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Proteomic analysis of a compatible interaction between *Pisum sativum* (pea) and the downy mildew pathogen *Peronospora viciae*

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Abstract A proteomic approach was used to identify host proteins altering in abundance during *Peronospora viciae* infection of a susceptible cultivar of pea (*Pisum sativum* cv. Livioletta). Proteins were extracted from fully developed pea leaflets at 4 days post-inoculation, before visible symptoms were apparent. Cytoplasmic proteins and membrane- and nucleic acid-associated proteins from infected and control leaves were examined using two-dimensional difference gel electrophoresis. The majority of proteins had a similar abundance in control and infected leaves; however, several proteins were altered in abundance and twelve were found to have increased significantly in the latter. These proteins were selected for either matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry or electrospray ionisation quadrupole time-of-flight tandem mass spectrometry analysis following trypsin digestion, with sequence identity being assigned to eight of the proteins. These included the ABR17 stress-response protein, the pathogen-induced PI176 protein, three photosynthetic proteins, a glycine-rich RNA binding protein and two glyceraldehyde 3-phosphate dehydrogenases (cytosolic and chloroplastic) which can be induced by a range of abiotic and biotic stresses in many plant species. The possible roles of these proteins in the response of the pea plant during *P. viciae* infection are discussed. This study represents the first proteomic analysis of downy mildew infection of pea leaves, and provides the basis for further work to elucidate molecular mechanisms of compatibility in *P. viciae* infections.

Keywords Electrophoresis · DIGE · MALDI-TOF · Mass spectrometry · Oomycete · Protein

Abbreviations

2-D DIGE	two-dimensional difference gel electrophoresis
dpi	days post-inoculation
ESI Q-TOF MS/MS	electro-spray ionisation quadrupole time-of-flight tandem mass spectrometry
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
MALDI-TOF MS	matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry

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Introduction

Downy mildew is the most common foliar disease of the pea crop (*Pisum sativum*) in the UK, with up to 55% losses in yield observed where plant resistance is

ineffective (Clark and Spencer-Phillips 2000). Downy mildew is also a significant problem in other parts of the world where peas are grown (Amey and Spencer-Phillips 2006).

Production of conidia by *Peronospora viciae* results in a substantial loss of photosynthate from the host to the pathogen, contributing to symptoms such as stunted growth, distortion and early death of the infected plant (Mence and Pegg 1971). This re-direction of photosynthates and the other effects are likely to be accompanied by changes in the abundance of certain host proteins. Proteomics not only has the potential to identify these proteins, but also to provide quantitative data which signify their relative importance to the process.

Proteomic technologies such as 2D-DIGE (Ünlü et al. 1997), mass spectrometry and bioinformatics are an effective and accurate way of identifying and measuring protein differences between cell types (Beranova-Giorgianni 2003). Typically, the proteome of a control cell type or tissue is compared to a treated or diseased cell type or tissue. Protein differences observed between the two samples are investigated further to identify protein function and origin. 2D-DIGE and mass spectrometry are being used increasingly to identify proteins that increase or decrease in abundance during plant-microbe interactions (Corbett et al. 2005; Coulthurst et al. 2006). At present, little is known about mechanisms of pathogenesis in *P. viciae* infections of pea, with very few host and pathogen factors explained at a biochemical and molecular level (Clark and Spencer-Phillips 2004). Proteomics provides a global approach to explore changes in abundance of specific components of the pathosystem proteome, and hence to identify specific proteins and processes likely to be central to the outcome of infection.

To date, few proteomic studies of oomycete pathogens of plants have been performed. Most have focused on *Phytophthora* species such as *P. infestans*, a devastating pathogen of solanaceous plants (Ebstrup et al. 2005; Grenville-Briggs et al. 2005), *P. nicotianae* which has a wide host range (Mitchell et al. 2002) and *P. palmivora*, a serious pathogen of tropical crops including cocoa (Shepherd et al. 2003). The latter study examined the proteome of the asexual spores at various stages of development and germination, and identified a number of proteins that may be specific to different phases of the asexual life-cycle. The study by Mitchell et al. (2002) examined proteins from zoospores and cysts, whereas Grenville-Briggs et al. (2005) examined the proteomes of mycelium, zoospores and germinating cysts with appressoria, and Ebstrup et al. (2005) compared cysts, germinated cysts and appressoria. This work, however, only provided information on pathogen proteins during pre-invasion stages of infection. In contrast, Colditz et al. (2004) examined the proteome of the roots of the legume *Medicago truncatula* during infection by the oomycete *Aphanomyces euteiches*, with the majority of induced proteins belonging to the pathogenesis-related (PR)-10 group of pathogenesis-related (PR) proteins.

Proteomics has been applied previously to the study of pea proteins, including two host genotypes inoculated with the powdery mildew pathogen *Erysiphe pisi* (Curto et al. 2006). These authors compared the proteomes of *E. pisi*-infected and non-inoculated control leaves, and identified seven and 16 proteins with increased abundance following infection in resistant and susceptible interactions, respectively. The proteins functioned in photosynthesis, carbon metabolism, energy production, stress and defence, protein synthesis, and degradation, and signal transduction. Other published data relate to abiotic stresses such as salinity (Kav et al. 2004), biotic stresses such as infection by the parasitic plant *Orobanche crenata* (Castillejo et al. 2004) or a combination of interactions such as in *Glomus mosseae*-inoculated pea roots treated with calcium (Repetto et al. 2003). Numerous studies have examined the proteomes of other legumes. These include the symbiotic relationships of *Medicago* with *G. mosseae* (Bestel-Corre et al. 2002) and *Sinorhizobium meliloti* (Djordjevic et al. 2003), and soybean infected by the bacterium *Bradyrhizobium japonicum* to gain further information about the processes involved in nodulation (Wan et al. 2005).

The aim of the present work was to generate fundamental information on the most abundant proteins specifically involved in a compatible *P. viciae*–pea interaction, and to compare this with data on proteins in compatible and resistant interactions between pea and *E. pisi* (Curto et al. 2006).

Materials and methods

Inoculation of pea with *P. viciae*

P. viciae isolate Nitouche (kindly provided by Dr David Kenyon, National Institute of Agricultural

Botany, Cambridge, UK) was maintained on a mixture of seven pea cultivars (*P. sativum* cvs Livioletta, Kelvedon Wonder, Maro, Krupp Pelushka, Early Onward, Solara and Progreta). For protein extractions, plants of *P. sativum* cv. Livioletta were cultivated from seed in compost (Levington F2S) in growth chambers (Sanyo; 16 h light at 20°C, 8 h dark at 14°C). At 10 days after sowing, the surfaces of fully developed leaflets were rubbed gently to flatten waxes before being inoculated with *P. viciae* conidia by the method of El-Gariani and Spencer-Phillips (2004). Control plants were inoculated with sterile distilled water only.

Protein extraction and preparation

Proteins were extracted from fully developed leaflets of healthy pea plants and from *P. viciae*-infected plants at 4 dpi according to the method of Giavalisco et al. (2003). Their method is claimed to result in three fractions, comprising enriched preparations of (I) cytosolic proteins, (II) membrane-bound proteins and (III) nucleic acid-associated proteins. Protein fractions II and III were pooled for analysis in the present investigation. In brief, leaves were ground in liquid nitrogen before addition of 0.125 (*v*/*w*) inhibitor mixture 1 (100 mM KCl; 20% *v*/*v* glycerol; 50 mM Tris, pH 7.1), including Complete Protease Inhibitor Cocktail Tablet (Roche, Germany) used according to the manufacturer's instructions, and 0.05 (*w*/*w*) of inhibitor mixture 2 (1 mM Pepstatin A, 1.4 mM PMSF). Samples were centrifuged for 60 min at 22,000 g at 4°C. The supernatant containing the soluble cytosolic protein (fraction I) was removed and stored at −80°C. The pellet was ground further in liquid nitrogen before addition of 0.125 (*v*/*w*) of inhibitor mixture 3 (200 mM KCl; 20% *v*/*v* glycerol; 100 mM phosphate buffer, pH 7.1; Complete Protease Inhibitor Cocktail, as before), one volume of buffer A (100 mM phosphate buffer, pH 7.1; 200 mM KCl; 20% *v*/*v* glycerol; 2 mM MgSO4; 4% *w*/*v* CHAPS) and 2% (*w*/*w*) ASB14 detergent (Calbiochem, UK). Samples were homogenised thoroughly before the addition of 0.025% (*v*/*w*) DNase and the resulting mix stirred at 4°C for 45 min. Subsequently, 23% *v*/*w* buffer B (700 mM 1,4-dithiothreitol (DTT), 7 M urea, 2 M thiourea) was added and the homogenate was stirred at room temperature for 45 min. The homogenate was then centrifuged for 60 min at 22,000 g at 17°C and the resulting supernatant containing membrane and nucleic acid-associated proteins (fractions II and III) was removed and stored at −80°C.

The proteins were prepared for 2-D DIGE using the 2D Clean-up kit (Amersham Biosciences), re-suspended in lysis buffer (30°mM Tris pH 8.5; 4% *w*/*v* 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulphonate) (CHAPS); 7 M urea; 2 M thiourea) and quantified using the 2D Quant kit (Amersham Biosciences) according to the manufacturer's instructions. Protein samples were stored at −80°C until further analysis.

CyDye labelling

Samples were labelled using fluorescent cyanine dyes (Amersham Biosciences) according to the manufacturer's protocols. The cyanine dyes were reconstituted in fresh 99.8% anhydrous dimethyl formamide. Aliquots of 50 μg of protein were labelled with 400 pmol of amine reactive CyDye for 30 min on ice in the dark, then 1 μl of 10 μM lysine was added to the tube and incubated on ice in the dark to halt the reaction. The samples were made up to 100 μl with rehydration solution (8 M urea; 2% *w*/*v* CHAPS; 0.002% *w*/*v* bromophenol blue; 0.2% *w*/*v* DTT; 2% *w*/*v* immobilised pH gradient (IPG) buffer (pH 3–10, Amersham Biosciences)).

2-D DIGE

Samples were subjected to isoelectric focusing (IEF) using IPG strips (24 cm, Amersham Biosciences) in the pH 3–10 non-linear range, with rehydration loading to separate proteins in the first dimension according to isoelectric point. The IPG strips were rehydrated overnight at room temperature in the protein sample made up to 450 μl with rehydration solution and covered with mineral oil. The strips were transferred to an Ettan IPGphor II (Amersham Biosciences) and IEF was performed with a 50 μA limit/IPG strip. IEF voltage conditions were 300 V step and hold for 3 h, 1,000 V gradient for 6 h, 8,000 V gradient for 3 h and 8,000 V step for 4 h 40 min.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was used to separate proteins in the second dimension according to molecular weight. Following focusing in the first dimension, each strip

was removed from the IEF unit and equilibrated in 15 ml equilibration buffer (50 mM Tris pH 8.8; 6 M urea; 30% *v/v* glycerol; 2% *w/v* SDS; 0.002% *w/v* bromophenol blue) amended with 150 mg DTT with gentle shaking for 15 min at room temperature. The strips were equilibrated further in 15 ml equilibration buffer amended with 375 mg iodoacetamide with gentle shaking for 15 min at room temperature in the dark. Finally, the strips were equilibrated in 10 ml equilibration buffer alone for 5 min at room temperature. The strips were loaded onto a 12.5% acrylamide gel (dimensions 24 cm×20 cm×1 mm) and overlaid with 1% agarose in SDS running buffer (25 mM Tris pH 8.3; 192 mM glycine; 0.1% SDS) amended with 0.002% (*w/v*) bromophenol blue. The gels were electrophoresed in SDS buffer at 2.5 W per gel for 30 min, followed by 100 W until the bromophenol blue dye front had run off the bottom of the gels. A minimum of three biological samples was used in these experiments, with two replicate gels produced for fraction I and the combined fractions II plus III of the extracted proteins from each sample. A minimum of three additional, non-CyDye-labelled gels were run for each sample for protein spot picking.

Image analysis

Gels were scanned on a Typhoon 9400 imager (Amersham Biosciences) to visualise CyDye-labelled proteins. Cy3 scans were obtained using a 532 nm laser and emission filter of 580 nm BP30. Cy5 scans were obtained using a 633 nm laser and a 670 nm BP30 emission filter. Scans were performed at 100 μm resolution with the photomultiplier tube voltage set for a maximum pixel intensity of 60 to 80,000 pixels. All images were cropped using ImageQuant V5.2 software prior to analysis to remove areas outside the gel. Analysis of each of the gels was performed with DeCyder Differential In-gel Analysis module software (V5.0; Amersham Biosciences) using the double detection setting and an estimated protein spot number of 2,500. Parameters for an exclusion filter were determined and applied according to the manufacturer's instructions, with resulting spots confirmed individually by visual inspection. Protein spots altering in abundance by at least two-fold consistently on all gels, and one protein that remained unchanged, were selected for analysis.

MALDI-TOF MS and ESI Q-TOF MS/MS analyses

Protein spots were excised from the gels using an Ettan Spot Picker (Amersham Biosciences) and subsequently digested using an Ettan Digester (Amersham Biosciences) with 10 μl trypsin (20 ng μl^{-1}; Promega Sequencing Grade Porcine Modified) in 20 mM ammonium bicarbonate (Sigma) overnight at room temperature. Following tryptic digestion, the peptides were extracted in 50% acetonitrile/0.1% trifluoroacetic acid to a clean microtitre plate and transferred to an Ettan Spotter (Amersham Biosciences). The peptides were mixed with matrix (10 mg ml^{-1} α-cyano-4-hydroxycinnamic acid in 50:50 *v/v* methanol/acetonitrile) for spotting onto Micromass target plates for analysis in a MALDI-TOF mass spectrometer (Waters-Micromass, UK). Peptide mixtures were analysed using a nitrogen UV laser (337 nm). MS data were acquired in the MALDI reflector positive ion mode in the mass range 800–3,500 Da. Identification of proteins from the mass fingerprints generated was performed using Proteinlynx Global Server software (V2.0.5, Waters-Micromass, UK) for searching against the SwissProt and National Centre for Biotechnology Information (NCBI, Bethesda, USA) databases. Search parameters included a peptide mass tolerance of 100 ppm, estimated calibration error of +0.025 Da, one missed cleavage per peptide, fixed carbamidomethylation of cysteine, and variable oxidation of methionine.

Nanoelectrospray ionization tandem mass spectra were acquired using a Q-TOF Micro mass spectrometer (Waters-Micromass, UK) coupled to a LC Packings capillary liquid chromatography system. Aliquots (15 μl) of peptide solutions prepared as before were injected using an auxiliary solvent flow of 30 μl min^{-1} and desalted on a C_{18} PepMap Nano-Precolumn (5× 0.3 mm internal diam (i.d.), 5 μm particle size; Dionex, Amsterdam, The Netherlands) for 4 min. Peptides were eluted and separated using a C_{18} PepMap100 nano column (15 cm×75 μm i.d., 3 μm particle size) with a gradient flow of 200 nl min^{-1} and solvent system of: auxiliary solvent, 0.1% HCOOH; solvent A, 5% *v/v* CH_3CN/95% *v/v* 0.1% *v/v* aqueous HCOOH; solvent B, 80% *v/v* CH_3CN/20% *v/v* 0.1% *v/v* aqueous HCOOH. The solvent gradient was 4 min at 5% aqueous solvent B, 5% to 55% B over 40 min, 55% to 80% B over 1 min, maintained at 80% B for 5 min, then reduced to 5% B in 0.1 min and the column

washed with solvent A for 9.9 min before the next sample injection. The column was connected to the nanosprayer of the Z-spray ion source using a short length of 75 μm i.d. capillary. Voltages used were 3,500 V for the capillary, 45 V for the sample cone and 2.5 V for the extraction cone. MS spectra were acquired throughout the chromatographic run, while MS/MS spectra were acquired in data-dependent mode on the most abundant ions having charge states of 2+, 3+ and 4+ between *m/z* 400–2,000. The collision cell was pressurised with 1.38 bar ultra-pure argon (99.999%, BOC) and collision voltages depended on the m/z and charge states of the parent ions. The mass spectrometer was calibrated daily using MS/MS fragment ions from [Glu^1]-fibrinopeptide B (Sigma). Processed data were submitted to ProteinLynx Global Server (V2.0.5) and also to MASCOT (Matrix Science) for searching against SwissProt and NCBI databases. Search criteria were: peptide tolerance of 100 ppm; fragment tolerance of 0.1 Da; two missed cleavages per peptide; fixed carbamidomethylation of cysteine and variable oxidation of methionine modifications.

Results

The proteins from pea plants inoculated with *P. viciae* and sterile distilled water (SDW) controls were visualised using 2D-DIGE, with each gel comprising two experimental samples labelled with Cy3 (control) and Cy5 (*P. viciae*-infected). DeCyder software detected between 977 and 1337 protein spots on two representative gel images (Table 1). The total number of spots detected on the gels varied by 19.7% between the two replicates of fraction I (enriched cytosolic soluble proteins), and by 26% between the replicates of combined fractions II plus III (enriched membrane-associated and nucleic acid-associated proteins). The proportion of proteins with decreased abundance following *P. viciae* infection was 1.7% and 3.5% for the replicates of fraction I, and 0.38% and 0.92% for the replicates of fractions II plus III. The proportion of proteins with increased abundance was 5.2% and 7.2% for fraction I, and 2.3% and 4.5% for fraction II plus III. Therefore the proteins for MALDI-TOF MS and ESI Q-TOF MS/MS were selected on the basis that their abundance altered significantly and reproducibly on all gel replicates of the different biological samples (two CyDye-labelled replicates plus a minimum of three additional unlabelled replicate gels) thus removing potential biological and gel artefacts from the analysis. This resulted in 12 proteins that increased in abundance during *P. viciae* infection as indicated in Fig. 1, a representative gel from this experiment. In contrast, no proteins decreased in abundance significantly (greater than two-fold) and consistently following infection. An additional protein (spot number 6) is indicated on the gel as an example of a protein that does not alter in abundance upon infection by *P. viciae*. The relative fold abundance of protein spots from infected versus SDW-inoculated leaves determined by DeCyder software, and selected individual spot images, are illustrated in Figs. 2 and 3. The molecular weight, pI, matched peptides, sequence coverage and score (either Proteinlynx Global Server or MASCOT) of each protein is indicated in Table 2.

Table 1 The relative abundance of proteins detected in 2D gels for fractions I (cytosolic proteins) and fractions II + III (enriched in membrane and nucleic acid-associated proteins) from pea leaves 4 dpi with conidia of *P. viciae*, compared to SDW, in two separate experiments (a and b)

Fraction	Relative abundance of proteins (no. of spots detected)			
	Decreased	Similar	Increased	Total
Ia	22	1,245	70	1,337
Ib	37	960	77	1,074
II + IIIa	5	1,285	30	1,320
II + IIIb	9	924	44	977

Protein 1 (Figs. 1 and 2) was identified as the disease resistance response protein PI176 from pea (accession number P13239). Compared to control plants, its abundance increased by 3.4 and 6.6-fold in fractions I and fractions II plus III respectively. The protein has similar predicted and observed molecular weights and iso-electric points. The three peptides matching published sequences of PI176 represented 16.4% of its amino acid sequence. Protein 2 was identified as abscisic acid responsive protein ABR17 from pea (accession number Q06931). The abundance of this protein resembled that of PI176, with increases of 2.9 and 5.8-fold for fractions I and II plus III respectively. Theoretical and observed values for molecular weight and pI are in accord and the seven matched peptides provided 51% coverage of the amino acid sequence. Protein 3 was matched following de novo sequencing by ESI Q-TOF MS/MS to a glycine-rich RNA binding protein from *Sinapis alba*

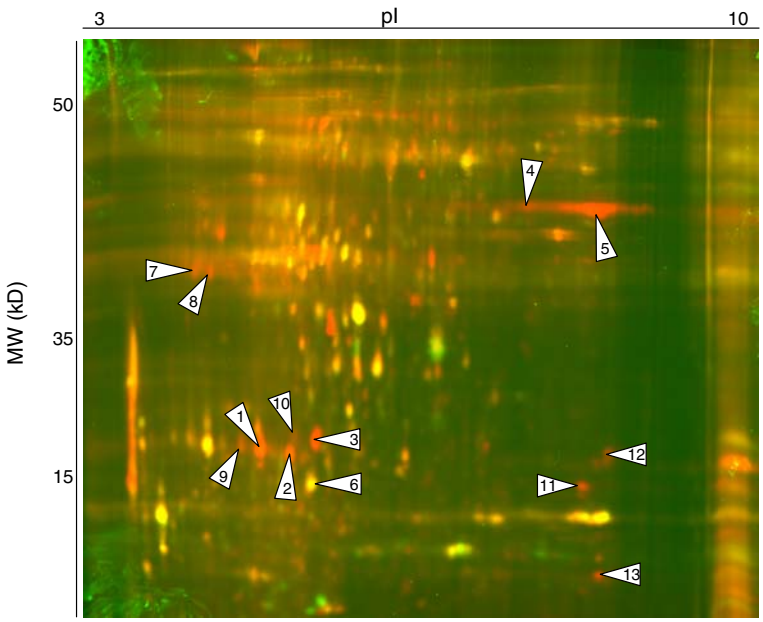

Fig. 1 A typical 2D-DIGE gel obtained from analysis of the pea leaf proteome during early stage infection (4 dpi) by *P. viciae*, compared to proteins from control leaves treated with SDW, for cytosolic fraction I. *Spot colour* indicates the effect of *P. viciae* on protein abundance: *red* = increased; *green* = decreased; *yellow* = no change. Proteins that increased in abundance reproducibly on all gels are indicated by the numbers *1–13*, except for *spot 6* which represents a protein showing no change in abundance during infection by *P. viciae* (see Fig. 2)

(accession number P49311) and, compared to controls, had increased abundances of 6.4 and 6.5-fold for fraction I and II plus III respectively. The theoretical and observed molecular weight and pI values agreed, and the single peptide covered 4.7% of the amino acid sequence. Protein 4 matched to cytosolic GAPDH from pea (accession number P34922). In comparison to control plants, the abundance of the protein increased by 6.1-fold in fraction I and by 6.3-fold in fractions II plus III. Predicted and observed molecular weight and pI values were similar, with the nine matched peptides covering 34.6% of the amino acid sequence. Protein 5 was identified as a chloroplastic precursor of GAPDH A from pea (accession number P12858). Compared to control samples, the protein increased in abundance by 7.7-fold for fraction I and 8.6-fold for fractions II plus III. Whilst 15 peptides were matched, covering 44.7% of the amino acid sequence, and the predicted and observed pI values were in accord, the observed molecular weight was significantly less than predicted. This suggests that processing of the precursor

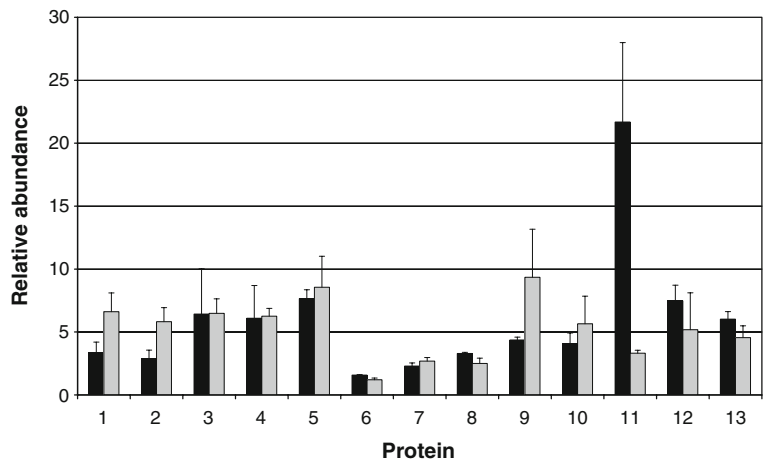

Fig. 2 Relative abundance of proteins *1–13* that increase in pea leaves at 4 dpi after inoculation with *P. viciae*, compared to proteins from control leaves treated with SDW. Protein abundances for fraction I (*black bar*, cytosolic proteins) and fractions II plus III (*gray bar*, enriched in membrane and nucleic acid-associated proteins) were calculated using DeCyder Software (Amersham Biosciences) from two replicate experiments. Standard errors are indicated

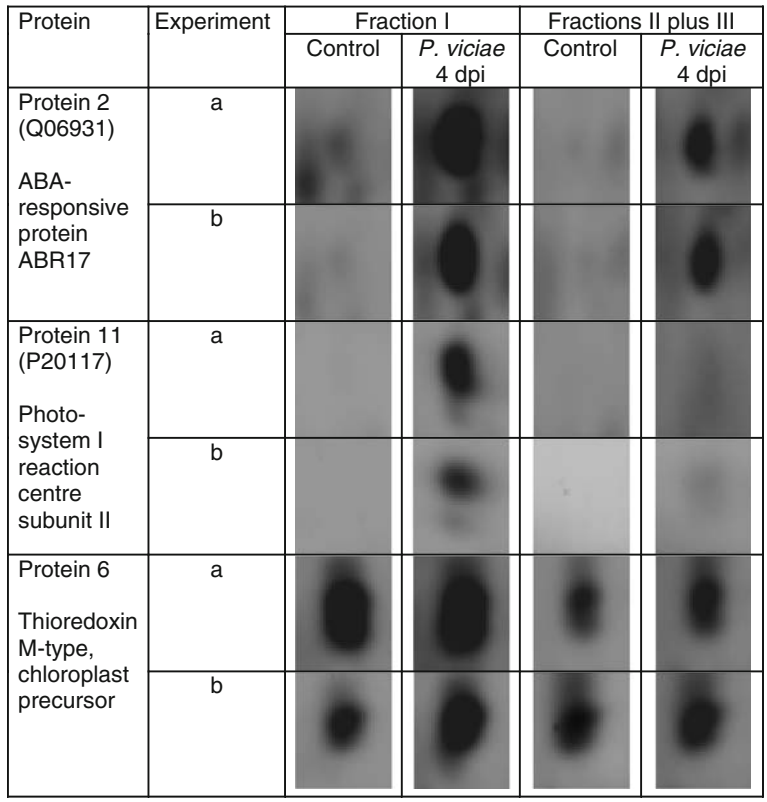

Fig. 3 Images of representative protein spots on 2D gels, from two replicate experiments (*a* and *b*). *Protein 2* (*ABR17*) increases in abundance during *P. viciae* infection of pea leaves in fractions I and fractions II plus III; *protein 11* (*photosystem I reaction centre subunit II*) increases to a greater extent in fraction I than fractions II plus III; *protein 6* (tentatively identified as *thioredoxin M-type, chloroplast precursor*) does not differ in abundance between control (SDW) and infected pea leaves

Table 2 Proteins identified using MALDI-TOF MS and ESI Q-TOF MS/MS that differ in abundance in response to infection by *P. viciae* at 4 dpi

Spot number	Matching protein	Protein accession no.	Observed/predicted mW (kDa)	Observed/predicted pI	Matched peptides	Sequence coverage	Score
1	PI176	P13239	17.0/16.9	5.1/5.1	3	16.4	11.2[b]
2	ABR17	Q06931	16.9/16.6	5.2/5.1	7	51.0	9.1[b]
3	Glycine-rich RNA binding protein	P49311	17.0/16.4	5.5/5.5	1[a]	4.7[a]	NA
4	Cytosolic GAPDH	P34922	36.0/36.6	7.0/7.0	9	34.6	11.6[b]
5	Chloroplastic GAPDH	P12858	36.0/43.3	8.5/9.0	15	44.7	11.9[b]
6	Thioredoxin M-type precursor	P48384	14.0/12.5	5.4/5.4	1	5.2	11.2[b]
11	Photosystem I reaction centre subunit II	Q9S7H1	14.0/23.1	8.0/9.8	3	10.1	121[c]
12	ATP synthase epsilon chain	P05039	15.0/15.2	8.6/6.6	3	15.3	99[c]
13	Photosystem I iron sulphur centre	P10793	9.0/9.2	8.5/7.5	3	17.3	89[c]

All proteins increased in abundance by more than two-fold (see Fig. 2), except protein 6 which was essentially unchanged between treatments. Spot numbers relate to Fig. 1; all accession species were *P. sativum*, except *S. alba* for spot 3 and *A. thaliana* for spot 11

NA Not applicable

[a] Protein matched by de novo sequencing using ESI Q-TOF MS/MS

[b] Proteinlynx Global Server

[c] MASCOT

may have occurred. Protein 6 was identified tentatively (only one matched peptide) as a thioredoxin M-type chloroplast precursor from pea (accession number P48384) and was selected as a protein that differed by less than two-fold compared to control plants. Indeed, in some gels (e.g. Fig. 1), its abundance appeared unchanged following *P. viciae* infection.

Protein 11 had the highest peptide match to the photosystem I reaction centre subunit II precursor from *Arabidopsis thaliana* (accession number Q9S7H1), and also matched to a partial sequence obtained for the same protein in pea (accession number P20117). The predicted molecular weight and pI values were different to those observed, both being larger than the values observed on the gel, and suggesting that spot 11 represents a fragment of this protein. The greatest increase in abundance was observed for this protein (Fig. 2), which increased by 21.7-fold in fraction I compared to an increase of 3.3 for fraction II plus III. Three peptides from protein 12 matched to an adenosine triphosphate (ATP) synthase epsilon chain from pea (accession number P05039), covering 15.3% of the amino acid sequence. The molecular weight for the protein observed on the gel matched the predicted, but the pI differed. Protein 13 matched to the photosystem I iron sulphur centre from pea (accession number P10793). Three peptides were matched, covering 17.3% of the amino acid sequence, with predicted pI and molecular weight values matching those observed.

The abundances of the unidentified proteins 7 and 10 (Figs. 1 and 2) increased similarly in both fractions. The abundance of protein 8 was greater in the soluble fraction I than the membrane and nucleic acid-associated proteins of fraction II plus III, having a 3.3 and 2.5-fold increase in abundance compared to control samples, respectively. In fraction II plus III, protein 9 increased in abundance by more than twice that observed in fraction I (9.3 and 4.3-fold respectively). Thus differences between the relative abundance of proteins isolated from the two fractions were apparent for four of the 13 proteins (Fig. 2).

Discussion

Eight proteins whose abundance was observed to have increased consistently by at least two-fold in 4 day-old *P. viciae* infections of pea were identified by either MALDI-TOF MS or ESI Q-TOF MS/MS. A further four proteins were observed to increase in abundance consistently during *P. viciae* infection, yet could not be identified using MS. The possible roles and functions of the identified proteins during the response to infection by biotrophic pathogens are discussed. It is notable, however, that none of these proteins were reported to increase in abundance following *E. pisi* infection (Curto et al. 2006), which suggests that they reflect a specific response to *P. viciae* in this compatible interaction. This study differs from previous proteomic investigations of oomycetes in that it examines the proteome of the plant-pathogen interaction in leaves.

It is believed widely that the majority of plant defence mechanisms, such as basal resistance and the hypersensitive response, are induced early during infection via a complex network of signals that is initiated following perception of the pathogen by host cells (Dangl and Jones 2001; Kamoun et al. 1999b; McDowell and Dangl 2000). The identification of proteins with potential and proven roles in plant defence in plants harvested at 4 dpi would correlate with these concepts. Proteins were harvested at this stage as well-developed colonies are present, even though external symptoms are not apparent (El-Gariani and Spencer-Phillips 2004). Indeed, conidiophore initials are first observed at 4.5 dpi in this host-pathogen system (Clark and Spencer-Phillips 2004), with sporulation visible macroscopically by 7 dpi. Further proteomic analyses of these later stages of infection are underway to identify additional proteins involved in the *P. viciae*-pea interaction.

The difference in numbers of proteins identified as increased or decreased in abundance between the two fractions implies that a slightly larger number of cytosolic proteins increase in abundance than in a fraction enriched in membrane plus nuclear-associated proteins (6.1% and 3.2%, respectively). However, it should be noted that the same proteins were identified in both fraction types, indicating that the method of extraction may not be effective at specifically selecting cytosolic, membrane-associated and nucleic acid-associated proteins for pea samples. Whilst it may be more appropriate to pool all fractions in further studies to facilitate comparison of proteomes, some proteins (e.g. protein 11) showed significant differential increases between the fractions.

Of the 12 proteins observed to have abundances significantly and consistently increased during the *P. viciae* interaction with pea, eight were matched to pea or other plant proteins in the SWISS-PROT database. The observed and theoretical isoelectric points and molecular weights of the proteins were mostly in accord, except for proteins 5 and 11 where the predicted molecular weight values were much greater than those observed. For protein 11, this may reflect the match to the *A. thaliana* protein rather than the pea protein which has not been sequenced fully. Additionally, this protein was identified as photosystem I reaction centre subunit II (Table 2) and therefore should be membrane bound, but it was mostly present in fraction I which Giavalisco et al. (2003) suggests should contain soluble proteins. Together with the discrepancy in the predicted and observed molecular weights, this suggests that a protein fragment has been identified. The match of protein 5 to a precursor of chloroplastic glyceraldehyde 3-phosphate dehydrogenase (GAPDH) may indicate that the processed protein was present here.

Even in transcriptional profiling studies, it is often the case that not every gene that alters in expression during a specific interaction can be identified from databases, as shown in the yeast *Saccharomyces cerevisiae* (Gygi et al. 1999) and in *M. truncatula* roots following infection by the oomycete *A. euteiches* (Colditz et al. 2004; Nyamsuren et al. 2003). Indeed, further studies are needed to identify the four unidentified proteins, as they are likely to play significant roles in this plant-pathogen interaction. Identification would provide information about probable function and thereby help elucidate the molecular mechanisms of pathogenicity and host response in downy mildew infections.

Three of the proteins with increased abundance are likely to be involved in initiating and maintaining a defence response by pea during *P. viciae* infection. Disease resistance response protein PI176 was identified originally by Fristensky et al. (1988), and is a member of the pathogenesis-related class 10 (PR10) family of proteins which are found exclusively in plants. This set of proteins generally is induced by abiotic and biotic factors such as wounding (Liu et al. 2003; Warner et al. 1992, 1993), salt stress (Moons et al. 1997), pathogenic infections (Fristensky et al. 1988; Liu et al. 2003; Matton and Brisson 1989; McGee et al. 2001; Pinto and Ricardo 1995; Schmelzer et al. 1989; Somssich et al. 1988), drought (Dubos and Plomion 2001), chemicals such as copper (Utriainen et al. 1998) and plant hormones (Moons et al. 1997; Wang et al. 1999a). PI176 is a variant of the pea PI49 protein, differing in four amino acid substitutions and one amino acid deletion (Fristensky et al. 1988). There are conflicting views on the potential biological action of the PR10 proteins. Some studies suggest they have RNase activity (Bantignies et al. 2000; Moiseyev et al. 1994; Park et al. 2004; Swoboda et al. 1996), with others (Biesiadka et al. 2002) suggesting that this is not the case, as the crystal structure of some PR10 proteins such as LIPR10.1A and LIPR10.1B from *Lupinus luteus* does not support RNase activity. Additionally, Biesiadka et al. (2002) noted that the *L. luteus* PR10 proteins studied have little or no RNase activity. This discrepancy in the literature may reflect the large number of PR10 protein homologues within plants. For example, 13 different PR10 cDNA clones have been identified in *Pinus monticola* (Liu et al. 2003) and, although some redundancy is likely to exist in the activity of these proteins, it is also possible that some of the proteins have significantly different functions within plants. Similar proteins in bean (*Phaseolus vulgaris*) are thought to act intracellularly as no signal peptides have been identified for the proteins (von Heijne 1985; Walter et al. 1990), whilst transcripts of the parsley (*Petroselinum crispum*) *PcPR1* gene accumulate rapidly and to a large extent in cells adjacent to the site of pathogen infection (Schmelzer et al. 1989; Somssich et al. 1988). From the literature and the protein abundance pattern of PI176 observed in this study, it appears that PI176 is important in the intracellular response of pea to infection by *P. viciae* in this compatible interaction, and further analysis of this family of proteins is merited.

Abscisic acid response protein 17 (ABR17) is an additional member of the pathogenesis-related class 10 (PR10) family and has a similar pattern of abundance to PI176. This has also been observed for *PR10* genes in other pea-microbe interactions, such as infection by the arbuscular-mycorrhizal fungus *G. mosseae* where transcript levels of the *PI49* and *PI176* genes increase in pea roots up to 15 dpi (Ruiz-Lozano et al. 1999), and for their proteins in *A. euteiches* infection of *M. truncatula* (Colditz et al. 2004). The gene for ABR17 is closely related to

ABR18 having significant similarities at the DNA and amino acid levels, a trait shared by abscisic acid-inducible proteins from other plant species such as alfalfa (*Medicago sativa*; Luo et al. 1992) and the barrel medic (*M. truncatula*; Colditz et al. 2004). Abscisic acid is thought to play a key role in mediating adaptive plant responses to environmental stress, plant development, seed dormancy and germination, as well as plant defence (Luo et al. 1992; Moons et al. 1995). It is thought that the ABR proteins in pea increase in abundance in response to both abscisic acid and environmental cues, with the present study indicating a role also in response to pathogens.

The increased abundance of both ABR and PI proteins would indicate that ABA-mediated signalling is important in the *P. viciae*–pea interaction. This notion concurs with Nyamsuren et al. (2003) who observed increased transcripts of these genes in *A. euteiches* infections of *M. truncatula*, a close relative of pea. Although ABA has been shown to play a role in signalling, Colditz et al. (2004) showed that of six PR10 proteins identified in *M. truncatula*, only three increased in abundance in response to ABA, and none altered in abundance in response to drought stress. Therefore, three of the PR10 proteins increased in abundance in response to *A. euteiches* alone, for the limited range of stresses assessed. It would seem that some proteins may play a specific role in response to biotic stress such as pathogen invasion, perhaps providing opportunities for the development of pathogen-tolerant crop species. Further studies should compare both plant-microbe interactions, especially as *A. euteiches* is also a serious pathogen of pea crops (Pfender 1989). This may determine whether the induction of the *ABA*-responsive genes is a general plant response to oomycete pathogens, resulting from increased ABA content as a side-effect of infection due to senescence, reduced water availability and cell death, or perhaps a combination of several factors.

Glycine-rich proteins (GRPs) have been implicated in numerous roles in plants. In pea, GRPs have been associated with dormancy and have similarity to proteins that are stimulated by auxin and numerous abiotic stresses (Luo et al. 1991; Laberge et al. 1993; Stafstrom et al. 1998). Structurally related GRPs are often components of the cell walls of higher plants (Showalter 1993), and accumulate in vascular tissues as part of the defence mechanism against pathogens and wounding (Mousavi and Hotta 2005). A second class of GRPs, the glycine-rich RNA-binding proteins (GR-RBP) such as protein 3 identified tentatively in the present study, are thought to play an important role in post-transcriptional regulation of gene expression. Some evidence exists for altered transcript and/or protein abundance of GR-RBPs in response to virus infection (Geri et al. 1999; Naqvi et al. 1998), acute hypersensitive response and salicylic acid treatment (Naqvi et al. 1998), abscisic acid treatment (Aneeta Sanan-Mishra et al. 2002; Baudo et al. 1999; Bergeron et al. 1993; Carpenter et al. 1994; Gomez et al. 1988; Kim et al. 2005) and methyl-jasmonate treatment (Richard et al. 1999), thus providing evidence of a role in the response of plants to pathogens. There are no previous reports regarding the response of these proteins to attack by oomycete pathogens, and the tentative identification in the present study indicates that further investigation is needed.

Cytosolic GAPDH (protein 4 in the present study) is one of three forms of GAPDH in plants, and is involved in the second phase of glycolysis, catalysing the conversion of D-glyceraldehyde 3-phosphate into 3-phospho-D-glyceroyl phosphate. Evidence for GAPDH having a role in defence is provided by Laxalt et al. (1996) who noted that cytosolic GAPDH transcripts accumulated in potato plants infected by *P. infestans*, and treated with both the *P. infestans* elicitor eicosapentaenoic acid and salicylic acid. Interestingly, although GAPDH transcripts increased to their highest levels at 2 dpi in potato, it was not until 3 dpi that a corresponding increase in enzyme activity was observed. This would indicate that there is a substantial time-lag between induction of genes involved in the pathogen response and the production of their corresponding proteins. It has been suggested that these proteins be given the term ‘stress-induced metabolic response’ proteins (Laxalt et al. 1996). The time-lag also suggests that the selection of 4 dpi as the time point for extracting proteins in the present study is suitable for investigating host-pathogen interactions in plants capable of adjusting their metabolism to survive various stresses in this short time period.

The possibility that the increase in GAPDH abundance is due simply to an increase in metabolic turnover of the protein, and not a specific stress response, should not be overlooked. The abundance of this protein and the other proteins, both identified and unidentified, in other plant parts such as roots,

stems and tendrils of pea is also worth investigating. Elevated levels of plant defence proteins in roots, for example, would help prevent downy mildew infection via soil-borne oospores. Investigations are in progress to establish whether the increase in GAPDH abundance is also evident in pea in response to abiotic stresses such as wounding and water-deficit.

The discovery that levels of a chloroplastic GAPDH A were also elevated in response to *P. viciae* infection (protein 5) is surprising, as to our knowledge there is no previous evidence for this in other biotrophic infections. It is likely that chloroplastic GAPDH plays a similar role to cytoplasmic GAPDH in that an overall increased metabolic state is induced during infection by *P. viciae*. Alternatively, GAPDH may have a role in plant signalling pathways, with increasing evidence indicating that plant enzymes may be multi-functional (Moore 2004). For example, GAPDH has been demonstrated to be an inhibitory target of hydrogen peroxide in *A. thaliana* with potential roles in mediating reactive oxygen species signalling (Hancock et al. 2005). Interestingly, mRNA and enzyme levels of GAPDH are also induced by abscisic acid treatment (Velasco et al. 1994), indicating that ABA signalling is important in the plant response to pathogens, especially as ABR17 protein levels also increased following *P. viciae* infection. GAPDH is also associated with the cell wall in *A. thaliana* (Chivasa et al. 2002), and found in the peribacteroid membrane of root nodules in *Lotus japonicus* (Wienkoop and Saalbach 2003). The specific function of GAPDH remains to be elucidated, and it seems that proteins involved in making adjustments to plant metabolism during stress are under-studied.

Three proteins increasing in abundance in response to *P. viciae* infection are involved in photosynthesis. Two are integral to photosystem I, the reaction centre subunit and the iron sulphur centre, whilst the third is involved in the synthesis of ATP (ATP synthase epsilon chain). Photosystem I is a chloroplastic, multimolecular complex that uses ferredoxin-like iron-sulphur cluster proteins as terminal electron acceptors. The ATP synthase epsilon chain is a small sub-unit of the chloroplast ATP synthase, which produces ATP from ADP in the presence of a proton gradient across the chloroplast thylakoid membrane (Hopkins and Hüner 2004). Whilst most studies indicate that infections by plant pathogens result in a reduced photosynthetic rate, transient increases in the rate of photosynthesis during infection by biotrophic pathogens have been observed (reviewed in Scholes 1992), which may reflect the increase in proteins of the photosynthetic apparatus observed in this study. In the biotrophic pathogen-plant interactions where photosynthetic rate initially increased during infection, an overall reduction in photosynthesis was eventually observed, when compared to control plants. An analysis of the proteins observed to alter in abundance at later stages of infection by *P. viciae*, along with experimental data on photosynthetic rates, would reveal if a similar pattern occurs. The hypothesis that a fragment of the photosystem I reaction centre subunit II has been identified in the present study, however, suggests that the intact protein has been degraded as a result of infection. This would be expected to cause a decrease, rather than an increase, in photosynthetic activity. The possibility that photosynthetic rates in adjacent, uninfected tissues and cells may increase to compensate for a reduction in colonised areas also merits investigation. These effects have been observed in *Botrytis fabae* infections of *Vicia faba* leaves, uninfected leaves on *P. vulgaris* plants infected by the rust fungus *Uromyces phaseoli*, and uninfected leaves of pea plants inoculated with powdery mildew spores (Lucas 1998).

Thus it would appear that pea leaves are capable of implementing a response to *P. viciae* infection through the up-regulation of various pathogen-related and metabolic pathway proteins. Whether these proteins act in concert rather than individually is not known, although previous studies have shown that plants synthesise a number of proteins in response to stresses that may work individually and/or in conjunction with other proteins (Luo et al. 1992).

It is noticeable that no *P. viciae* proteins were identified in this study, which may be because there is very little database coverage of DNA and proteins from this pathogen. This was also the case with the proteomic study of *A. euteiches* infection of *M. truncatula* (Colditz et al. 2004), where no *A. euteiches* proteins were found. Reasons given included the restricted amount of pathogen-infected root cells and the restricted growth of *A. euteiches* in host cells. The difficulty in identifying oomycete proteins was illustrated by Shepherd et al. (2003), who identified just three *P. palmivora* proteins from 2D gels loaded with proteins extracted from hyphae grown in vitro, all of which corresponded to actin. Indeed it can be difficult to identify genes specifically expressed during infec-

tion by oomycetes (Beyer et al. 2002 and references therein). Despite improved techniques, up to 70% of gene sequences cannot be identified using genomic studies (Beyer et al. 2002), and house-keeping genes are more easily identified than those that may be involved in infection. Proteomic approaches will always tend to be more successful if supported by extensive and complete genome databases. *P. infestans, Phytophthora sojae* and *Hyaloperonospora parasitica* genome projects are currently underway or are near completion (Beynon, personal communication; http://www.pfgd.org/; Kamoun et al. 1999a; Qutob et al. 2000) which may enhance the success of proteomic analyses of oomycetes. Indeed, the peptide mass fingerprints obtained for the unidentified proteins in this study may yield matches when used to search these new data sets, and also any proteins identified from analysis of the *P. viciae* conidial proteome (Chuisseu Wandji et al. 2007).

To our knowledge, the data presented here represent the first application of 2-D DIGE to investigate a plant–oomycete pathogen interaction, and indicate the sensitivity and accuracy of this method for identifying proteins that are important to the plant's response to infection. An increased understanding of these mechanisms may accelerate progress in developing novel control strategies for downy mildew diseases. Indeed, the DRR49 protein from pea has been used to increase potato tuber production in the presence of pathogen-infested soil (Chang et al. 1993), and to increase levels of resistance to *Leptosphaeria maculans* in canola (Wang et al. 1999b). It is therefore possible that the proteins identified in the current study may be of use in the development of novel plant lines with enhanced resistance to biotic and abiotic stresses.

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