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ARTICLE

Two-Dimensional Electrophoresis Based Proteomic Analysis of the Pea (*Pisum sativum*) in Response to *Mycosphaerella pinodes*

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Responses to *Mycosphaerella pinodes* in pea were studied by using a proteomics approach. Two-dimensional electrophoresis (2-DE) was used in order to compare the leaf proteome of two pea cultivars displaying different phenotypes (susceptible and partial resistance to the fungus), as well as in response to the inoculation. Multivariate statistical analysis identified 84 differential protein spots under the experimental conditions (cultivars/treatments). All of these 84 protein spots were subjected to MALDI-TOF/TOF mass spectrometry to deduce their possible functions. A total of 31 proteins were identified using a combination of peptide mass fingerprinting (PMF) and MSMS fragmentation. Most of the identified proteins corresponded to enzymes belonging to photosynthesis, metabolism, transcription/translation and defense and stress categories. Results are discussed in terms of responses to pathogens.

KEYWORDS: Pea−*Mycosphaerella pinodes* interaction; pea proteome; plant responses to pathogens

INTRODUCTION

Aschochyta blight, caused by *Mycosphaerella pinodes* (Berk & Blox) Vesterg, the teleomorph of *Ascochyta pinodes* Jones, is the most important foliar disease of pea crop (*Pisum sativum* L.) worldwide. *M. pinodes* causes necrotic spots on all aerial parts of the pea plant and is responsible for important yield and seed quality losses (*1*). Genetic resistance appears to be the most practical method of control (*2*). However, although extensive searches have been carried out, only moderate resistance is available in pea cultivars, and this has been inadequate to control the disease (*3*−*6*). Higher levels of resistance have been identified in wild species of *Pisum* (*7*, *8*), but still have not been efficiently used in breeding programs.

Quantitative trait loci (QTL) analysis has identified numerous genomic regions involved in partial resistance to *M. pinodes* in pea (*9*−*13*). Physiological and biochemical studies of the pea−*M. pinodes* interaction reported that *M. pinodes* elicitor (*14*) induces some defense responses in pea, such as accumulation of the phytoalexin pisatin (*15*), activation of the genes encoding phenylalanine ammonia-lyase (PAL) and chalcone synthase (*16*), activation of PR proteins (β-1,3-glucanase, chitinases) (*17*), generation of superoxide anion (*18*), enhancement of ATPase activity (*18*), and activation of the polyphosphoinositide metabolism (*19*).

A large number of plant proteomic papers are collected in two recent reviews (*20*, *21*). To our knowledge most proteomic studies in legumes published to date have dealt with the model system *Medicago truncatula* while few proteomics studies have been reported with other legume species (*20*), a small number of them dealing with the responses to pathogens in pea (*22*−*27*). We will focus on those dealing with pathogenic fungi. Thus, Curto et al. (*25*) reported changes in the leaf proteome of two pea genotypes differing in their resistance to *Erysiphe pisi*. Using a 2-DE/mass spectrometry strategy, differentially regulated proteins between genotypes and treatments (control and infected leaves) were identified. The identified proteins mainly belong to three functional categories: photosynthesis, carbohydrate catabolism and stress/defense responses. Authors concluded that an increased activity of the energy metabolism in resistant plants occurred to compensate for the cost of constitutive resistance. Wen et al. (*26*) studied the reaction of pea to *Nectria hematococca* using a multidimensional protein identification technology, concluding that the root cap secretes a complex mixture of proteins that appear to function in protection of the root tip from infection. Finally, Amey et al. (*27*) used a 2-DE/mass spectrometry strategy to identify host proteins altering in abundance during *Peronospora viciae* infection of a susceptible pea cultivar. Among them they found proteins belonging to photosynthesis, carbohydrate metabolism and stress/defense-related category.

In the present study we aim to analyze the leaf pea proteome in response to *M. pinodes* inoculation. Based on previous investigation carried out to assess the partial resistance to *M. pinodes* in field pea (*28*), we have selected two cultivars displaying differential response to *M. pinodes*. Differences in the 2-DE map between the two cultivars, as well as in response to parasite infection, were analyzed, and some of the differential proteins were identified by MALDI-TOF/TOF mass spectrometry. Thirty-one proteins were identified, most of them belonging to the functional category of photosynthesis, metabolism, transcription/translation and defense and stress-related proteins.

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MATERIALS AND METHODS

Plant Material and Inoculation. Two pea cultivars, 'Messire' and 'Radley', were used in the experiment. Messire cultivar is highly susceptible to *M. pinodes* while Radley has shown incomplete resistance that is still the highest level of resistance available so far in a pea cultivar (*5*, *28*, *29*).

Pea seeds were germinated in Petri dishes on wet fiber papers and kept in the dark at 20 °C for five days. When the root reached 4−5 cm length, plants were transferred to individual pots containing 250 cm^3 of a 1:1 sand−peat mixture and grown in a controlled environment (20 ± 2 °C with a 12 h dark/12 h light photoperiod, at 250 μmol m^{-2} s^{-1}). At 3−4 leaf stage (approximately 14 days after planting) half of the plants of each accession (8 of Messire and 12 of Radley) were inoculated with *M. pinodes*, whereas the remaining plants were inoculated with sterile water and used as controls. For inoculation the monoconidial *M. pinodes* isolate Co-99, derived from an isolate obtained from infected pea material collected in commercial fields at Córdoba (Spain), was used. A spore suspension was prepared by flooding the surface of 12−14 day old cultures with sterile water, scraping the colony with a needle and filtering the suspension through two layers of sterile cheesecloth. The concentration of spores in the solution obtained was further determined with a hemocytometer and adjusted to 5×10^5 spores per mL. Finally, Tween-20 (120 μL per 100 mL of suspension) was added as a wetting agent. The spore suspension was applied extending the suspension over the leaf surface using a small paintbrush. After inoculation high humidity was ensured during the first 24 h by ultrasonic humidifiers operating for 15 min every two hours. After that period the humidifiers were turned off. Controls, noninoculated plants, were maintained in the same environmental conditions as inoculated plants.

Leaves from inoculated and noninoculated plants were harvested 48 h after inoculation (hai), frozen in liquid nitrogen and stored at −80 °C until protein extraction.

Protein Extraction and Two-Dimensional Gel Electrophoresis. Proteins were extracted according to the TCA−acetone precipitation protocol (*30*) with some minor modifications. Leaf samples (ca. 2 g fresh weight) from three independent replicates per treatment and cultivar were ground with liquid nitrogen in the presence of glass powder by using a precooled mortar and pestle. The powder was suspended in −20 °C cold acetone containing 10% v/v TCA and 0.07% w/v DTT (4 mL per g of fresh tissue) and sonicated for 10 min on ice at 50 MHz by using an ultrasonic homogenizer 4710 series (Cole-Parmer). After standing for 1 h at −20 °C, the samples were centrifuged at 48400*g* for 30 min at 4 °C. The pellet was washed twice by resuspension in cold (−20 °C) acetone containing 0.07% w/v DTT, placing at −20 °C for 30 min and centrifuged at 27200*g* for 15 min at 4 °C. The resulting pellet was lyophilized for 10 min and resuspended in sample buffer, containing 8 M urea, 2% w/v CHAPS, 0.5% v/v Bio-Lyte 3-10 carrier ampholytes, 20 mM DTT and Bromophenol blue traces. Samples were sonicated for 5 min in an ultrasonic bath and incubated for 1 h at 35 °C. Samples were centrifuged at 27200*g* for 15 min at room temperature, and soluble proteins were determined by RCDC Protein Assay Kit (BioRad), according to the manufacturer's instructions.

IPG strips (BioRad) 7 cm wide pH gradients for analytical and 17 cm 5−8 pH gradient for preparative purposes were used. Strips were passively rehydrated for at least 12 h with 125 μL (7 cm) or 300 μL (17 cm) of sample buffer, containing 150 and 500 μg of protein, respectively. Strips were loaded onto a PROTEAN IEF System (BioRad, Hercules, CA, USA) and focused at 20 °C with increasing linear voltage according to the manufacturer's instructions: 250 V-4000 V until reaching 10000 V h for 7 cm IPG strips and 250 V-10000 V until reaching 40000 V h for 17 cm IPG strips. After IEF, strips were equilibrated by soaking first for 10 min in 375 mM Tris-HCl buffer pH 8.8, 6 M Urea, 2% SDS, 20% glycerol, solution and then for 10 min in the same solution containing 135 mM iodoacetamide.

Second dimension SDS−PAGE was performed using the Mini-Protean 3 System (7 cm strips), or the PROTEAN IIxi Cell System (BioRad) for the 17 cm strips. Electrophoresis was carried out at 20 °C in 12% house-made polyacrylamide gels until bromophenol blue reached the end of the gel, by using a constant voltage of 200 V (60 min) for small gels, and 30 mA (60 min) plus 50 mA (240−300 min) for preparative gels. Broad molecular range markers (BioRad) containing myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovoalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa) were loaded beside the strip.

Small size gels were Coomassie stained according to Neuhoff et al. (*31*). Preparative gels were stained with CBB G-250 according to the procedure reported by Mathesius et al. (*32*). Gel images were captured with a GS800 imaging densitometer (BioRad), and initially analyzed with the PDQuest Advanced version 8.0.1 software (BioRad) using 10-fold over background as a minimum criterion for presence/absence. This software assigns unique standard spot numbers to each protein spot, termed SSP. Spot detection and matching errors were corrected manually based on the respective group consensus data. Normalized spot volumes (individual spot intensity/normalization factor) calculated for each gel based on total quantity in valid spots were determined, and these values used to designate the differential protein spots.

Mass Spectrometry Analysis and Database Searching. Mass spectrometry was performed at the Proteomics Facility, SCAI (University of Córdoba). Spots were excised manually from gels and digested with modified porcine trypsin (sequencing grade; Promega) by using a ProGest (Genomics Solution) digestion station. The digestion protocol used was that of Schevchenko et al. (*33*), with minor variations. Gel plugs were destained by incubation (twice for 30 min) with a solution containing 200 mM ammonium bicarbonate in 40% acetonitrile at 37 °C, then being subjected to three consecutive dehydration/rehydration cycles with pure acetonitrile and 25 mM ammonium bicarbonate in 40% acetonitrile, respectively, and finally dried at room temperature for 10 min. Then, 20 μL of trypsin, at a concentration of 12.5 ng/μL in 25 mM ammonium bicarbonate, was added to the dry gel pieces and the digestion proceeded at 37 °C for 12 h. Peptides were extracted from gel plugs by adding 10 μL of 1% (v/v) trifluoracetic acid (TFA) and incubating for 15 min. Peptide fragments from digested proteins were then crystallized with α-cyano-4-hydroxycinnamic acid as a matrix. The MS analysis was performed in a MALDI-TOF/TOF (4700 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA) mass spectrometer in the *m*/*z* range 800 to 4000, with an accelerating voltage of 25 kV. Spectra were internally calibrated with peptides from trypsin autolysis ($M + H^+ = 842.510$, $M + H^+ = 2211.105$). The three most abundant peptide ions were then subjected to fragmentation analysis, providing information that can be used to determine the peptide sequence.

A combined peptide mass fingerprinting (PMF) tandem MSMS search was performed using GPS Explorer software v. 3.5 (Applied Biosystems) over nonredundant NCBI database, using the MASCOTsearch engine (Matrix Science Ltd., London; http://www.matrixscience.com). The following parameters were allowed: taxonomy restrictions to *Viridiplantae*, a minimum of four peptides matches, a maximum of one miscleavage, 50 ppm mass tolerance, and peptide modifications by carbamidomethylcysteine and methionine oxidation were accepted. The confidence in the PMF matches was based on the score level (only scores greater than 67 are significant, $p < 0.05$) and confirmed by the accurate overlapping of the matched peptides with the major peaks of the mass spectrum.

Statistical Analysis of Protein Abundance Data. For statistical treatment and cluster analysis of protein abundance values, the web-based software NIA array analysis tool was utilized (*34*); available at http://lgsun.grc.nia.nih.gov/anova/index.html. This software tool selects statistically valid protein spots based on analysis of variance (ANOVA). After uploading the data table (spreadsheet in the Supporting Information) and indication of biological replications, the data were statistically analyzed using the following settings: error model "max (average, actual)", 0.01 proportion of highest variance values to be removed before variance averaging, 10 degrees of freedom for the Bayesian error model, 0.05 FDR threshold, zero permutations. First, hierarchical clustering was performed to check the entire data set, and the results were represented in dendrograms using the cluster function of the software. Second, the entire data set was analyzed by PCA using the following settings: covariance matrix type, three principal components, 1-fold change threshold for clusters, 0.6 correlation threshold for clusters. PCA results were represented as a biplot, with proteins more abundant in those experimental situations located in the same area of the graph. Protein spots data for this analysis were recorded (Supporting Information). Third, pairwise comparisons of protein spot mean abundance values were performed with the software tool using the following settings: 0.05 FDR, 1-fold change threshold. Fourth, histograms representing log average protein spot values were downloaded using the software.

Figure 1. Symptoms observed about one week after *M. pinodes* inoculation on the lower leaves of susceptible Messire (left) and incomplete resistant Radley (right) pea cultivars.

RESULTS

Two-Dimensional Gel Electrophoresis and Mass Spectrometry Analysis. Symptoms observed about one week after *M. pinodes* inoculation are shown in **Figure 1**, although these were not yet visible at the time of leaf sampling for protein extraction (48 hai).

According to the results of small size 2-DE gels (Supporting Information), a deeper analysis was performed by using the narrowest pH gradient, 5 to 8 and 17 cm in length. For each of the conditions analyzed (cultivars and treatments), three replicates corresponding to independent protein extracts were made. As determined following the CBB staining of the gels and the use of the PDQuest software, an average of 315 ± 92 spots were resolved (**Figure 2a**,**b**). After normalization of protein spot images and manual verification, 84 differential protein spots were detected. The following criteria were used for considering a spot as being variable: (i) consistently present or absent in all three replicates; (ii) display cultivars- or treatment-ratios differing at least 1.5-fold; (iii) differences statistically significant ($p < 0.05$) between cultivars or treatments. To illustrate this, **Figure 2a** shows all of the differential protein spots on virtual gel and **Figure 2c** shows SSPs 1104 and 6408 on real gels. **Table 1** summarizes the features of the experiment.

Statistical Analysis of Protein Abundance and Expression Cluster Analysis. Abundance data of all of the 84 differential protein spots were analyzed using the web-based NIA array analysis software tool developed by Sharov et al. (*34*). This software uses analysis of variance (ANOVA) for statistical analysis of a large data set with multiple variables and subjects. Expression cluster analysis can be performed by a variety of methods including principal component analysis (PCA), obtaining a more accurate grouping of the samples and determining the most discriminant spots (*34*).

First, a hierarchical clustering of biological experiments and their repetitions were performed. We found that the experimental conditions could be divided into two large clusters in a dendrogram, namely, cluster 6 (Radley-inoculated and Radley-noninoculated) and cluster 5 (Messire-inoculated and Messire-noninoculated) (**Figure 3a**). This clustering indicated that Messire plants (clustering closest together) had protein abundance profiles similar and lightly different from those shown by Radley plants (clustering closest together) grown under the two experimental conditions (noninoculated and inoculated) across the experiments. The hierarchical clustering of biological repetitions confirmed that the data were reproducible for the experiments (Supporting Information).

Protein Spot Identification and Expression Pattern Analysis. The 84 differential protein spots were analyzed by MALDI-TOF/TOF after tryptic digestion, and the MS spectra were used to screen a *Viridiplantae* index of the nonredundant NCBI database (**Table 2**). Of the 84 protein spots analyzed, 31 were successfully identified with a high probability score and matched peptides for most of them, except spot 3103 with 2 matched peptides, which was accepted like valid identification due the high sequence coverage for a protein of low molecular weight. Photosynthetic, metabolic, transcriptional/translational and defense/stress-related proteins, with approximately 72% of identified proteins, dominated the 2-DE profile of leaf tissue. Only one protein (oxygen-evolving enhancer protein 1, gi|131384) was represented by more than one spot (3301 and 3305) with slightly different M_r and p*I* values (**Table 2**), hence corresponding to isoform or multiple forms/posttranslational modification variants of the same gene product.

Proteins identified were classified in the following functional categories: photosynthesis, glycolysis/glyconeogenesis, citrate cycle, glutamine biosynthetic process, metabolic process, protein binding, nucleic acid binding, transcription/translation, defense and stress related proteins, cellular processes and unknown. Considering the PCA analysis (**Figure 3b**), photosynthetic proteins were found in the categories of proteins correlated with PC1 (positive and negative direction) and PC2 (positive direction). Metabolic proteins were found in the categories of proteins correlated with PC1 (positive and negative direction) and PC3 (positive direction). Proteins belonging to transcription/translation category, as well as nucleic acid binding proteins, were found in PC1 and PC2 (positive direction). Defense and stress-related proteins were found in the categories of proteins correlated with PC1, PC2 (positive direction) and PC3 (negative direction). Proteins belonging to the cellular processes category were found in PC1 and PC3 (positive direction). The degree of protein abundance change within a specific PC was measured by the slope of regression of log-transformed protein abundance versus the corresponding eigenvector multiplied by the range of values within the eigenvector (*34*) (**Figure 3c**). **Figure 4** shows mean log abundance intensities for all 31 protein spots identified. We can appreciate associations with protein spot abundance patterns in the majority of the identified proteins.

A high proportion of photosynthetic (4/5), metabolic (4/6) and transcriptomic/translational, as well as nucleic acid binding (7/7) proteins, were found more abundant in Messire than Radley cultivar, when both inoculated and noninoculated plants were compared (**Figure 5**). Stress and defense-related proteins were found in all of the pairwise comparisons of protein abundance, with a clear trend to increase in inoculated plants. In general, a trend toward greater abundance of proteins is observed under inoculation in both cultivars.

DISCUSSION

In order to increase our current knowledge of the pea response mechanisms activated in response to *M. pinodes*, the leaf pea proteome was analyzed in noninoculated and inoculated plants of two cultivars displaying different phenotypes (susceptible and incomplete resistance). We used Radley as resistant, which was considered a good line to study the different mechanisms of resistance to *M. pinodes* as had previously shown the highest levels of incomplete resistance to *M. pinodes* available in pea germplasm (*5*, *28*, *29*).

Previous histological studies comparing *M. pinodes* development in Messire and Radley concluded that Radley possessed several mechanisms of resistance (*35*), some stopping the development of the pathogen at the epidermis and others restricting its growth in the mesophyll. Thus, resistance to *M. pinodes* in Radley was characterized by a lower success in colony establishment, associated with the rapid death of the epidermal cell being attacked by *M. pinodes* and by a smaller colony size (**Figure 1**).

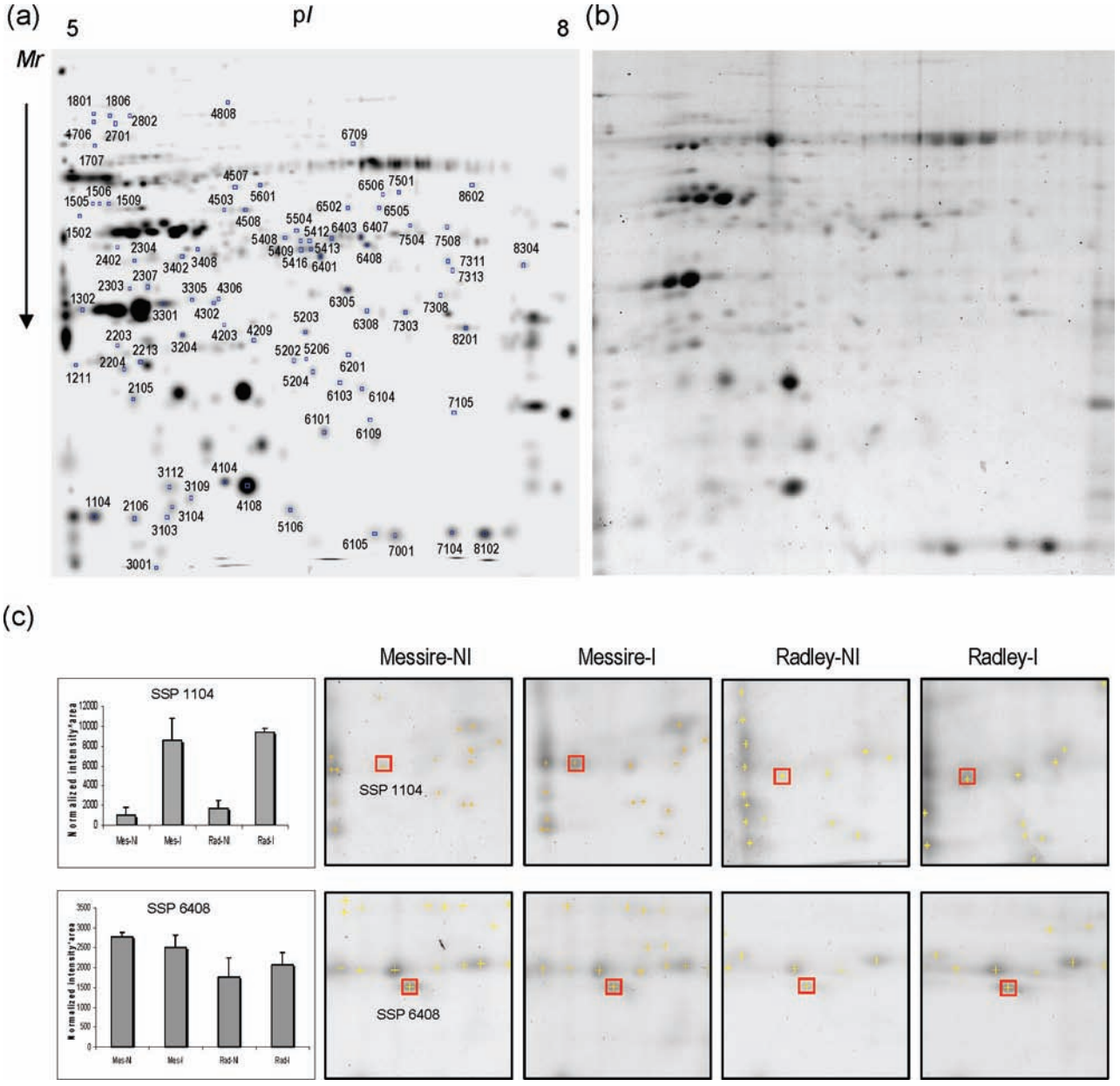

Figure 2. Location of 84 variable protein spots on a virtual two-dimensional gel (**a**). Representative Coomassie-stained 2-D protein gel of Messire cultivar (**b**). Representative protein spot images (**c**). Two protein spots were selected as examples to illustrate differential expression profiles: SSP1104 (up) and SSP6408 (down). On the left, graphs of mean abundance values for the respective protein spot are shown (standard deviations were calculated from the means of the three repetitions). Close-up regions of the Coomassie-stained 2-D gels are shown (from the left to right: Messire-NI, Messire-I, Radley-NI and Radley-I). The positions of the protein spots on the gels are marked by cross hairs. The respective differential protein spots, matched throughout all presented gels, are marked by boxed cross hairs.

The proteomic study was performed 48 hai, as at this time the pathogen had reached the pea leaf mesophyll and the different mechanisms of resistance could be acting. By using 2-DE and mass spectrometry we aim to identify key elements involved in the pea response to *M. pinodes*. Several proteins were identified differentiating cultivars, as well as in response to inoculation, although the results discussed here are limited to a small soluble fraction of the whole proteome, determined by the extraction protocol and the 2-DE separation technique utilized, with 5−8 p*I* and 10−100 kDa M_r as the best range to resolve most of the solubilized proteins, and above the detection limit for Coomassie staining. Under our experimental conditions, an average of 315 spots were resolved, this being the distribution pattern observed in leaf tissue from other plant species (*36*−*39*).

A multivariate analysis of data to detect outliers as well as clustering among the 2-DE gels was performed. Principal component analysis offers a strong approach to obtaining an overview of the main variation and of inter-relations between spots in the protein patterns (*40*). To perform this statistical cluster analysis, we employed a software tool designed for the analysis of biological gene chip data (*34*), but that has also been successfully used in the analysis of protein abundance data. This software identifies patterns in our data set and exploration of associations between protein spots and experimental conditions using PCA.

We found a similar protein abundance profile in the three replicates from each experimental condition, pointing out the reliability of our data. Multivariate analysis showed 84 differential protein spots, and all of them showed reproducible differential abundance behavior in pairwise comparisons of experimental conditions. All of the 84 protein spots were analyzed by mass spectrometry, and only 31 could be matched against the NCBI database. This low percentage of identified proteins is expected

Table 1. Summary of the Features of the Proteomic Experiment

features	number of protein spots
average of total spots detected in the Coomassie stained gels	315 ± 92
average of spots consistently present in all three replicates	154 ± 39
differential protein spots[a]	
total	84 (27% of spots detected)
between noninoculated cultivars	49
between inoculated cultivars	53
in Messire in response to inoculation	30
in Radley in response to inoculation	23
total identified spots	31
unique proteins[b]	30

[a] The 84 differential abundant proteins were selected using the PDQuest software. [b] Number of different proteins identified.

for those species which are absent or underrepresented in public databases as pea. About 52% of identified proteins belonged to legumes species, of which 39% corresponded to *Pisum sativum*. In all the pea-specific matches, theoretical and experimental p*I* and M_r were in good agreement, encouraging confidence in the identifications. Those cases in which differences between these values were observed could be interpreted in terms of protein degradation (lower experimental than theoretical M_r values), or may account for different post-translational protein modifications.

We discuss each functional group and the behavior pattern observed for the conditions studied (cultivars and response to *M. pinodes* inoculation). Among proteins differentiating cultivars were photosynthetic (spots 2105, 3301, 3305, 5202 and 7501), metabolic (spots 5408, 5413, 5504, 6408 and 7311), stress-related proteins (spots 5204 and 6403), and transcription/translation proteins (spots 2307, 3103, 4508, 6305, 6505, 6506 and 7313).

Photosynthetic proteins, as oxygen-evolving enhancer protein 2 (OEE2, spot 2105), OEE1 (spot 3305), light harvesting protein (spot 5202) and large subunit of RubisCo protein (spot 7501), were identified in larger amount in Messire when cultivars were compared. The OEE protein/complex has been implicated in photosynthetic oxygen evolution and is associated with the PSII complex, the site of the oxygen evolution in all higher plants and algae (*41*). We can associate the higher photosynthesis rate in Messire cultivar to a higher production of biomass (**Figure 1**).

Two enzymes belonging to energetic metabolism, such as malate dehydrogenase (MDH) (spots 6408 and 7311) and fructose-bisphosphate aldolase (spots 5413 and 7508), were identified displaying similar abundance patterns when cultivars and treatments were compared. These enzymes were more abundant in the susceptible noninoculated cultivar, and increased in the resistant cultivar in response to inoculation.

Spot 5504 was identified as an enzyme belonging to amino acid metabolism (glutamine-ammonia ligase or glutamine synthetase; GS). We found that this protein increased in Radley cultivar but decreased in Messire in response to inoculation. This enzyme has been found extensively related to abiotic stresses, such as salinity in potato (*42*), tomato (*43*), wheat (*44*), drought and high temperature in perennial grass (*Leymus chinensis*) (*45*), etc. GS can be associated with amino acid conversion, and amino acid composition might be altered due to several stresses, which could promote stress-resistance (*46*).

The biological interpretation of the differences observed when noninoculated cultivars were compared is a matter of speculation; however, taking into account physiological differences between both cultivars, we can postulate that it could be related to differences in the efficiency in energy utilization for growth and fitness purposes. As has been reported in other biological systems (*37*, *39*, *47*), a similar behavior for some of the photosynthetic and metabolic proteins identified in this work was observed in response to biotic and abiotic stress, with a clear trend to decrease in the susceptible and increase in the resistant genotype in response to the stress. This behavior suggests that differences observed could be related to efficiency in energy utilization as a consequence of the sink effect of the pathogen on the host plant.

Protein spot 5408 was identified as pyridoxal 5-phosphate (PLP)-dependent enzyme. PLP, the biologically active form of vitamin B6, has multiple roles as a versatile cofactor of enzymes that are mainly involved in the metabolism of amino acid compounds (*48*). We found the PLP dependent enzyme decreased in both cultivars after inoculation, but its relation with the response to *M. pinodes* is unclear.

Two nucleic acid binding proteins, namely, glycine-rich RNA binding protein (GRP, spot 3103) and far-red impaired response protein (spot 6505), displayed a similar behavior. Both proteins were found in different amounts when cultivars were compared, with a higher amount in the susceptible cultivar. Under inoculation a trend to increase in the susceptible cultivar was observed. Recent works provide evidence of the GRP role in the response of plants to pathogens. Thus, an increase of this protein in pea plants has been reported in response to *Peronospora viciae* inoculation (*27*). In a recent study we have found differential abundance of GRP in three *M. truncatula* genotypes in response to rust inoculation (*39*), supporting the hypothesis of its role in the response of plants to pathogens. Hudson et al. (*49*) found in *Arabidopsis* mutants in far-red impaired response (*far1*) a reduced responsiveness to continuous far-red light, which implies a specific requirement for FAR1 in phytochrome A signal transduction. In other work differences in the expression of this gene have been found in *Arabidopsis* plants infected with *Agrobacterium tumefaciens* (*50*). The relation of this protein in our work is uncertain.

Another binding protein, DNA-binding protein (spot 2307), as well as two transcriptional/translational proteins, reverse transcriptase (spot 6506) and elongation factor Tu (EF-Tu, spot 4508), were identified in higher amount in the susceptible noninoculated cultivar, with a trend to decrease in response to inoculation. Differential expression of this gene has been recorded in response to various abiotic stresses in pea plants, being downregulated in response to salinity and ABA treatment. This suggests that regulation of this gene may have an important role in plant adaptation to environmental stresses (*51*).

Proteins of defense response such as disease resistance response protein (PR10, spot 1104) and ABA-responsive protein (ABR17, spot 2106) were identified. Pathogenesis-related (PR) proteins are found in virtually all plants in response to pathogen infection and, in many cases, in response to abiotic stresses as well and include the PR10 family. PR10 proteins, having ribonuclease activity, were first described in *P. sativum* inoculated with *Fusarium solani* (*52*) and have been subsequently described to be induced in many species against abiotic and biotic extresses (*53*) including lentil against *Ascochyta lentis* (*54*). It has been reported that ABR17, a member of the group 10 family of pathogenesis-related proteins (PR 10), mediated stress tolerance to multiple abiotic stresses such as salinity, cold temperature, freezing (*55*). In a previous study ABR17 was identified in pea plants in response to the parasitic plant *Orobanche crenata* (*23*). Recently, several disease resistance genes were isolated and mapped in genomic regions of two pea genotypes containing QTLs for resistance to *M. pinodes* (*56*). In the present work, we found that ABR17 and PR10 proteins increased after inoculation in both cultivars, which suggests the involvement of these proteins in the defense response against *M. pinodes*.

Two stress response proteins were identified differentiating cultivars, with a higher amount in noninoculated Messire. These were

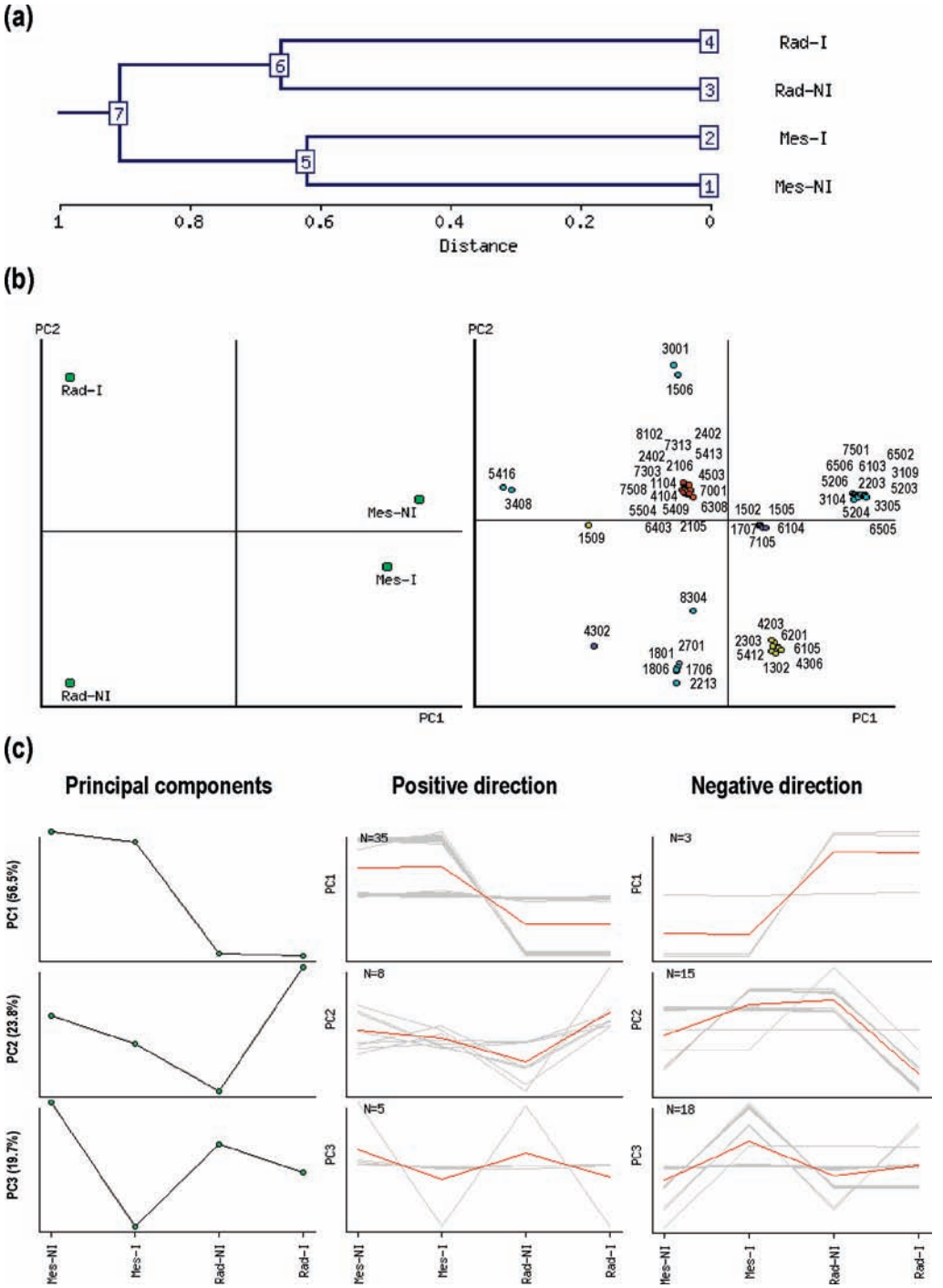

Figure 3. Statistical protein abundance cluster analysis of cultivars and response to *M. pinodes* inoculation using the ANOVA-based NIA array analysis tool (*34*). (**a**) Dendrogram showing hierarchical clustering of experimental conditions. The expression clusters are numbered from 1 to 7. (**b**) Two-dimensional biplots showing associations between experimental samples and protein spots generated by principal component analysis (PCA). Samples (left) and protein spots (right) were plotted in the first two component space. A short distance between samples and protein spots in the component space is indicative of similarity in expression profiles. (**c**) Protein spot abundance clustering based on PCA. For each PC, two clusters of proteins were identified that were positively and negatively correlated with the PC. Protein clustering was performed sequentially starting from the first PC. Proteins that were already clustered with a PC were not included in the clusters associated with subsequent PCs. Protein spots identified in this analysis are recorded in the Supporting Information.

annexin-like protein (spot 5204) and heat shock protein binding (Hsp, spot 6403). They also were found in different amounts in both cultivars in response to inoculation. Annexins constitute a ubiquitous family of more than 15 structurally related, membrane-binding proteins present in eukaryotic cells (*57*). Evidence has been reported for a potential role of the annexin-like protein in response to oxidative stress in bacteria and plants, suggesting that it may play a role in plant defense against various types of biotic as well as abiotic stresses (*58*). The generation of oxidative burst is one of the earliest responses to attempted pathogen attacks. Accumulation of reactive oxygen species (ROS) is associated with the occurrence of hypersensitive response, is effective against biotrophic fungi (*59*), can be toxic and inhibit fungal growth (*60*) and can act as signaling agents in plant defense (*61*). We found in our study that an annexin-like protein increased in both cultivars after inoculation, although it was more significant in the susceptible cultivar.

Table 2. Identification of Differential Spot Proteins by MALDI-TOF/TOF and Classification According to Their Functions

spot[a]	protein name	score	species (accession no.)	PM[b]/coverage %	M_r/p*I* exptl (theor)[a]	functional category	more/less abundance change ratio (FDR)[c]
2105	oxygen-evolving enhancer protein 2, chloroplast precursor (OEE2)	116	*Pisum sativum* (gil131390)	12/46	27.5/5.5 (28.2/8.3)	photosynthesis	0.4 R_I/M_I (0.05)
3301	oxygen-evolving enhancer protein 1, chloroplast precursor (OEE1)	227	*Pisum sativum* (gil131384)	11/35	36.4/5.7 (35.1/6.3)	photosynthesis	4.8 R_I/R_{NI} (0)
3305	oxygen-evolving enhancer protein 1, chloroplast precursor (OEE1)	189	*Pisum sativum* (gil131384)	14/62	36.9/5.9 (35.1/6.3)	photosynthesis	3.4 M_I/M_{NI} (0.001), 0 R_{NI}/M_{NI} (0)
5202	light harvesting protein	68	*Pisum sativum* (gil309673)	5/22	30.6/6.4 (29.6/8.8)	photosynthesis	0.5 R_{NI}/M_{NI} (0.0375)
7501	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	79	*Polypodium furfuraceum* (gil42541540)	12/32	56.6/7.0 (49.1/7.0)	photosynthesis	0 R_{NI}/M_{NI} (0)
5413	fructose-bisphosphate aldolase 2, chloroplast	223	*Pisum sativum* (gil461501)	13/33	43.1/5.5 (38.0/5.5)	glycolysis/glyconeogenesis	2.1 R_{NI}/M_{NI} (0.0244), 0.4 R_I/R_{NI} (0.0007)
7508	fructose-bisphosphate aldolase, cytoplasmic isozyme 2	232	*Pisum sativum* (gil1168410)	15/55	47.1/7.3 (38.6/6.8)	glycolysis/glyconeogenesis	2.2 R_I/R_{NI} (0.0219)
6408	malate dehydrogenase	165	*Glycine max* (gil3193222)	8/29	43.7/6.8 (27.5/5.6)	citrate cycle	0.5 R_{NI}/M_{NI} (0.0302)
7311	malate dehydrogenase	223	*Pisum sativum* (gil37725953)	14/40	41.5/7.3 (37.4/7.0)	citrate cycle	0.5 R_{NI}/M_{NI} (0.0382)
5504	glutamate-ammonia ligase (EC 6.3.1.2) 3A, cytosolic	102	*Pisum sativum* (gil2129882)	6/15	46.1/6.4 (37.6/5.8)	glutamine biosynthetic process	1.9 R_I/M_I (0.0262)
5408	pyridoxal-5-phosphate-dependent enzyme, beta subunit	71	*Medicago truncatula* (gil92876186)	9/28	44.7/6.4 (41.0/6.5)	metabolic process	0.5 R_I/M_I (0.0452)
2307	DNA-binding protein	89	*Arabidopsis thaliana* (gil601843)	10/44	38.4/5.6 (21.1/7.7)	protein binding	0.5 R_{NI}/M_{NI} (0.049)
3103	glycine-rich RNA binding protein	75	*Medicago sativa* (gil6273331)	2/20	19.8/5.7 (10.8/4.5)	nucleic acid binding	0 R_{NI}/M_{NI} (0)
6505	far-red impaired response protein	89	*Oryza sativa* (gil42407459)	16/17	52.1/6.9 (82.0/8.9)	nucleic acid binding	0 R_{NI}/M_{NI} (0)
4508	elongation factor Tu, chloroplast precursor (EF-Tu)	223	*Pisum sativum* (gil6015084)	13/31	51.5/6.1 (53.1/6.6)	transcription/translation	0.5 R_{NI}/M_{NI} (0.033)
6305	retrotransposon protein, putative, unclassified	95	*Oryza sativa* (gil110288963)	24/16	38.0/6.7 (17.1/8.8)	transcription/translation	0.5 R_{NI}/M_{NI} (0.0375)
6506	reverse transcriptase	88	*Oryza sativa* (gil20279456)	21/17	55.9/6.9 (171.6/8.8)	transcription/translation	0 R_{NI}/M_{NI} (0)
7313	50S ribosomal protein L1, chloroplast precursor (CL1)	188	*Pisum sativum* gil1350625	10/57	40.4/7.3 (23.5/10.2)	transcription/translation	3.8 M_I/M_{NI} (0.0001), 0.34 R_I/M_I (0.0014)
1104	disease resistance response protein Pi49 (PR10)	359	*Pisum sativum* gil118933	12/72	19.8/5.3 (16.8/4.9)	defense response	5.5 R_I/R_{NI} (0)
2106	ABA-responsive protein ABR17	91	*Pisum sativum* (gil1703042)	8/57	19.7/5.5 (16.6/5.1)	defense response	7.1 R_I/R_{NI} (0)
5204	annexin-like protein	75	*Arabidopsis thaliana* (gil51969286)	9/31	29.7/6.5 (36.6/7.7)	stress response	5.7 M_I/M_{NI} (0.0006), 0.1 R_I/M_I (0)
6403	heat shock protein binding	71	*Arabidopsis thaliana* (gil15231204)	8/42	44.6/6.6 (17.5/10.0)	stress response	2.1 R_I/R_{NI} (0.0317), 0.4 R_{NI}/M_{NI} (0.0007)
2304	myosin class II heavy chain (ISS)	88	*Ostreococcus tauri* (gil116057040)	20/17	41.7/5.5 (134.3/5.9)	cellular processes	2.2 M_I/M_{NI} (0.0059), 0.5 R_I/M_I (0.0215)
3104	myosin class II heavy chain (ISS)	76	*Ostreococcus tauri* (gil116057804)	32/8	20.4/5.7 (468.5/6.2)	cellular processes	77.4 M_I/M_{NI} (0), 0 R_{NI}/M_{NI} (0)
1707	unknown protein	74	*Arabidopsis thaliana* gil15223730	22/10	76.1/5.3 (217.2/4.8)	unknown	∞ M_I/M_{NI} (0), 0 R_I/M_I (0)
2701	unnamed protein product	94	*Ostreococcus tauri* (gil116054844)	19/16	91.3/5.4 (108.8/5.4)	unknown	∞ M_I/M_{NI} (0), 0 R_I/R_{NI} (0)
3112	hypothetical protein	77	*Oryza sativa* (gil50508931)	13/29	21.5/5.7 (29.6/5.8)	unknown	2.4 M_I/M_{NI} (0.01), 0.4 R_{NI}/M_{NI} (0.0044)
3408	hypothetical protein MtrDRAFT_AC140774g5v1	74	*Medicago truncatula* (gil92870352)	6/48	43.1/5.9 (13.7/5.1)	unknown	∞ R_{NI}/M_{NI} (0)
4306	unknown protein	86	*Arabidopsis thaliana* (gil15238179)	14/25	37.0/6.0 (63.0/7.6)	unknown	0 R_I/M_I (0)
5416	hypothetical protein	76	*Oryza sativa* (gil53793527)	16/16	43.1/6.5 (100.5/10.7)	unknown	∞ R_{NI}/M_{NI} (0)
8201	hypothetical protein	96	*Arabidopsis thaliana* (gil4467113)	23/24	33.8/7.4 (174.7/5.1)	unknown	0 R_{NI}/M_{NI} (0)

[a] Experimental mass (M_r, kDa) and p*I* were calculated with PDQuest software (BioRad) and standard molecular mass markers. Theoretical values were retrieved from the protein database (NCBI). The software assigns a standard spot number to each spot (SSP). [b] PM: number of matched peptides with the homologous protein from database. Proteins with fragmented peptides sequences are given in boldface (see Supporting information containing data of peptide sequences). [c] The most drastic (more/less abundant) changes are given as normalized volume (calculated with PDQuest software) ratios: M_{NI} (Messire noninoculated), R_{NI} (Radley noninoculated), M_I (Messire inoculated), R_I (Radley inoculated). In brackets appears false discovery rate (FDR) values (0.05 threshold).

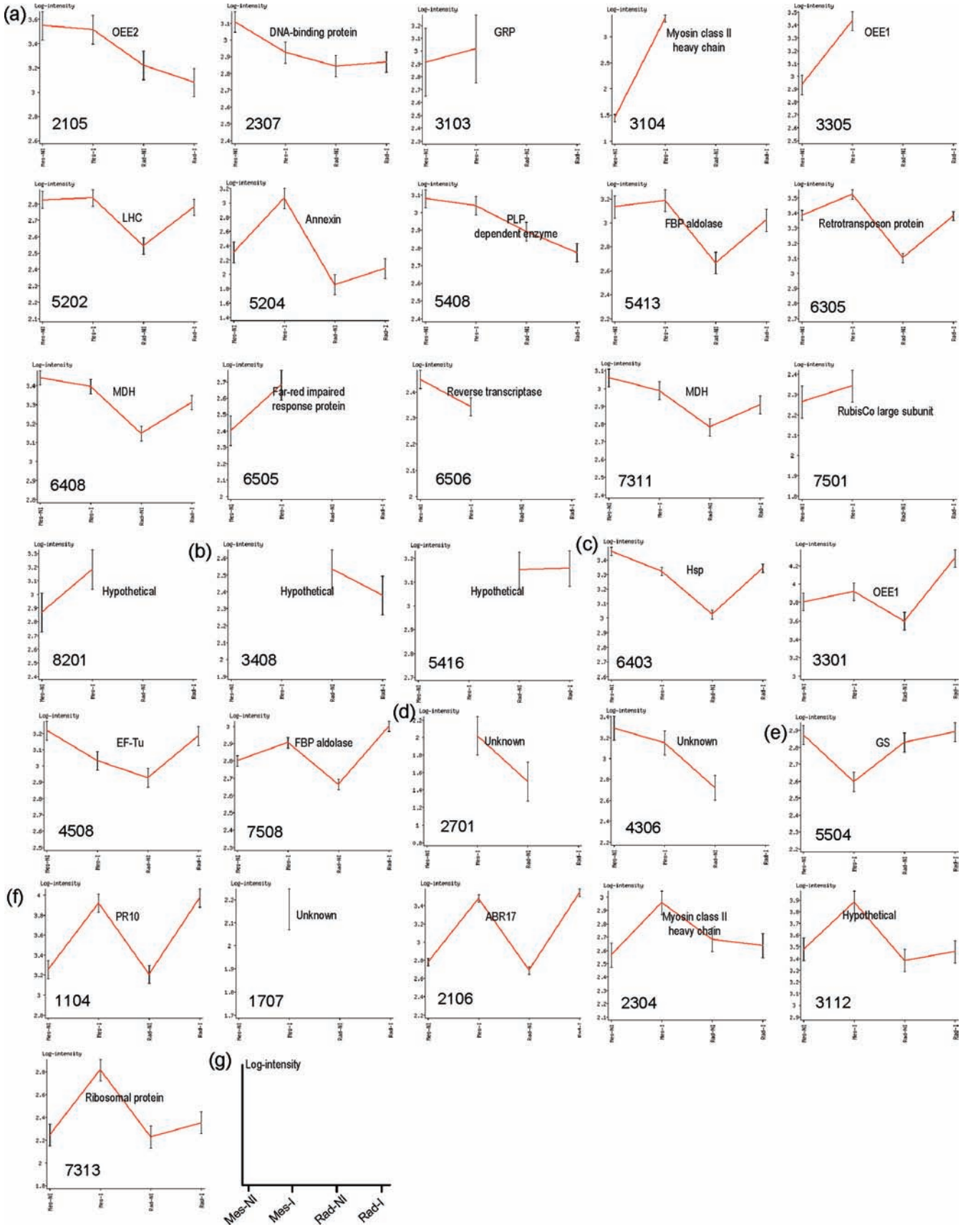

Figure 4. Mean log abundance intensities for protein spots identified by PCA and pairwise comparisons. (**a**) Protein spots positively correlated with PC1. (**b**) Protein spots negatively correlated with PC1. (**c**) Protein spots positively correlated with PC2. (**d**) Protein spots negatively correlated with PC2. (**e**) Protein spots positively correlated with PC3. (**f**) Protein spots negatively correlated with PC3. (**g**) Legend for graphs in panels a−f. The mean log intensity values were calculated from the sample replications.

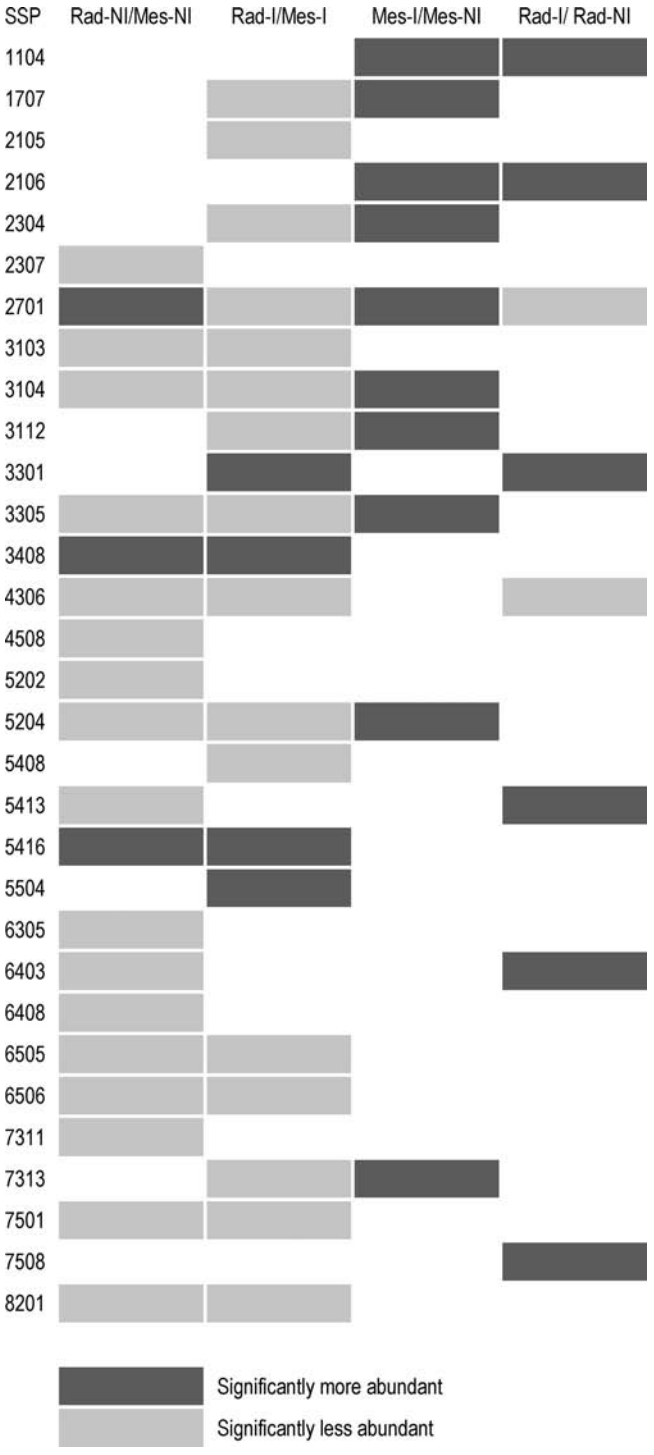

Figure 5. Expression patterns for 31 protein spots based on pairwise comparisons of their protein abundance values. Four pairwise comparisons were selected from hierarchical clustering results. The shading illustrates whether protein spots were more and less abundant. Two shades of gray were used to illustrate the significantly abundant (more or less abundant), or not significantly changed (white) in the respective pairwise comparison. The first column represents protein abundance changes when comparing both noninoculated cultivars, the second column comparing both inoculated cultivars, the third column inoculated versus noninoculated Messire cultivar, and the fourth column inoculated versus noninoculated Radley cultivar.

Molecular chaperones, also known as "heat-shock proteins" (Hsps), promote the correct folding and maturation of many other proteins in the cell. Small heat shock proteins (sHSPs) ranging from approximately 12 to 42 kDa, serve as a first line of defense against stress-induced cell damage by binding and maintaining denaturing proteins in a folding-competent state (*62*). We have identified an Hsp of 17.5 kDa that is more abundant in noninoculated Messire plants than in Radley, but that surprisingly increased in resistant plants after inoculation. The differential behavior of these stress-related proteins in both cultivars could contribute to different reaction mechanisms against *M. pinodes* inoculation.

Other proteins identified in this work correspond to different functional groups, and their relation to plant responses to parasitic plants is unclear. These are two myosin class II heavy chain (spots 2304 and 3104) belonging to the cellular processes category, which was found increased in inoculated susceptible plants. Myosins in plant cells are related to cell division, movement of mitochondria and chloroplast streaming, rearrangement of transvacuolar strands and statolith positioning (*63*). Recent proteomic studies have identified a myosin heavy chain-like protein accumulated in *in vitro* palms inoculated with the pathogen *Phoenix dactylifera* (*64*). Two other proteins belonging to the translation category, such as retrotransposon protein (spot 6305) and 50S ribosomal protein (spot 7313), were found more abundant in Messire cultivar. Finally, seven proteins were not able to be assigned to a known function (spots 1707, 2701, 3112, 3408, 4306, 5416 and 8201).

Based on the results obtained, we conclude that differences observed between noninoculated cultivars could be related to efficiency in energy utilization for growth and fitness purposes. With regard to the abundance pattern from differential identified proteins under inoculation we observed an increase of proteins involved in energetic and amino acid metabolism in the resistant cultivar, as well as a general increase in the amounts of proteins belonging to the defense and stress related category in both cultivars at the earliest stages of infection, coinciding with the moment the pathogen had reached the pea leaf mesophyll. A clear distinction between both cultivars cannot be made based on the defense and stress-related proteins identified in this study. However, most differences observed were related to efficiency in energy utilization, probably to compensate the cost of resistance, such as has been described in previous plant−pathogen interactions.

Data obtained in this work could help to study molecular aspects of the defense and resistance responses of legumes to Aschochyta blight, as well as being used in programs aimed at improving new crop varieties by means of plant breeding and biotechnology. Nevertheless, an integration of physiological and biochemical (genomics and high-throughput proteomics) approaches to study this plant−pathogen interaction, using a large collection of candidates, will be essential to elucidate target key elements of defense for *M. pinodes* infection.

ABBREVIATIONS USED

ABR, ABA-responsive protein; ANOVA, analysis of variance; CBB, Coomassie Brilliant Blue; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate; CHCA, α-cyano-4-hydroxycinnamic acid; DTT, dithiothreitol; EF-Tu, elongation factor Tu; FDR, false discovery rate; GRP, glycine-rich RNA binding protein; GS, glutamine synthetase; Hsp, heat shock protein; IEF, isoelectric focusing; IPG, immobilized pH gradient; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight; MDH, malate dehydrogenase; MS, mass spectrometry; OEE, oxygen-evolving enhancer protein; PCA, principal component analysis; PLP, pyridoxal 5-phosphate; PMF, peptide mass fingerprinting; PR, pathogenesis-related; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TFA, trifluoracetic acid; 2-DE, two-dimensional electrophoresis.

Supporting Information Available: A spreadsheet containing the data set of protein abundance intensity values for protein spots selected due to prospective differential expression behavior, a table containing the data of protein abundance cluster analysis based on PCA, a table containing the peptide sequences of identified proteins, and a representative 2-DE gel CBB stained from Messire plants in the 3−10 pH range. Most proteins were concentrated in the 5 to 8 pH range. This material is available free of charge via the Internet at http://pubs.acs.org.

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