Mb respectively (Spanu et al., 2010). The genome of *E. necator* was also estimated to be 126 ± 18 Mb by Jones et al. (2014). The studies of fungal genomes reveal major gene losses despite their large genome size which accounts to be more than four times greater in comparison to the median of other ascomycete members. The *Blumeria* genome showed only 5854 curated genes and reduced number of protein-coding genes (~6500 annotated genes in *Bgh* and *Bgt*) in spite of their large genome size (120–160 Mbp) owing to genome expansion resulting from

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proliferation of transposable elements (TE). The presence of TE has been associated with the appearance of virulent pathogenic forms (Hacquard et al., 2013; Spanu et al., 2010; Wicker et al., 2013).

The garden pea-powdery mildew pathosystem is largely unexplored, therefore, the identified proteins provide an insight into the framework of the *E. pisi* proteome. In this study, the proteome of *E. pisi* pathogen has been identified using a sensitive and reliable nano LC–MS/MS approach and the findings are presented.

2. Materials and methods

2.1. Plant material and fungal isolation

Pea seeds were sterilized using 0.1% Sodium hypochlorite for 5 min. followed by washing once with 70% ethanol for 30 s. The seeds were then washed with sterile distilled water 4–5 times followed by soaking in autoclaved distilled water for 12–24 h and sown in pots/ containers filled with soil and manure in 3:1 ratio. The plants were maintained under greenhouse conditions at a temperature of 25 ± 3 °C for the experiments Bheri et al., 2016. The powdery mildew isolates, Ep01 (GenBank acc. Nos. - KM096758, KM189823) and Ep03 (GenBank acc. Nos. - KM096760, KM189825) were collected, based on different host compatibilities, from well separated Arkel and JI2302 pea plants respectively, maintained under greenhouse conditions in the plant culture facility of Department of Plant Sciences, University of Hyderabad [Lat. and Long.- 17° 27′ N / 78° 19′ E].

2.2. Inoculation of pea plants

Pea plants of Arkel var. were inoculated with the two isolates, Ep01 and Ep03 in isolation. The experiments were carried in two sets for each of the isolates. Atleast 200 plants were tested in each of the four biological replications. Thirty-day old plants were inoculated by dusting fresh conidia and the fungal mycelium was harvested at the 10 dpi stage. The mock or un-inoculated control plants of Arkel were free of powdery mildew infection and served as negative control for the disease. The mycelium was washed with water (HPLC grade, Sigma) twice by vortexing at 12,000 rpm for 5 min. The supernatant was discarded and the mycelium was frozen using liquid nitrogen and stored at −80 °C till extraction.

For qRT- PCR, Arkel and JI2480 were used as the susceptible and resistant type respectively. 10 mg of conidia was dusted on the leaves of the two cultivars with three plants each and plants were maintained in growth chamber conditions at 25 °C. Infected leaves were collected at different infection time points from 6, 12, 24 and 48 h post-infection (hpi) to analyze gene expression changes in response to powdery mildew infection. Healthy leaf was used as a control. Samples were immediately frozen in liquid nitrogen and stored at −80 °C for total RNA extraction. Three biological replicates were used for all treatments.

2.3. Protein extraction and nano LC–MS/MS

Protein extraction was carried out according to Noir et al. (2009). The fungal mycelium was ground finely in 1 ml of extraction buffer [50 mM Tris-Base pH 8.0; 10 mM EDTA; 1% (CHAPS); 10 mM (DTT); protease inhibitor cocktail]. The lysate was briefly sonicated with short pulses of 30 s. at 70% amplitude for 3 min. The sample was spun down. The protein concentration in the supernatant was estimated using 2D Quant Kit (GE Healthcare) and the quality was checked on SDS-PAGE. The supernatant was lyophilized and stored until further processing. The sample was reconstituted in 20% Acetonitrile made with 100 mM Ammonium bicarbonate. The protein concentration was determined using Bicinchoninic Acid Protein Assay Kit (Sigma). The proteins were precipitated from the lysates using precipitation buffer (50% Acetone, 50% Ethanol and 0.1% Acetic acid) and washed three times with ice-cold acetone.

2.4. In-solution digestion and nano LC–MS/MS

The in-solution digestion protocol followed was as per Shevchenko et al. (2006) and Wiese et al. (2007). Digested peptides were vacuum dried and reconstituted in 15 µl of 2% ACN (LC/MS grade –Fluka) with 0.1% Formic acid (LC/MS grade –Fluka). The complex tryptic peptide mix was separated on 1200, 1D nano-LC (Agilent) on a 180-minute RPLC gradient. The NanomateTriversa was used as an automated nano-ESI source to generate ions for later mass spectrometric analysis. This was followed by the acquisition of data through MS/MS analysis using LTQ-Orbitrap Discovery (Thermo). 20 fmoles of Standard BSA digest was analyzed in parallel to check the performance of the instrument.

2.5. Protein identification, categorization and network analysis

The generated data was searched for protein identity using MASCOT 2.4 as the search engine on Proteome discoverer 1.4. The data was searched against Uniprot Swiss-Prot database (non-redundant database with reviewed proteins), Uniprot/TrEMBL (database with unreviewed proteins) and *Erysiphe* database downloaded from NCBI (National Center for Biotechnology Information). A minimum of two high confident peptides was used as a prerequisite to identify the proteins. The target false discovery rate, FDR (Strict) was 0.01 while Target FDR (Relaxed) was 0.05. The functional categorization was carried out manually based on the Gene Ontology (biological process) and KEGG entries. The network analysis was carried out using STRING version 10.5 using *Magnaporthe oryzae,* an ascomycete phytopathogen, as the reference organism (Szklarczyk et al., 2017).

2.6. RNA isolation and cDNA synthesis

Total RNA was extracted from all the infected samples and control collected at different time points post inoculation using TRI-reagent (Sigma-Aldrich). The leaf tissue was homogenized in TRI reagent followed by addition of Chloroform. The samples were shaken and allowed to stand for 2–15 min, followed by centrifugation at 12,000 rpm for 15 min. at 2–8 °C. The upper aqueous phase containing RNA was transferred to a fresh tube and 0.5 ml of 2-propanol per ml of TRI Reagent used was added and mixed. The samples were centrifuged at 12,000 rpm for 10 min.at 2–8 °C. The RNA precipitate was air dried and dissolved in DEPC treated water. RNA integrity and its quality were checked by formaldehyde gel electrophoresis. Quantity and purity testing was done by NanoDrop 2000 spectrophotometer (Thermo Scientific). First strand cDNA synthesis was performed using 2 µg/µl total RNA per sample using cDNA synthesis kit (Takara) as per instructions.

2.7. Quantitative real-time PCR (qRT- PCR) analysis

Primers were designed by using OligoAnalyzer 3.1 bioinformatics tool for the genes encoding Cyclophilin, ABC transporter, G-protein beta subunit and *Actin* (Internal control), detected in the proteomic data for quantitative gene expression study. The primers were designed with expected amplicon size ranging from 125 to 225 bp. Primers were checked for self-annealing sites, hairpin loop formation and heterodimer. Yeast *Actin* gene was used as internal control for normalization of gene expression data. The lists of primers used for quantitative-reverse transcriptase (qRT) along with annealing temperatures are provided in Table 1. The qPCR was performed on 96-well PCR plate (Applied Biosystems) on Eppendorf gradient. The 10 µl reaction mix contained 5.2 µl of 1X SYBR mix, 0.5 µl of 0.25 µM of each primer, 1 µl of 1:5 diluted cDNA and 2.8 µl of water to make up the volume. The PCR profile used was initial denaturation of 95 °C for 3 min followed by 95 °C for 15 s, annealing temperature for 30 s, 72 °C for 20 s followed by

Table 1
The list of three selected genes detected in proteomic data through Real-Time Quantitative-Reverse Transcriptase (q-RT) PCR along with their annealing temperatures and biological function.

S. No.	Name	Function	Primer Sequence	Annealing Temperature
1	*CYP*	Cyclophilin	F:CCTAAAACAGTGGAGAACTT R:AGTGCCATCACCTTTGGTA	56 °C
2	*ABC*	ABC transporter	F:GCCACTGACGAGGAAATCTA R:CTCTGTCTCTGAATCTAGGG	56 °C
3	*G-BETA*	G-protein beta subunit	F:GTGATGCGTTCTCTAAGCTG R:CCCCATATTGATTAAGTTCTCG	56 °C
4	Yeast *Actin*	*Actin* gene-Internal control	F:GTAACATCGTTATGTCCGGTGGTAC R:CCAAGATAGAACCACCAATCCAGAC	50 °C

40 cycles of 95 °C for 15 s and 60 °C for 15 s. A melting curve was performed at the end of qPCR run. During qRT analysis, all the samples were set in triplicates including no-template DNA control (NTC). Threshold cycle value (Ct) for each sample was determined from the realplex software (Eppendorf) for pathogenicity-associated and housekeeping genes.

2.8. Expression stability analysis and statistics analysis

The relative quantification of fungal genes was done by threshold cycle model (Ct value) which describes number of cycles required for the amplification of any genes to reach a threshold level. The level of gene expression was normalized by subtracting the Ct value of internal control genes from Ct value of target gene named as ΔCt, for each repetition of samples in every run. Furthermore, the expression fold change was calculated by using $2^{\hat{}-\Delta Ct}$ for all pathogenicity genes for both genotypes (Livak et al., 2001). Statistical analysis was done using SigmaPlot 11.0 and RT-qPCR was plotted to compare gene expression using student-t test at different time points.

3. Results

3.1. Protein profiling

The prime goal of the study is to provide an insight into the proteome of *E. pisi* pathogen to detect virulence and pathogenesis-related proteins. A reference proteome of *E. pisi* obtained through host-pathogen interaction involving pea and *Erysiphe* pathogen would provide a basis to understand the mechanism of pathogenesis and virulence. The protein profiling was carried by isolating proteins from fungal mycelium collected from *Erysiphe*-inoculated leaves of the susceptible plant, Arkel. The data generated on LTQ-Orbitrap-MS was searched using MASCOT 2.4 as search engine. The data was searched against UniProtSwiss-Prot database (non-redundant database with reviewed proteins), UniProtTrEMBL (database with unreviewed proteins) and *Erysiphe* database downloaded from NCBI. Fungal species like *Puccinia triticina*, *Blumeria graminis*, *Monilinia laxa*, *Aspergillus Fumigatus*, *Magnaporthe* species were also included in the database search. The standard BSA (20 fmoles) was successfully found back with an area of 4.646E8 and sequence coverage of 48.6%. A total of 1748 protein accessions were obtained, from which, 328 distinct proteins were identified and categorized through Gene ontology on the basis of their biological role. The number of identified proteins belonging to different functional categories based on Gene Ontology are listed in Table 2 and Table S1. The proteins were categorized based on their involvement in metabolism of nucleosides and nucleotides, amino acids, carbohydrates, lipids, proteins (metabolism, modification, targeting and localization), secondary metabolite biosynthetic process, cytoskeletal organization, fungal cell wall organisation, replication and repair, regulation of cell growth, RNA binding and modification, oxidative phosphorylation, cell redox homeostasis, signal transduction, transport and response to stress. In addition, 350 predicted and hypothetical proteins were also identified (Table S2). The graphical representation of the functional categorization of the *E. pisi* proteome, is depicted in Fig. 1. The hypothetical proteins have been excluded to present the categorization of distinctly identified polypeptides. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Deutsch et al., 2017) via the PRIDE (Vizcaíno et al., 2016) partner repository with the dataset identifier PXD010238 and 10.6019/PXD010238. The functional classification of protein accessions revealed proteins related to signal transduction, secondary metabolite formation and stress which might be involved in virulence and pathogenesis. Network analysis using STRING version 10.5 showed the predicted functional associations with PPI enrichment p-value < 1.0e-16, based on experimental evidence, curated databases, predicted interactions and other evidences such as text-mining, co-expression and protein homology. A representative visualization is presented in Fig. 2 and the functional enrichment of the analysis is presented in Table 3. Some of the potential proteins that might be involved in pathogenicity and virulence of *Erysiphe* spp. based on earlier studies are listed in Table 4.

Table 2
The list of proteins categorized into functional groups on the basis of their biological role.

Protein description	No. of proteins
Nucleoside and nucleotide metabolism	5
Amino acid metabolism	12
Carbohydrate metabolism	21
Lipid metabolism	21
Secondary metabolite biosynthetic process	1
Protein metabolism and modification	117
Protein targeting and localization	7
Cytoskeletal organization	8
Fungal cell wall organization	2
Replication and repair	14
Regulation of cell growth	11
RNA binding and modification	35
Oxidative phosphorylation	19
Cell redox homeostasis	3
Signal transduction	24
Transport	21
Response to stress	3
Unclassified proteins	4
Total	328

3.2. Relative gene expression of putative pathogenicity-related genes

The validation of the three genes encoding Cyclophilin, ABC transporter, G-protein beta subunit along with *Actin* (internal control), detected in proteome profiling was done through qRT-PCR. The qRT-PCR analysis was conducted with Arkel as susceptible and JI 2480 as resistant genotype at different time point of 6, 12, 24, 48 h post inoculation (hpi) and control of both genotypes. The qRT-PCR data was normalized by using yeast-*Actin* gene expression. Relative fold change in qRT-based gene expression levels was calculated and the graphs were plotted to estimate the mRNA transcript accumulation in both

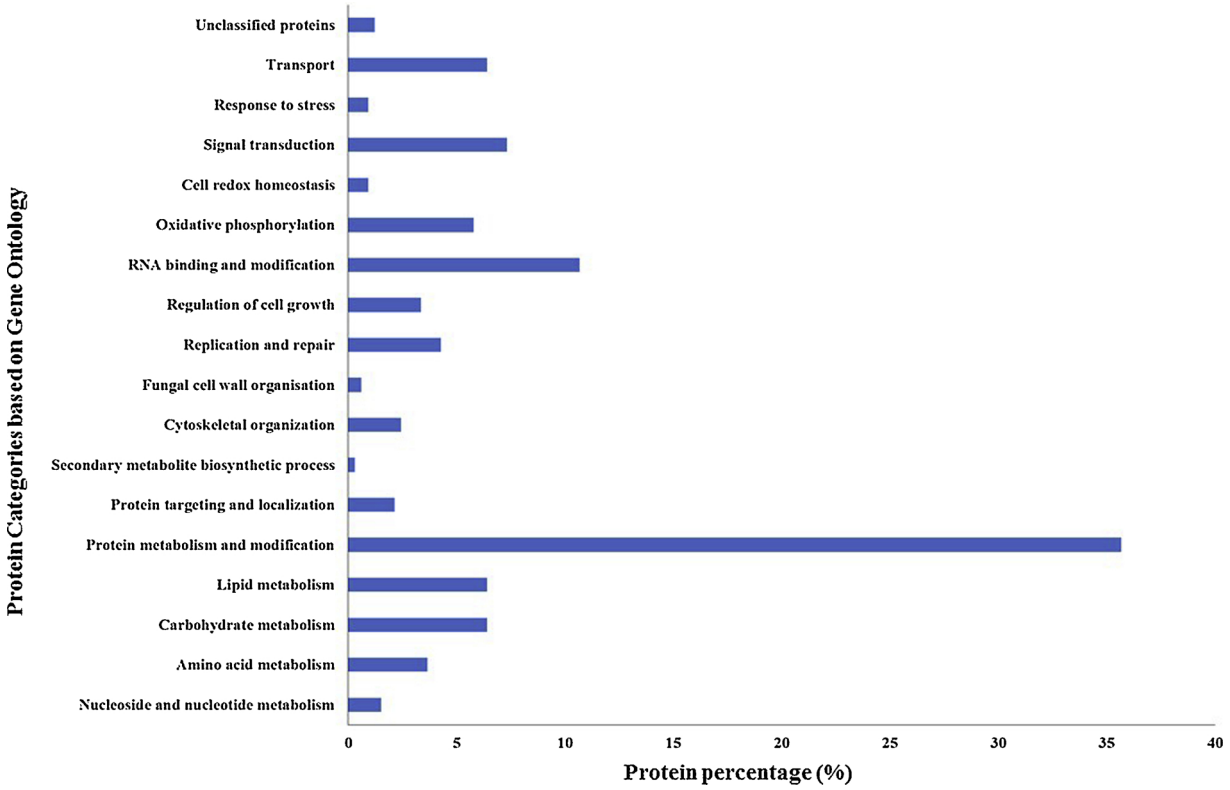

Fig. 1. The graph shows the functional categorization of proteins identified from *E. pisi* proteome.

genotypes at different time points (Fig. 3).

The mRNA transcripts of heterotrimeric G-protein beta subunit (*G-β*) gene were observed to be higher in susceptible Arkel var. in comparison to JI2480 post infection while the susceptible control showed minimal expression unlike the resistant healthy controls. The transcripts were found to gradually increase till 24 h in Arkel with sudden decrease in later time points of infection while in JI2480 the highest gene expression occurred at 12 hpi compared to resistant and susceptible controls. The highest expression of *G-β* gene was observed in susceptible genotype at 24 hpi with an expression level of ~4 fold change in response to infection. Transcripts of ABC transporter (*ABC*) gene were strongly accumulated at 24 h post-inoculation with ~4 fold change expression followed by quick decrease at 48 h post-inoculation in Arkel genotype. In case of JI 2480, transcript highest peak was observed at 12 hpi which further completely declined at 24 hpi and further increased at 48 hpi. The highest transcript accumulation was observed in Arkel var. and was much higher than the JI2480 expression. Cyclophilin (*CYP*) transcripts had shown less accumulation in both the resistant and susceptible genotypes with the change in expression being ~0.8 fold, observed at 12 hpi. *CYP* gene was observed to be down-regulated after 12 hpi.

4. Discussion

Among the fungal pathogens which infect crop plants, powdery mildews are predominant and cause severe crop losses. *Erysiphe pisi*, being an adapted pathogen of garden pea has a narrow host range. In addition, owing to its biotrophic mode of living, the pathogen poses a huge challenge in carrying out studies focusing on the mechanism of its survival as a biotroph as well as its virulence as a well-adapted pathogen to garden pea. Consequently, fewer studies are reported regarding the mechanisms which have enabled their existence and pathogenicity. It is intriguing to understand the regulatory processes which confer the ability of biotrophic pathogen systems to survive/overcome the host surveillance mechanisms and proliferate. The adapted host-pathogen system comprising of *E. pisi* and pea and their interactions could be utilized to gather an insight into the mechanisms of pathogenesis.

Most of the proteomic studies involving powdery mildew fungi have targeted differentially regulated proteins expressed during compatible interactions between the host and the pathogen at various stages of interaction leading to the identification of fewer fungal proteins, although limited studies have been carried out to identify their proteomes (Bindschedler et al., 2011, 2009; Curto et al., 2006; Godfrey et al., 2009; Marsh et al., 2010; Noir et al., 2009). Proteomic studies have been carried out on phytopathogenic fungi during *in planta* colonization, particularly, the powdery mildew pathogens using the *Bgh* system, however, only a few reports are available in *Erysiphe* spp. of garden pea. Curto et al. (2006) reported studies in garden pea-powdery mildew system, based on the interactions between *P. sativum-E. pisi* using JI2480 and Messire genotypes. It was observed that the resistance to *E. pisi* in JI2480 plants is based on constitutive defense responses as evidenced from the detection of a number of proteins involved in stress and defense responses, carbohydrate catabolism and photosynthesis. This may result in an increased energetic metabolism in the case of the resistant plants to support constitutive resistance. Therefore, a proteomic study based on *Erysiphe* pathogen to establish a reference proteome map for the identification of putative proteins involved in pathogenesis and virulence is required. Proteins involved in pathogenesis and virulence have been identified through homology searches with similar proteins in other phytopathogenic fungi, with the number of peptides representing protein abundance (Bindschedler et al., 2009). Noir et al. (2009) reported the first functionally annotated proteome of a powdery mildew fungus using ungerminated conidia of *Bgh* by 2D gel electrophoresis with MALDI-TOF MS and MALDI-TOF/TOF MS/MS. The 123 distinct proteins identified from 180 spots belonging to various metabolic pathways such as lipid, carbohydrate, proteins as well as protein processing indicated that the protein machinery of the conidia is suitable for meeting the needs as a storage structure as well as germination processes for the propagation of the pathogen. Godfrey et al.

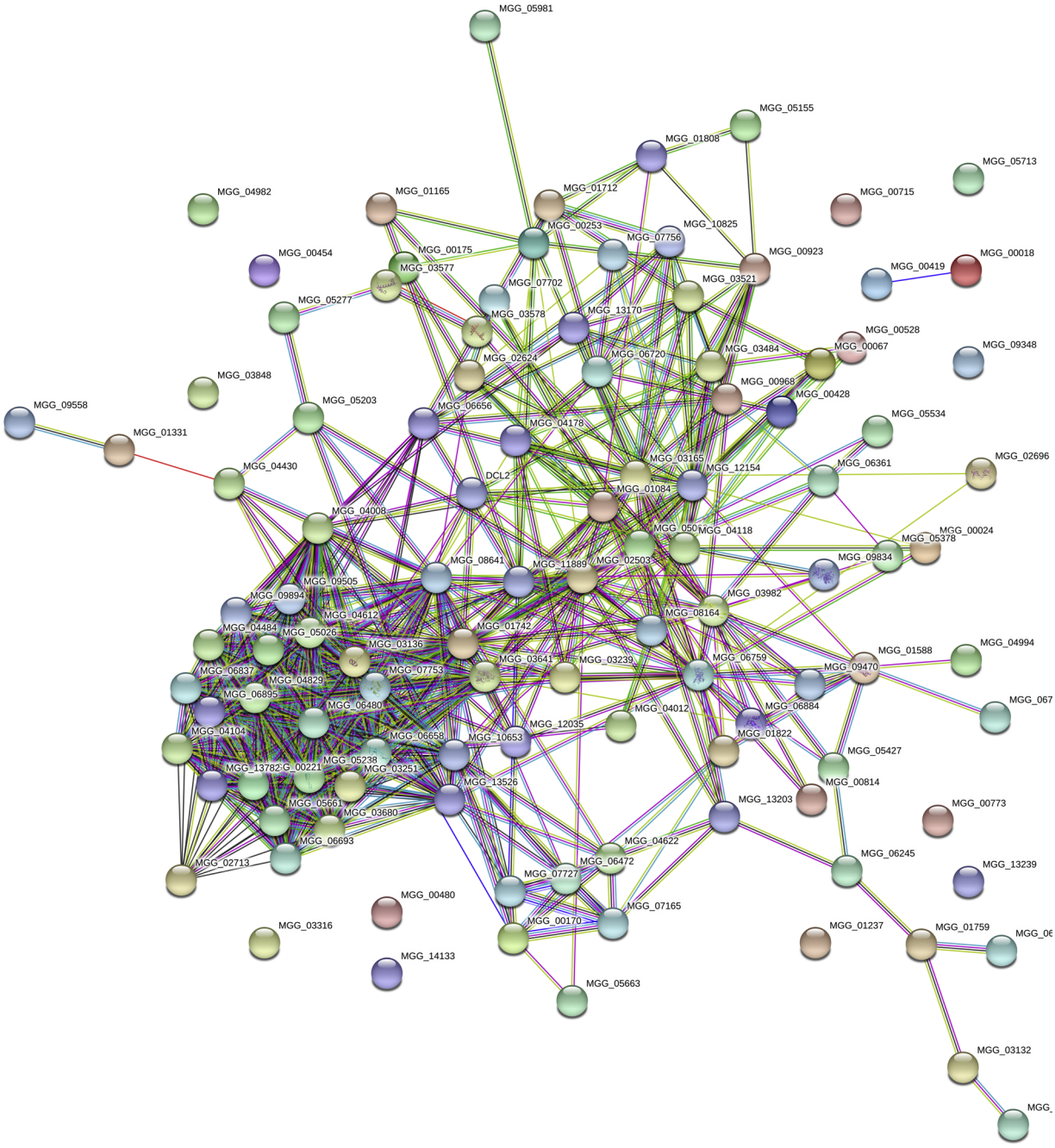

Fig. 2. The figure shows a STRING network visualization using STRING version 10.5, with 111 nodes and 618 edges. The avg. local clustering coefficient = 0.534 and PPI enrichment p-value < 1.0e-16. The nodes represent proteins and the edges represent the predicted functional associations. The colored nodes indicate query proteins and first shell of interactors; white nodes indicate second shell of interactors; empty nodes indicate proteins of unknown 3D structure and filled nodes indicate predicted or proteins with known 3D structure. The associations were inferred from the following evidence: experimental evidence (purple line); curated databases (light blue line); predicted Interactions based on gene neighbourhood (dark green line), gene fusions (red line) and gene co-occurrence (dark blue line); other evidences like text-mining evidence (light green line), co-expression (black line) and protein homology (violet line).

(2009) reported the *Bgh* haustorial proteome with 204 proteins involved in protein metabolic pathways and energy production using LC–MS/MS technique.

Though the *E. pisi* nucleotide sequence is available in public domain as draft sequence (Panstruga and Spanu, 2014), information about its protein profiling is limited. Moreover, proteome-based analysis has been limited in case of organisms which have not been explored much at the genetic level (Bregar et al., 2012). However, the information at the genetic level being very vast, protein-based studies help in faster identification of key proteins involved in pathogenesis and virulence through host-pathogen interactions. Up to 491 candidate secreted effector proteins (CSEPs) involved in pathogenesis were identified in the *Blumeria* genome (Pedersen et al., 2012). However, a previous study to detect effector proteins in *Bgh* showed that out of 248 candidate effectors, as few as ten proteins were reported to be conserved among the three mildew causing fungi analyzed, suggesting that the effectors might be conferring species-specific adaptations (Spanu et al., 2010).

The proteome of *E. pisi* isolates showed a high proportion of proteins pertaining to protein-machinery. The detection of heat shock proteins (HSP) which form a vital component of cell regulatory machinery indicate their significant role in survival and spread of the biotrophic *Erysiphe* pathogen. The Hsp's are involved in a variety of important

Table 3
The functional enrichments in the STRING retrieved network are categorized on the basis of Biological Process (GO), Molecular Function (GO), Cellular Component (GO), KEGG Pathways, PFAM Protein Domains as well as INTERPRO Protein Domains and Features.

S. no	pathway ID	pathway description	count in gene set	false discovery rate
Biological Process (GO)				
1	GO:0044237	cellular metabolic process	11	0.00105
2	GO:0007275	multicellular organismal development	5	0.00211
3	GO:0010468	regulation of gene expression	5	0.00211
4	GO:0034641	cellular nitrogen compound metabolic process	8	0.00211
5	GO:0043170	macromolecule metabolic process	9	0.00211
6	GO:0043581	mycelium development	5	0.00211
7	GO:0044238	primary metabolic process	10	0.00211
8	GO:0071704	organic substance metabolic process	10	0.00211
9	GO:0010467	gene expression	7	0.00242
10	GO:0050789	regulation of biological process	6	0.00242
11	GO:0051252	regulation of RNA metabolic process	4	0.00242
12	GO:0051171	regulation of nitrogen compound metabolic process	5	0.00253
13	GO:0044260	cellular macromolecule metabolic process	8	0.00285
14	GO:0044699	single-organism process	8	0.00301
15	GO:0080090	regulation of primary metabolic process	5	0.00373
16	GO:0031323	regulation of cellular metabolic process	5	0.00388
17	GO:0090304	nucleic acid metabolic process	6	0.00388
18	GO:0008150	Biological process	9	0.00832
19	GO:2000112	regulation of cellular macromolecule biosynthetic process	4	0.0127
20	GO:0006417	regulation of translation	3	0.015
21	GO:0016070	RNA metabolic process	5	0.015
22	GO:0006406	mRNA export from nucleus	2	0.029
23	GO:0045787	positive regulation of cell cycle	2	0.029
24	GO:0071427	mRNA-containing ribonucleoprotein complex export from nucleus	2	0.029
25	GO:0071702	organic substance transport	4	0.033
26	GO:0044271	cellular nitrogen compound biosynthetic process	4	0.036
27	GO:0044710	single-organism metabolic process	5	0.036
28	GO:0006950	response to stress	4	0.0393
29	GO:0033036	macromolecule localization	4	0.0414
30	GO:0071166	ribonucleoprotein complex localization	2	0.0469
Molecular Function (GO)				
31	GO:0005488	binding	10	0.000251
32	GO:0003674	Molecular function	11	0.000377
33	GO:0097159	organic cyclic compound binding	8	0.000377
34	GO:1901363	heterocyclic compound binding	8	0.000377
35	GO:0003676	nucleic acid binding	6	0.00396
36	GO:0000166	nucleotide binding	5	0.00519
37	GO:0046872	metal ion binding	5	0.00519
38	GO:0043167	ion binding	6	0.00728
39	GO:0003723	RNA binding	4	0.0297
40	GO:0003824	catalytic activity	6	0.0402
Cellular Component (GO)				
41	GO:0005622	intracellular	10	0.00142
42	GO:0005623	cell	10	0.00142
43	GO:0005634	nucleus	8	0.00142
44	GO:0044424	intracellular part	10	0.00142
45	GO:0005575	Cellular component	10	0.00182
46	GO:0043231	intracellular membrane-bounded organelle	9	0.00182
47	GO:0032991	macromolecular complex	7	0.00183
48	GO:0043234	protein complex	6	0.00255
49	GO:0000786	nucleosome	2	0.00544
50	GO:0044444	cytoplasmic part	7	0.0141
51	GO:0005737	cytoplasm	7	0.0183
52	GO:0005829	cytosol	5	0.0183
53	GO:0030529	ribonucleoprotein complex	4	0.0206
54	GO:0005758	mitochondrial intermembrane space	2	0.0412
55	GO:0043232	Intracellular non-membrane-bounded organelle	4	0.0412
56	GO:0000785	chromatin	2	0.049
KEGG Pathways				
57	3010	Ribosome	20	1.63e-19
58	1200	Carbon metabolism	10	8e-07
59	1100	Metabolic pathways	23	2.28e-06
60	1120	Microbial metabolism in diverse environments	11	1.71e-05
61	1230	Biosynthesis of amino acids	9	1.71e-05
62	3050	Proteasome	5	0.000202

(*continued on next page*)

Table 3 (*continued*)

KEGG Pathways				
63	20	Citrate cycle (TCA cycle)	4	0.00117
64	1110	Biosynthesis of secondary metabolites	10	0.00336
65	61	Fatty acid biosynthesis	2	0.0142
66	1210	2-Oxocarboxylic acid metabolism	3	0.0398
PFAM Protein Domains				
67	PF00227	Proteasome subunit	5	0.000284
68	PF10584	Proteasome subunit A N-terminal signature	3	0.0359
INTERPRO Protein Domains and Features				
69	IPR001353	Proteasome, subunit alpha/beta	5	0.000492
70	IPR029055	Nucleophile aminohydrolases, N-terminal	5	0.011
71	IPR000426	Proteasome alpha-subunit, N-terminal domain	3	0.0312
72	IPR023332	Proteasome A-type subunit	3	0.0312

cellular functions including stress responses, pathogen virulence (Neckers and Tatu, 2008), adhesion in the process of host cell invasion or as signaling molecules (Pizarro-Cerdá and Cossart, 2006). Cyclophilins having Peptidyl-prolyl cis-transisomerases activity have been reported to act as virulence determinant (CYP1) in *Magnaporthe grisea*, the causal agent of rice blast disease (Viaud et al., 2002). Also, the 40S ribosomal protein S19 and telomere and ribosome-associated protein, Stm1 are reported to be 6 and 8-hour developmental stage candidate biomarkers respectively identified in germination of *A. fumigatus* conidia (Suh et al., 2012).

The presence of a high number of metabolic proteins not only indicate a highly active protein machinery but also, a well supportive nuclear machinery represented by proteins involved in nucleoside, nucleotide and nucleic acid metabolism, replication and repair as well as RNA binding and modification. The massive expansion of powdery mildew genomes and the presence of QDE2 proteins which indicate active gene silencing mechanisms may possibly be one of the regulatory mechanisms in powdery mildew genomes (Bindschedler et al., 2009).

The polypeptides involved in carbohydrate and lipid metabolism meet the energy requirements of the fungus. However, Phosphogluconate dehydrogenase with an ability to act as an adhesin and immunogen in mice has demonstrated its role in bacterial pathogenesis in case of *Streptococcus pneumoniae* and *S. suis* (Daniely et al., 2006; Tan et al., 2008). The identified polypeptides involved in cytoskeletal and fungal cell wall organization support the structural and functional organization of the fungus. Though the *C. albicans* CaEcm33 protein is involved in the fungal cell wall organization, its absence shows decreased virulence in *CaEcm33*-deleted mutants (Martinez-Lopez et al., 2006, 2004). The *AfuEcm33* in *A. fumigatus* leads to increase in conidial germination, cell adhesion, virulence and resistance to the antifungal drug, caspofungin (Romano et al., 2006). Similarly, polypeptides related to oxidative phosphorylation, cell redox homeostasis and signal transduction are important for pathogen growth and survival. Filamentous fungi produce a number of secondary metabolites which have been implicated in pathogenesis. The polyketide synthase involved in conidial yellow pigment biosynthesis was identified in Ep03 isolate. Polyketide synthases and modular Non-ribosomal peptide synthetases are reported to be encoded in *Blumeria* while hydrophobins could not be identified in the study (Spanu et al., 2010).

The *E. pisi* genes, Cyclophilin, ABC transporter and G-protein beta, identified from proteomic data were evaluated for expression with Arkel and JI2480 genotypes as susceptible and resistant types through qRT-PCR at 6 h, 12 h, 24 h and 48 h post inoculation. These genes were found to be involved in pathogenesis and virulence of ascomycete fungi, *Ustilago maydis, Blumeria graminis* and *Magnoporthe oryzae*. Higher transcript accumulation of *G-β* gene at 6, 12 and 24 hpi was observed in Arkel indicating its role in regulation of early pathogenesis pathways. G-β role has been reported in *M. grisea* during appressorium formation while in *B. graminis*, it is involved in acquiring susceptibility by invading the host plant (Nishimura et al., 2003; Hoefle et al., 2011). Gene transcript accumulation and elevation of G-protein in chestnut blight fungus, *Cryphonectria parasitica* was found to be regulating cAMP levels, thereby, aiding in signal transduction cascades during pathogenesis (Chen et al., 1996). The observations suggest that *G-β* gene may play a major role in initiation of cAMP-PKA pathway. ABC transporters have also been reported to play an important role in plant-microbe interactions and in secretion of host-specific toxins for protection against plant defense compounds (Andrade et al., 2000; de Waard, 1999). Elevated peak of ABC transcript at early infection time point of infection at 24 h, followed by sudden decrease at later time points in susceptible

Table 4
A few of the putative proteins that might be involved in pathogenicity and virulence of *Erysiphe* spp.

Protein	Predicted Function of the protein in *Erysiphe* sp.	References
Phosphogluconate dehydrogenase	Adhesion of *S. pneumoniae* and *S. suis* to host cells	(Daniely et al., 2006; C. Tan et al., 2008)
Cyclophilin/Peptidyl prolyl cis-transisomerase	Virulence determinant in *M. grisea*	(Viaud et al., 2002)
G-protein β sub-unit	Appressorium formation in *M. grisea* and acquiring susceptibility in *B. graminis*	(Nishimura et al., 2003; Hoefle et al., 2011)
ABC transporters	Secretion of host-specific toxins for protection against plant defense compounds in *Aspergillus nidulans*	(Andrade et al., 2000; de Waard, 1999).
40 S ribosomal Protein, S19 and Telomere and ribosome associated protein, Stm1	Developmental stage candidate biomarkers	(Suh et al., 2012)
Neuronal Calcium Sensor 1 (NCS1)	Calcium tolerance and regulation of sporulation	(Burgoyne, 2007)
QDE2	Gene silencing in *B. graminis* f. sp. *hordei*	(Bindschedler et al., 2009)
Molecular chaperones, BipA	Unfolded protein response and ER stress	(Kohno et al., 1993)
Hsp90	Fungal signaling networks	(Leach et al., 2012)
Ecm33	Conidial germination, antifungal drug resistance and hypervirulence in *A. fumigatus*	(Romano et al., 2006)

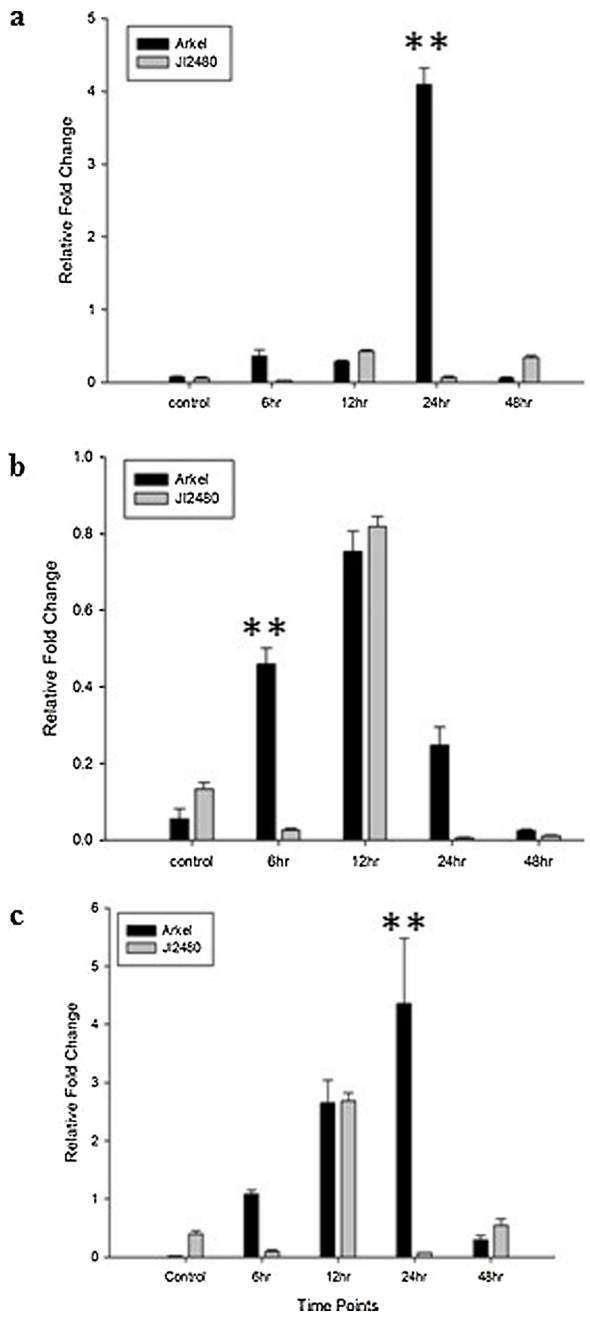

Fig. 3. The figure shows relative gene expression of (a) ABC transporter, (b) Cyclophilin and (c) G-protein beta subunit in Arkel and JI 2480 genotypes as susceptible and resistant 528 genotypes through qRT-PCR at 6 h, 12 h, 24 h and 48 h post inoculation. The qRT-PCR data was normalized by using yeast-Actin gene expression as an internal control. Statistical analysis was carried using student-t test at different time points.

genotype hints at its role against plant defense compounds and declined transcript levels in resistant genotype, JI2480 provide further evidence. *CYP*, found to be down-regulated during infection in both susceptible and resistant genotype, may not be involved actively in pathogenesis by *E. pisi* fungus. It has been reported that *CYP* acts as a virulence determinant during plant infection. *Cyp1* mutants in *Magnoporthe grisea* have been shown to exhibit reduced virulence and impaired associated functions, such as penetration peg formation and appressorium turgor generation (Viaud et al., 2002). *CYP* gene expression in case of *E. pisi* infection may be visible in later time points of infection thereby, actively participating in generating fungal virulence.

Proteomic studies have been carried out in case of other pathogens of garden pea as well. The expressed proteome in hyphae and germinating cysts of the closely related pathogens, *P. pisi* and *P. sojae*, was studied using tandem mass spectrometry. The comparative proteomic analysis showed that fifty-eight orthologous proteins were more abundant in germinated cysts of both pathogens. The pathogens utilize stored lipid compounds for energy generation during cyst germination as observed by the induction of proteins involved in β-oxidation, the carnitine shunt and the glyoxylate cycle. Also, several candidate pathogenicity factor proteins such as serine proteases, a ricin-B lectin protein, a dioxygenase, ABC and MFS transporters were identified in germinating cysts of both pathogens. Twenty-three orthologous proteins, identified as candidate proteins for vegetative growth, were observed to be more abundant in hyphae of both pathogens (Hosseini et al., 2015). The proteome-wide analysis of Pea seed-borne mosaic virus (PSbMV)-infected *Pisum sativum* L. cultivars, resistant (B99) and susceptible (Raman) was carried out through LC–MS profiling. The comparison of PSbMV-resistant and susceptible cultivars showed significant increase in the amino acid biosynthesis, carbohydrate metabolism and redox signaling, particularly in the increased jasmonic acid levels as well as lipoxygenase levels and activity (Cerna et al., 2017). Proteomic studies in case of pea-*Fusarium oxysporum* f. sp. *pisi* (Fop) interactions showed a differential expression of proteins involved in carbohydrate and energy metabolism, signal transduction and cellular process, redox and homeostasis, nucleotides and amino acid metabolism, folding and degradation, defense, biosynthetic process and transcription/translation. The most susceptible genotypes showed increased levels of enzymes involved in the pentose shunt in addition to ROS burst and an increase in PR proteins, in contrast to the resistant genotype which showed proteins involved in alterations in the membrane and cell wall composition (Castillejo et al., 2015). Responses to *Mycosphaerella pinodes* in susceptible and partially resistant pea cultivars showed differential expression of proteins involved in photosynthesis, metabolism, transcription/translation and defense and stress-related proteins (Castillejo et al., 2010). Proteomic analysis in case of *Peronospora viciae* infection of a susceptible cultivar of pea (*Pisum sativum* cv. Livioletta) revealed significant increase in the abundance of the pathogen-induced PI176 protein, the ABR17 stress-response protein, a glycine-rich RNA binding protein, three photosynthetic proteins as well as cytosolic and chloroplastic glyceraldehyde 3-phosphate dehydrogenases in the infected leaves (Amey et al., 2008).

A study of *E. pisi* proteome is vital to understand not only the obligate biotrophic pathogen but also for the host because it is an outcome of an interaction between the host and the pathogen system which enables the survival of the latter. The proteome identified in this study clearly indicates a highly active, self-sufficient system, supported by a protein and nucleic acid machinery. This indicates that the pathogen has been able to conserve the transcriptional and translational mechanisms irrespective of the genome expansions and the consequent gene losses. The identification of proteins involved in metabolism of lipids, carbohydrates, amino acids, nucleosides and nucleotides as well as the essential processes of growth, signal transduction and redox regulatory pathways shows that though the pathogen is a biotroph, it has the means to carry out metabolic processes to assimilate the nutrients it derives from the host system and survive. The proteome also suggests that proteins need to be critically studied for their role in virulence and pathogenicity due to their multi-functional nature. Also, the unclassified proteins and a large number of hypothetical/predicted proteins may serve as a repertoire for further gene function studies. The gene expression observed in case of Cyclophilin, ABC transporter and G-protein beta also indicates the importance of these genes in the early stages of pathogenesis. Our study suggests that the powdery mildews have a limited and a self-sufficient proteome that has enabled the pathogen to survive despite its biotrophic mode of nutrition.

5. Conclusion

E. pisi proteome analyzed in the study reflects an outcome of host-pathogeninteraction at molecular level. Since *E. pisi is*a biotroph and a well-adapted pathogen, it is intriguing to understand the regulatory processes that enable it to survive and overcome the host surveillance mechanisms and proliferate. The identified proteins need to be critically studied for their role in virulence and pathogenicity due to their multi-functional nature. Also, the unclassified proteins and a large number of hypothetical/predicted proteins may serve as a repertoire for further gene function studies. The adapted host-pathogen system comprising of *E. pisi* and pea and their interactions could be utilized to gather an insight into the mechanisms of pathogenesis in biotrophs.

Direct link to deposited data and information to users

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010238 and 10.6019/PXD010238.

Declaration of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.02.006.

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