Proteomic Analysis of *Phytophthora infestans* Reveals the Importance of Cell Wall Proteins in Pathogenicity*

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The oomycete *Phytophthora infestans* is the most harmful pathogen of potato. It causes the disease late blight, which generates increased yearly costs of up to one billion euro in the EU alone and is difficult to control. We have performed a large-scale quantitative proteomics study of six *P. infestans* life stages with the aim to identify proteins that change in abundance during development, with a focus on preinfection life stages. Over 10,000 peptides from 2061 proteins were analyzed. We identified several abundance profiles of proteins that were up- or downregulated in different combinations of life stages. One of these profiles contained 59 proteins that were more abundant in germinated cysts and appressoria. A large majority of these proteins were not previously recognized as being appressorial proteins or involved in the infection process. Among those are proteins with putative roles in transport, amino acid metabolism, pathogenicity (including one RXLR effector) and cell wall structure modification. We analyzed the expression of the genes encoding nine of these proteins using RT-qPCR and found an increase in transcript levels during disease progression, in agreement with the hypothesis that these proteins are important in early infection. Among the nine proteins was a group involved in cell wall structure modification and adhesion, including three closely related, uncharacterized proteins encoded by PITG_01131, PITG_01132, and PITG_16135, here denoted *Piacwp1*–3. Transient silencing of these genes resulted in reduced severity of infection, indicating that these proteins are important for pathogenicity. Our results contribute to further insight into *P. infestans* biology, and indicate processes that might be relevant for the pathogen while preparing for host cell penetration and during infection. The mass spectrometry data have been deposited to ProteomeXchange via the PRIDE partner repository with the data set identifier PXD002446. *Molecular & Cellular Proteomics* 16: 10.1074/mcp.M116.065656, 1958–1971, 2017.

*Phytophthora infestans* is a devastating oomycete plant pathogen that causes the disease late blight on potato and several related plants. It originated in South or Central America and reached Europe during the middle of the nineteenth century. The first described major outbreak culminated in the Great Irish Famine of 1845–1852 (1). Since then, it has been the most damaging pathogen for potato growers in Europe and North America. The costs of reduction in yield due to late blight and of measures to control the disease have been estimated to more than one billion euro in the EU alone (2). Current strategies for late blight control largely depend on regular application of a combination of fungicides. Developing high quality potato varieties with durable resistance has not been very successful. *P. infestans* has the capacity to rapidly change its repertoire of effectors and thereby escape recognition by resistant potato varieties.

*Phytophthora infestans* belongs to the oomycetes, a class in the stramenopile lineage that together with the alveolate and Rhizaria lineages forms SAR, a supergroup that is distinct from animals, plants and fungi (3). The asexual life cycle of *P. infestans* comprises six life stages (Fig. 1A) (4). Hyphae grown on agar medium or colonizing an infected leaf produce sporangiophores with air-borne sporangia, which in turn release motile zoospores. When the zoospores touch a barrier, they encyst. The cysts germinate, and when the tip of the germ tube encounters a hydrophobic surface like a leaf surface, an appressorium is formed. The appressorium adheres to the surface and is triggered to form a penetration peg that pierces the cuticle and penetrates the epidermal cell where an infection vesicle is formed. Thereafter, hyphae grow into the mesophyll layer, where they form an intercellular hyphal network.
and produce feeding structures called haustoria that penetrate the mesophyll cells to maximize nutrient uptake from the host. As the lesion expands *P. infestans* switches to a more destructive necrotrophic mode of growth in the center of lesion, while continuing to grow as a biotroph at the edges. Depending on the conditions, it takes about 4–7 days before sporangiophores emerge from the stomata and produce sporangia, which will spread and initiate new infections.

The fact that oomycetes and fungi, two major groups of filamentous plant pathogens, are not closely related implies that comparative analyses of oomycetes and fungi is limited in drawing conclusions about the molecular mechanisms underlying infection. This makes it important to acquire new information about the infection process at a molecular level. An improved understanding of the infection process is useful for the development of cultivars with durable resistance as well as new fungicides. A characterization of mRNA or protein abundance in different life stages can contribute important information about what differentiates the life stages from each other, and thus indicate which properties are critical during infection. Judelson et al. (5) performed a microarray analysis of several life stages using a set of expressed sequence tags (ESTs)** generated by Randall et al. (8). They found differences in expression of genes with several different functions, such as metabolism, regulation of DNA synthesis, cellular structure, pathogenicity, as well as several genes encoding known effector proteins.

Previous proteomics studies focused on *P. infestans* have been rather small-scale 2D gel based experiments. In two studies, screening of ~200 spots allowed identification of a small number of specific proteins that were more abundant during appressorium formation and were involved in amino acid biosynthesis or cellulose synthesis or that exhibited changes in abundance during preinfection stages (7, 8). In another study Grenville-Briggs et al. (9) specifically analyzed proteins from the cell wall of sporulating mycelium, nonsporulating mycelium and appressoria (9) using LC-MS/MS and could identify four proteins as unique to the *P. infestans* appressorium cell wall.

Proteomics studies on other *Phytophthora* spp. include the study of Savidor et al. who used a label-free approach to compare mycelium and germinating cysts in *P. sojae* and *P. ramorum* and could identify several candidate proteins distinguishing the preinfection stage from the mycelial stage (51). In addition, Meijer et al. performed an analysis of proteins from isolated *P. ramorum* cell walls. They identified 17 proteins, mostly mucins or mucin-like proteins, and glycose hydrolasers, but also two transglutaminases and one elicin (10).

These studies on *P. sojae* and *P. ramorum* did not consider appressorium.

To acquire a larger data set of proteins spanning the major stages of development in *P. infestans*, we have performed a large-scale quantitative analysis of *P. infestans* proteins. In this study, we compare the abundance of more than 2000 proteins in six life stages. By combining proteomics with techniques for validation using RT-qPCR and RNA interference, we further confirm the abundances and functional role of selected proteins.

**EXPERIMENTAL PROCEDURES**

*P. infestans* Cultivation and Isolation of Tissue—*P. infestans* strain 88069 was cultivated on rye sucrose medium (11). Hyphae, sporangia, zoospores, cysts, germinated cysts and appressoria were isolated essentially as described by Resjø et al. (12) and Grenville-Briggs et al. (8). Hyphae were grown for 5 days in liquid rye sucrose medium, harvested and washed briefly with water before freezing in liquid nitrogen. To produce sporangia, zoospores, cysts, germinated cysts, and appressoria, *P. infestans* was grown on rye sucrose agar plates. Sporangia were harvested after 14 days by flooding the plates with cold water and gentle rubbing. The sporangial suspension was removed and filtered through a 40-μm nylon mesh to remove hyphal remnants. Zoospore release was induced by incubation of sporangia at 10 °C for 2–3 h. Encystment was induced by vortexing for 2 min. Sporangia, zoospores and cysts were harvested by centrifugation (13,000 × g, 1 min). Cysts were incubated at 10 °C in Petri dishes to induce germination and formation of appressoria. Germinated cysts were harvested by centrifugation after 3–4 h of incubation. Appressoria were harvested after 24 h by removing the water, adding 1 ml of extraction buffer and scraping with a cell scraper. All samples were frozen after isolation.

Protein Extraction and Tryptic Digestion—Hyphal samples were ground in a mortar with liquid nitrogen. The other life stages were ground with a plastic pestle in an Eppendorf tube. Proteins from all *P. infestans* preparations were extracted in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.25% SDS, 50 mM sodium pyrophosphate, 1 mM sodium fluoride, 50 μM sodium orthovanadate, 5 nM Calycin A, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 20 μM leupeptin, and separated on a short SDS-PAGE. The lane was excised, washed and the proteins digested with trypsin (Promega Trypsin Gold, Mass Spectrometry Grade). The tryptic digest was desalted using 200 μl C18 StageTips (Proxeon Biosystems) according to the manufacturer’s instructions.

Mass Spectrometry—The tryptic digest, corresponding to 10 μg protein loaded on the preparative SDS-PAGE, was subjected to HPLC-MS/MS analysis using an Eksigent nanoLC2D HPLC system coupled to an LTQ Orbitrap XL ETD. The peptides were loaded onto a precolumn (Agilent Zorbax 300SB-C18, 0.3 mm ID, 5 mm, 5 μm particle size) connected to an in-house packed picofrit column (Agilent Zorbax 300SB C18, 75 μm ID, 150 mm, 3.5 μm particle size). The analytical column was equilibrated for 10 min using buffer consisting of 0.1% formic acid (FA), 5% ACN at a flow rate of 15 μl/min and the peptides were separated in an 0.1% FA buffer using a 55 min linear gradient from 5% to 40% ACN followed by a 5 min linear gradient from 40% to 80% ACN, at a flow rate of 350 nl/min. The eluted peptides were analyzed online using an LTQ Orbitrap XL ETD. The Orbitrap was operated in data dependent mode to automatically perform Orbitrap-MS and LTQ-MS/MS analysis. Survey scan spectra (400–2000 Da) were acquired using the Orbitrap mass analyzer with the resolution r = 60,000. Automatic gain control was enabled (Target value for LTQ MSn was 1 × 10⁴ and the target value for FT MS was...
The seven most intense ions were selected for fragmentation in the LTQ, using a mass window of 2 Da for precursor ion selection. The precursor ions were fragmented with a normalized collision energy of 35 (with activation Q set to 0.25 and an activation time of 30 ms). Dynamic exclusion was enabled with a repeat count of 2, a repeat duration of 20 s, an exclusion duration of 120 s, an exclusion list size of 499 and a 10 ppm exclusion mass width relative to both low and high.

**Experimental Design and Statistical Rationale**—Biological replicates were from separate cultures, totaling six hyphal, four sporangial, four zoospore, four cyst, three germinated cyst and six appressorial biological replicates. Each biological replicate was analyzed once, except for one of the hyphal samples, which was reanalyzed because of suspected poor HPLC performance, and all available replicates were used for peptide identification and feature matching. For the quantitative analysis, three samples were excluded because of poor HPLC performance, low protein content and contamination, respectively. Further details about the reason for excluding these files can be found in [supplemental Table S6](#). Given this design, and the label-free workflow with MS1 precursor matching that was used, there was no need for repeated technical replicates.

The statistical analysis of the proteomics data was performed using the software programs Qlucore Omics Explorer 3.0 (Qlucore, Lund, Sweden) and DanteR (13). Qlucore was used as a tool to identify abundance profiles among the MS peptide features. Feature data was imported into Qlucore, log transformed and a heat map was generated in which samples and MS features were ordered by hierarchical clustering. Peptides from different life stages were analyzed using Qlucore’s Multi Group comparison, (ANOVA with an F-test), followed by the adjustment of the p values for multiple comparisons using the Benjamini-Hochberg procedure for calculating the q-value (14, 15). Only peptides with q < 0.01 were used for the heat map. Protein-level statistical analysis was performed in DanteR. The method called “Model Based Filter/Impute/Anova” in Dante R is a statistical model for filtering and imputation of missing values, followed by ANOVA (16).

The method named “Protein-level Anova” in DanteR is an ANOVA-based method that combines peptide data to generate p values at the protein level and is described in detail in (17). ANOVA-based statistics were used because log-transformed peptide intensities in general follow a normal distribution.

**Analysis of Proteomics Data**—The raw data from the Orbitrap were converted to Mascot Generic Format (MGF) and mzML (18) using ProteoWizard (version 2.1.2430) (19). The Proteo software environment (2.20 dev build 4523) (20) was used to search the MGF files with Mascot (version 2.3.01) and X!Tandem (“Jackhammer” 2013.06.15) against a database consisting of all *P. infestans* proteins in UniProt as of 2010–04-22, concatenated with an equal size decoy database (random protein sequences with conserved protein length and amino acid distribution, in total 36,512 target and decoy protein entries) (21). Search tolerances were set to 7 ppm for MS and 0.5 Da for MS/MS. The enzyme used to generate the peptides was trypsin and one missed cleavage was allowed. Carbamidomethylation of cysteine residues was selected as a fixed modification and oxidation of methionine residues was selected as a variable modification. Search results were exported from Mascot as XML, and results, including the top ranked peptide for each spectrum, were imported to Proteios, where the peptide-spectrum level search results from X-tandem and Mascot were combined, and q values were calculated using the target-decoy method as described by Käll et al. (22). The search results were then filtered at a q-value of 0.01, to obtain a peptide-spectrum-match (PSM) false discovery rate of 1% in the filtered list. The mass, charge and peptide identification scores for all identified peptides can be found in [supplemental Table S5](#). The MS proteomics data have been deposited to the ProteomeXchange Consortium ([http://proteomcentral.proteomexchange.org](http://proteomcentral.proteomexchange.org)) (23) via the PRIDE partner repository with the data set identifier PXD002446.

For quantitative analysis, a label free approach based on precursor intensities was used (24) with all data processing steps performed within Proteios. The feature detection step was performed on mzML files using msinspect (25) and subsequent feature matching and alignment between LC-MS/MS runs with a previously described workflow (26). The quantitative, un-normalized data for all peptides used for the quantitative analysis is shown in [supplemental Table S1](#). The resulting peptide data was linearly normalized against the median sum of all features in all conditions. The normalized data was analyzed using the software programs Qlucore Omics Explorer 3.0 (Qlucore, Lund, Sweden) and DanteR (13). Qlucore was used as a tool to identify abundance profiles among the MS features. Feature data was imported into Qlucore, log transformed and a heat map was generated in which samples and MS features were ordered by hierarchical clustering. Peptides from different life stages were analyzed using Qlucore’s Multi Group comparison, (an Anova with an F-test), followed by the adjustment of the p values for multiple comparisons using the Benjamini-Hochberg procedure for calculating the q-value (14, 15). Only peptides with q < 0.01 were used for the heat map. The resulting heat map (shown in Fig. 1B) was then manually inspected to select abundance profiles of interest. Eight profiles that were considered of biological interest, or that were highly abundant were selected for protein level analysis. The abundance profiles and the rules for including a protein in them are described in detail in Table I. The DanteR software was used for protein-level analysis. In DanteR, the data was log transformed and then subjected to two different analyses. First, the “Model Based Filter/Impute/Anova” feature of DanteR was used. Because this procedure has been shown to filter out proteins that are unique to one condition (27) a second analysis was carried out in which all missing values were imputed to 1. A “protein-level Anova” was then performed (order peptides using median, minimum number of peptides = 2, maximum number of peptides = 10, no weighting). For both procedures, p values were adjusted using the Benjamini-Hochberg procedure for calculating the q-value (14, 15). In both analyses, peptides were grouped into proteins based on the first listed peptide ID if the peptide could be assigned to more than one protein. The lists of proteins identified as belonging to a given abundance profile by Model Based Filter/Impute/Anova or protein-level Anova were then combined. The profiles are listed in [supplemental Table S2](#).

**Functional Classification of Proteins**—The proteins in profile 8 were manually assigned to different functional groups based on their UniProt protein name (as in the database used for searching proteomics data, described above) (28). When insufficient information was available the domains identified by the InterPro domain search was used (version 60.0) (29).

**RNA Extraction**—RNA extraction was performed as described previously (9). Briefly, total RNA was extracted from frozen samples ground in liquid nitrogen using a Qiagen RNeasy Plant Mini kit following the manufacturer’s protocol. Samples were derived from a time-course of potato leaves (cultivar Bintje) inoculated with *P. infestans* strain 88069 or from leaf discs inoculated with individual *P. infestans* lines produced from regenerated dsRNA-treated protoplasts. Before cDNA synthesis all samples were DNase treated using the Ambion Turbo DNA-free kit, according to the manufacturer’s protocol. RNA samples were assessed for purity and integrity by agarose gel electrophoresis and Nanodrop Spectrophotometry. First strand cDNA was synthesized from 20 μg total RNA by oligo(dT) priming using the BioRad iScript cDNA synthesis kit according to the manufacturer’s recommendations.

**SYBR Green Quantitative RT-qPCR Assays**—Primer pairs ([supplemental Table S4](#)) were designed to anneal specifically to each of the
ten transcripts from *P. infestans* in *vitro* and *in planta* quantitative RT-qPCR analysis. RT-qPCR assays were performed as described previously (7, 9). Template cDNA was derived from mycelium grown for 72 h in liquid pea broth as well as from potato leaves inoculated with *P. infestans*. Samples were taken at 6, 12, 24, 36, 48, 60, and 72 h post inoculation (hpi). The actA and ef1α genes from *P. infestans* were used as constitutively expressed endogenous controls (30) and the abundance of each transcript in mycelium was determined relative to both the actA and the ef1α transcripts as described previously (8). Relative transcript abundance was determined independently for each housekeeping gene (actA and ef1α) and each analysis yielded similar results. The results of the RT-qPCR assays were analyzed using the modified Delta-Delta Ct method as described in (30). As described previously (9), transcript abundance across the infection time-course was determined by comparison of the level of expression of each gene with a calibrator sample, which was mycelium and which was assigned the value of 1.0 for each gene, to allow comparisons to be made across the time course, and between genes. RT-qPCR assays for the expression of genes throughout the lifecycle time-course were performed using three biological replicates, each containing three technical replicates. RT-qPCR assays for the expression of genes in silenced lines were performed on individual dsRNA-treated lines. As described previously (44), given the small amounts of biological material available from each dsRNA-treated line, the individual lines formed the biological replicates for the experiment, while two technical replicates were performed for each real-time RT-PCR assay. Transcript abundance in each of the dsRNA-treated lines was compared with a calibrator sample (C1), which was assigned a value of 1.0 to allow relative expression levels to be directly compared between individual dsRNA-treated lines.

**RNA Interference and Pathogenicity Assays—** Oligonucleotide primers were designed to amplify 200–250 bp amplicons from a family of three closely related genes (PITG_01131, PITG_01132, and PITG_16135), encoding a family of *Phytophthora infestans* Appressorium Cell Wall Proteins (here denoted Piacwp1–3). These three genes are the only members of an uncharacterized gene family in *P. infestans* and share 99% sequence similarity at the DNA level. Therefore, it was not possible to design gene specific RNAi amplicons or RT-qPCR primers to distinguish these three genes. Instead amplicons were unique to the three genes with no off-targets elsewhere in the genome. RNA interference (RNAi) assays were performed as described previously (7). Briefly, sufficient PCR reactions were performed to yield 5 μg of Piacwp1–3 PCR product to use in dsRNA synthesis. Synthesis of dsRNA was performed using the Megascript RNA interference kit (Ambion) according to the manufacturer’s protocol. Proteolysing and transfection were performed as described previously (7). After treatment with dsRNA (Piacwp1–3 or non-endogenous GFP control), single colonies from regenerates (typically 5–6 days after treatment) protoplasts were transferred to rye agar medium. Fourteen days after the exposure of protoplasts to dsRNA, these colonies were flooded with cold sterile distilled water to induce zoospore formation. After 3 h at 4 °C, the suspension was collected and used for leaf inoculations. Eight 10 μl droplets from each independent dsRNA-treated line were inoculated onto 3 detached potato leaves. The remainder of the zoospore suspension was incubated in Petri dishes at 11 °C for 16 h to induce appressorium formation in *vitro* for phenotypic analysis. Silenced lines were examined using standard light microscopy (inverted microscope) and cysts were counted and assessed for the production of normal or aberrant germ-tubes and appressoria.

For each independent dsRNA-treated line, four separate 7 mm leaf discs covering the site of four separate inoculation droplets were excised at 12 hpi (15 days after exposure to dsRNA) for RNA extraction. Subsequently real-time RT-PCR was used to determine the level of gene silencing in the Piacwp1–3 dsRNA-treated lines, relative to the level of Piacwp1–3 gene expression in the control (GFP dsRNA-treated) lines. Macrosopic disease symptoms were recorded and photographed 6 days post inoculation. Symptoms were scored on a scale of 0–4, modified from the established field scoring scale used to assess late blight symptoms in *planta* (31), with 0 representing no visible disease symptoms; 1 representing very low-level infection (0.01–1% of each leaf infected); 2 representing moderate (1–20%) infection; 3 representing substantial (25–50%) infection and 4 representing severe (more than 50%) infection. Dunn’s test was performed using R.

**RESULTS AND DISCUSSION**

**Proteome Changes in *P. infestans* Life Stages—** We analyzed protein abundance in *P. infestans* hyphae, sporangia, zoospores, cysts, germinated cysts and appressoria. The quantitative analysis included 2061 proteins. The peptide sequences and quantified intensities are shown in supplemental Table S1. Protein abundance profiles were identified after manual inspection of the heat map shown in Fig. 1B. We identified eight abundance profiles (Table I, supplemental Table S2). Out of the 2061 proteins analyzed, 618 proteins were allocated to one of the abundance profiles. Since we are particularly interested in the first point of direct contact between host and pathogen i.e. the initiation of infection, we decided to focus on profile 8, which contains 59 proteins more abundant in germinating cysts (G) and appressoria (A) (Table II).

**Proteins With Increased Abundance in Germinating Cysts and Appressoria—** The proteins in profile 8 display a general trend where most proteins that are highly abundant in appressoria begin to increase already in germinating cysts. This is clearly visible in Fig. 2A, which shows the intensities of all peptides from the 59 proteins significantly more abundant in germinating cysts and appressoria. The reason that the proteins in profile 8 tend to start increasing in abundance in germinated cysts and then increase even more in appressoria is possibly that expression of the genes encoding proteins involved in appressorial function starts in the preceding tissue, germinating cysts. Classification based on predicted functions revealed that the most common roles of the 59 proteins were transport and energy metabolism. Other well-represented classes are those involved in cell wall synthesis and redox maintenance and signaling (Fig. 2B and Table II). Out of the 59 proteins, 20 were identified as phosphoproteins in our previous analysis (12). This is a rather large fraction, considering that not all proteins are phosphorylated and that the proteins in the previous analysis were identified after a phosphopeptide enrichment step, something that greatly affects which subset of the proteome will be identified. The overlap between the proteins we have identified as highly abundant in germinating cysts and appressoria, and proteins identified in previous proteomics studies of *P. infestans* is limited to five proteins involved in the structure and remodel-
ing of the cell wall. These proteins and other selected proteins among those upregulated in appressoria are discussed in the following sections.

**Transport and Energy Metabolism**—Two of the largest groups of proteins were those related to energy metabolism or transport. We discuss these groups together here, since it is likely that many of them are expressed as a reaction to the starvation conditions necessary for the induction of appressorium formation in vitro. This starvation will trigger the synthesis of a number of proteins as an adaptation to the lack of nutrients. It is reasonable that proteins that help *P. infestans* import and metabolize nutrients will increase in abundance for this reason. Nevertheless, it is plausible that some of these proteins are upregulated during the early stages of plant infection, since germinating cysts are effectively under starvation before entering the plant when host resources are not yet accessible for the pathogen. During infection, germinating cysts and appressoria need to mobilize stored energy reserves, export cell wall components and defend against host derived toxic compounds. Transport proteins may be involved in all such processes. Intriguingly, most of the proteins that were found to be phosphorylated in our previous study were derived from the metabolism or membrane transport groups. This probably reflects the high abundance of these proteins and the need for precise regulation of their activity.

**Amino Acid Metabolism**—Six proteins involved in amino acid metabolism were upregulated in germinating cysts and appressoria. The corresponding genes are predicted to encode anthranilate phosphoribosyltransferase (PITG_17032), serine hydroxymethyltransferase (PITG_06427), asparagine synthetase (PITG_13399), glyoxylate/hydroxypyruvate reductase A (PITG_16076), mitochondrial glycine dehydrogenase (PITG_15850), and a putative uncharacterized protein (PITG_08178), which we classify as being involved in amino acid metabolism since an Interpro (https://www.ebi.ac.uk/interpro/) search reveals that it contains a tyrosinase domain. This is consistent with previous observations that free amino acids accumulate during *P. infestans* appressorium formation, and that several proteins involved in amino acid biosynthesis are also upregulated at this stage of the lifecycle (8).

**Redox Sensing and Defense**—Another distinct group of proteins with increased abundance in the preinfectious life stages were those linked to the sensing of and defense against reactive oxygen species (ROS) (Fig. 2). Like the proteins involved in transport and energy metabolism, it is possible that these proteins are induced by starvation, since genes involved in the defense against ROS have been shown to be upregulated in response to starvation and other types of stress (32). Moreover, the plant hypersensitive response, a defense mechanism, will involve the production of reactive oxygen species (33), and pathogens will need to detect and defend themselves to infect successfully. One of these proteins (PITG_14492) is a member of the NmrA-like family. Members of this family act as redox sensors and regulators of...
transcription (34, 35), but have also been shown to be induced by nitrogen starvation (36). Thus, NmrA-like proteins might detect the degree of hypersensitive response and contribute to the regulation of the pathogen’s defense against it, but it is also possible that they are induced by starvation. Three proteins with antioxidant activity that also showed higher abundance were pyridine nucleotide-disulfide oxidoreductase (PITG_04690), thioredoxin-like protein (PITG_06657) and glutathione peroxidase (PITG_18316). They may serve as a defense against the oxidative burst by reducing ROS. Finally, the set included two proteins with glutathione S-transferase domains (PITG_12959 and PITG_12958). Glutathione S-transferase conjugates reduced glutathione to oxidation products and xenobiotics (37). These proteins may therefore play a role in cellular detoxification of proteins damaged by the oxidative burst.

Pathogenicity—Among the pathogenicity-related proteins that were more abundant in our analysis is a single RXLR effector (PITG_09218). P. infestans has 563 genes predicted to encode RXLR effectors (38). These effectors consist of a conserved N-terminal RXLR domain that is required for translocation into the host cell and a C-terminal domain that is highly diverse and shares no homology with known proteins. So far, only a few RXRL effectors have been associated with a biochemical or cellular function (38). All P. infestans avirulence genes discovered so far encode RXLR proteins. The higher abundance of the RXLR effector PITG_09218 in the preinfection life stages indicates that it is an early effector. However, its function remains to be elucidated. Interestingly, we did not identify any other RXLR effector, despite the large number of genes. There are probably two reasons for this. Firstly, not all RXLR effectors will be expressed during the preinfectious life stages. Secondly, many RXLR effectors have comparatively low abundance, making them difficult to detect with a shotgun proteomics approach such as ours (38). Among the other putative pathogenicity-related proteins there

| Profile number | Profile name                                      | Criteria for selection                                                                 | Number of proteins |
|----------------|---------------------------------------------------|----------------------------------------------------------------------------------------|--------------------|
| 1              | Up in hyphae                                       | Higher levels of protein in hyphae than in all other life stages, with q-values for the comparisons < 0.05 | 134                |
| 2              | Down in hyphae                                     | Lower levels of protein in hyphae than in all other life stages, with q-values for the comparisons < 0.05 | 298                |
| 3              | Down in sporangia                                  | Lower levels of protein in sporangia than in all other life stages, with q-values for the comparisons < 0.05 | 5                  |
| 4              | Up in cyst (compared to hyphae, sporangia and zoospores) | Higher levels of protein in cysts than in hyphae, sporangia and zoospores, with q-values for the comparisons < 0.05 | 20                 |
| 5              | Down in hyphae and sporangia                       | Lower levels of protein in hyphae and sporangia combined (i.e. sporangia and hyphae were treated as one group in the comparison) than in all other life stages, with q-values for the comparisons < 0.05 | 64                 |
| 6              | Up in sporangia and zoospores                     | Higher levels of protein in hyphae and sporangia combined (i.e. sporangia and hyphae were treated as one group in the comparison) than in all other life stages, with q-values for the comparisons < 0.05 | 107                |
| 7              | Down in sporangia and zoospores                   | Lower levels of protein in sporangia and zoospores combined (i.e. sporangia and zoospores were treated as one group in the comparison) than in all other life stages, with q-values for the comparisons < 0.05 | 21                 |
| 8              | Up in appressoria and germinated cysts             | Higher levels of protein in appressoria than in hyphae, sporangia, zoospores and cysts, with q-values for the comparisons < 0.05 | 59                 |
| UniProt ID  | Protein name                                      | Gene ID        | Classification |
|------------|--------------------------------------------------|----------------|----------------|
| D0N036     | Endomembrane protein 70-like protein, putative   | PITG_03376     | Adhesion       |
| D0N085     | Thrombospondin-like protein                      | PITG_16981     | Adhesion       |
| D0RM15     | Croquemort-like mating protein, putative         | PITG_22562     | Adhesion       |
| D0N4U8     | Serine hydroxymethyltransferase (EC 2.1.2.1)     | PITG_06427     | Amino acid metabolism |
| D0N9N0     | Putative uncharacterized protein                 | PITG_08178     | Amino acid metabolism |
| D0NL58     | Asparagine synthetase (EC 6.3.5.4)               | PITG_13399     | Amino acid metabolism |
| D0N841     | Glycine dehydrogenase, mitochondrial             | PITG_15850     | Amino acid metabolism |
| D0M5U7     | Putative uncharacterized protein                 | PITG_01131     | Cell wall      |
| D0N0L9     | Glycosyl transferase, putative                   | PITG_04125     | Cell wall      |
| D0N841     | Exoglucanase 1                                   | PITG_06788     | Cell wall      |
| D0N9N0     | Cell 5A endo-1,4-betaglucanase                   | PITG_08613     | Cell wall      |
| D0N1H2     | Transglutaminase elictor M81D                    | PITG_16959     | Cell wall      |
| D0N841b    | Translocation protein SEC63                     | PITG_02467     | Cellular trafficking |
| D0L9C      | Ubiquitin-ribosomal fusion protein, putative     | PITG_09555     | Cellular trafficking |
| D0L9C      | Putative uncharacterized protein                 | PITG_13373     | Cellular trafficking |
| D0L9C      | Putative uncharacterized protein                 | PITG_13374     | Cellular trafficking |
| D0L9C      | Lipase, putative                                 | PITG_01186     | Energy/metabolism |
| D0N5G      | Glycerol-3-phosphate dehydrogenase               | PITG_04065     | Energy/metabolism |
| D0N841     | Glucokinase, putative                           | PITG_06016     | Energy/metabolism |
| D0N841     | Mitochondrial Carrier (MC) Family               | PITG_12854     | Energy/metabolism |
| D0N841     | UDP-glucose 6-dehydrogenase (EC 1.1.1.22)       | PITG_18375     | Energy/metabolism |
| D0L9C      | Haustorium-specific membrane protein, putative   | PITG_00375     | Other          |
| D0N841     | Pyridoxal biosynthesis lyase pxDS                | PITG_00471     | Other          |
| D0L9C      | L-gulonolactone oxidase, putative                | PITG_05465     | Other          |
| D0L9C      | Quinolinate synthetase A protein, putative       | PITG_06783     | Other          |
| D0R3A5     | 12-oxyphytodienoate reductase, putative          | PITG_08491     | Other          |
| D0MIX1     | Oligopeptidase A, putative                       | PITG_02711     | Pathogenicity  |
| D0N841     | Secreted RXLR effector peptide protein, putative | PITG_09218     | Pathogenicity  |
| D0N841     | IpiB3-like protein                               | PITG_11341     | Pathogenicity  |
| D0N841     | Serine protease family S10, putative             | PITG_11525     | Pathogenicity  |
| D0MST4a    | Putative uncharacterized protein                 | PITG_00070     | Redox          |
| D0N1T7     | Pyridine nucleotide-disulphide oxidoreductase,    | PITG_04690     | Redox          |
| D0N841     | Thioredoxin-like protein                         | PITG_06657     | Redox          |
| D0N841     | Putative uncharacterized protein                 | PITG_12958     | Redox          |
| D0N841     | Putative uncharacterized protein                 | PITG_12959     | Redox          |
| D0N841     | NmrA-like family protein, putative               | PITG_14492     | Redox          |
| D0N841     | Glutathione peroxidase                           | PITG_18316     | Redox          |
| D0MIX2     | Putative uncharacterized protein                 | PITG_01973     | Signaling      |
| D0N841     | Putative uncharacterized protein                 | PITG_04908     | Signaling      |
| D0N841     | Putative uncharacterized protein                 | PITG_07041     | Signaling      |
| D0N841     | Putative uncharacterized protein                 | PITG_11676     | Signaling      |
| D0N841     | Putative uncharacterized protein                 | PITG_02409     | Transport      |
| D0N841     | Putative uncharacterized protein                 | PITG_03822     | Transport      |
| D0N841     | Vacuum amino acid transporter, putative          | PITG_06611     | Transport      |
| D0N841     | Major Facilitator Superfamily (MFS)              | PITG_06673     | Transport      |
| D0N841     | ATP-binding Cassette (ABC) Superfamily           | PITG_07177     | Transport      |
| D0N841     | Glucose transporter, putative                    | PITG_13003     | Transport      |
| D0N841     | ATP-binding Cassette (ABC) Superfamily           | PITG_13575     | Transport      |
| D0N841     | ATP-binding Cassette (ABC) Superfamily           | PITG_13579     | Transport      |
| D0N841     | Putative uncharacterized protein                 | PITG_07390     | Unknown        |
| D0N841     | Putative uncharacterized protein                 | PITG_07409     | Unknown        |
| D0N841     | Putative uncharacterized protein                 | PITG_11060     | Unknown        |
| D0N841     | Putative uncharacterized protein                 | PITG_11189     | Unknown        |
is a putative oligopeptidase (PITG_02711) and a serine protease (PITG_11525) that might be capable to degrade apoplastic host proteins during the first stages of infection. There is also an IpiB3-like protein (PITG_11341). IpiB proteins are glycine-rich proteins that were already discovered in 1994, in a screen for genes upregulated in the early stages of the \textit{P. infestans}-potato interaction, so during or shortly after infection (39, 40). As yet, the function of IpiB proteins is not known, but based on their glycine-rich nature it is tempting to speculate that they are structural proteins that play a role in establishing infection structures during the early stages of infection.

Cell Wall Structure and Adhesion—A large group of identified proteins are those related to membrane maintenance, cell wall synthesis and cellular adhesion. Germination of cysts and the formation of appressoria require extensive structural changes of both the cell wall and the membrane (7, 41). The appressorium adheres to the leaf surface. It acts as a focal point to secrete cell wall degrading enzymes to be able to penetrate the leaf surface (5, 7) and the cell wall must be adapted to these functions. This makes the proteins in this group particularly interesting, since they might be important for the ability of a hemibiotrophic pathogen like \textit{P. infestans} to infect its host.

Endomembrane protein 70 (EMP70) (PITG_03376) belongs to the TM9 family. This protein family is important for cellular adhesion in many biological systems, and has been proposed to control the level of adhesion proteins present at the cell surface in the amoebae \textit{Dictyostelium} (42, 43). Thrombospondin-like protein (PITG_16981) has been shown to be involved in adhesion in \textit{P. cinnamomii} (44), and is predicted to be secreted (45). This protein was also among the proteins found to be phosphorylated in our previous study (12), although the functional role of the phosphorylation has not been investigated. Croquemort-like mating protein (PITG_22562) contains a CD36 domain. CD36 is a so-called scavenger receptor which recognizes lipid and lipoprotein components of cell walls and mediates the uptake of cells and molecules as well as acting as a receptor for thrombospondin 1 (46). Thus, croquemort-like mating protein may act as a receptor for secreted thrombospondin-like protein or for molecular patterns on the leaf surface. The haustorium-specific membrane protein Pihmt1 (PITG_00375) was first described by Avrova \textit{et al.}, as one of the putative pathogenicity factors discovered in a suppression subtractive hybridization cDNA library (47). Using transient silencing, they demonstrated that this protein is critical for successful infection. Although the exact function is unknown it is believed to provide physical stability to the plasma membrane of \textit{P. infestans} infection structures (47).

One putative uncharacterized protein (PITG_01973) containing a lectin domain was also identified. Because lectin domains are carbohydrate binding, it is possible that this protein may be involved in adhesion to the host cell wall. Because the well-known effector CBEL also contains a lectin domain we aligned the protein sequence of PITG_01973 with that of CBEL from \textit{Phytophthora parasitica} (UniProt ID O42830). The alignment revealed only 3.4% identity and 9.0% similarity, but among the identical amino acids were F367 and Y368 from PITG_01973, which are homologous to F187 and Y188 of \textit{P. parasitica} CBEL. These two amino acids are in one of the cellulose binding domains (CBD) of CBEL and mutating Y188 to alanine has been demonstrated to reduce CBEL necrosis-inducing activity in planta (48), indicating that these residues are important for the ability of CBEL to function as an elicitor. However, a search of the SMART database (http://smart.embl-heidelberg.de/) identified neither a CBD, nor an Apple domain, the two domains comprising CBEL (49). In our opinion this makes it less likely that PITG_01973 has the same biological role as CBEL.

Several proteins with putative roles in cell wall synthesis and remodeling were also identified as upregulated during cyst germination and appressorium formation. In a previous \textit{P. infestans} cell wall proteomics study aimed at identifying specific components of the germinating cyst and appressorium cell wall proteome (9), five proteins were identified namely glycosyl transferase (PITG_04125), cell 5A endo-1,4-beta-glucanase (PITG_08613), exoglucanase 1 (PITG_06788), transglutaminase elicitor M81D (PITG_16959), and an uncharacterized gene family (PITG_01131, PITG_01132, PITG_16135.2). Detailed information regarding this can be found in supplemental Table S1.
observed later in the infection cycle (Fig. 3). Interestingly, one of these, the uncharacterized protein PITG_13374, was also found to be phosphorylated in our previous study, together with the closely related PITG_13373 (12). The function of these proteins is not known, but they contain a PX domain which is a phosphoinositide-binding structural domain involved in targeting of proteins to cell membranes (10).

**Transient Silencing of Genes Encoding Putative Cell Wall Proteins that are Abundant in Appressoria**—To investigate the biological importance of selected proteins for pathogenicity, we used RNA interference to transiently silence three genes that belong to the same family (PITG_01131, PITG_01132, and PITG_16135) and encode putative cell wall proteins that are highly abundant in germinating cysts and appressoria. We have named these proteins PiACWP1, PiACWP2, and PiACWP3 respectively. These proteins are small, acidic (35 kDa; pl 4.62) and identified as appressorium proteins both in the current study and a previous study (9). They are uncharacterized but because the previous study included purification steps to enrich for cell wall proteins we assume they are cell wall associated. Similarity and domain-profile searches indicate no matches to proteins or domains of known function. However, significant sequence similarity was observed with carbohydrate binding proteins from appressoria producing fungi and oomycetes (9). Therefore, we hypothesize an important structural role in the formation or function of *P. infestans* appressoria.

*P. infestans* protoplasts were transfected with dsRNA targeted to all three *Piacwp* genes simultaneously and as a negative control lines were generated with treatment with dsRNA from GFP. Fourteen lines treated with *Piacwp1–3* dsRNA and 8 lines treated with GFP dsRNA were generated, checked for gene silencing by RT-qPCR and assessed for their ability to cause infection in detached leaf assays (Fig. 4A, 4B). The majority (11) of the lines treated with *Piacwp1–3* dsRNA displayed significantly lower expression of *Piacwp1–3* than the control lines (Fig. 4A) indicating successful silencing had taken place. Within those eleven independent lines, gene expression was reduced by between 48 and 95% compared with the calibrator control sample (Fig. 4A). Three independent *Piacwp1–3* dsRNA treated lines showed similar *Piacwp1–3* expression levels to the control lines (Fig. 4A).

A scoring scale, from 1 (very low infection) to 4 (severe infection), was used to assess the pathogenicity of each independent line. At 6 dpi, all control (GFP dsRNA-treated) lines showed substantial or severe infection and were thus scored either 3 or 4 respectively (Fig. 4B). In contrast, eleven of the independent lines treated with *Piacwp1–3* dsRNA showed only low to moderate infection while only three (lines 12, 13, and 14) exhibited infection at a similar level to that seen in the control lines (Fig. 4B). Interestingly, these three lines were the same lines that showed similar *Piacwp1–3* transcript abun-
dance as control lines (Fig. 4A). The severity of infection was significantly different between leaves infected with \textit{P. infestans} lines with low levels of expression compared with leaves infected with lines with high levels of expression. (Fig. 4C).

The impaired ability of the \textit{P. infestans} lines with low levels of expression to infect potato leaves indicates a role for these proteins in the infection process.

To further investigate if this role is mediated by a contribution to the formation of germinating cysts and appressoria, we observed the morphology of germinating cysts and appressoria from each of the independent lines (Fig. 5A). In the lines treated with Piacwp1–3 dsRNA we observed that a proportion of the cysts showed abnormal phenotypes either in germinating cysts and/or during appressorium formation (Fig. 5B, 5C).

In several of the Piacwp1–3 dsRNA treated lines we observed varying proportions of cysts which produced either multiple germ tubes, or germ tubes that exhibited altered polarity of growth, appearing twisted and misdirected (Fig. 5A). Neither of these abnormal germinating cysts went on to produce appressoria in vitro. We also observed that a fraction of the cysts that could germinate normally, produced appressoria that were also altered in their polarity and which for example
often appeared twisted (Fig. 5A), or resembled the appressorium-like structures produced after silencing of the CesA (cellulose synthase) gene family. CesA plays an important role in the structural integrity of the appressorium cell wall (7). Lines treated with GFP dsRNA did not produce any abnormal appressoria and only a small number of abnormal cysts were

Fig. 4. Reduction in infection severity after transient silencing of the genes Piacw1–3. Photographs were taken after allowing cysts to germinate for 16 h in water at 11 °C. A, Normalized relative expression of Piacw1–3 in GFP dsRNA-treated lines (C1-C8) and 14 independent Piawp1–3 dsRNA-treated lines. B, Severity of infection of the individual lines. Each line was used to infect three separate leaves and the severity of infection was scored, with 1 representing very low levels of infection and 4 representing severe infection. A representative picture of each line is shown. C, Normalized relative expression value for each line by disease score. Red dots in the scatterplot correspond to Piawp1–3 dsRNA-treated lines and green dots correspond to GFP dsRNA-treated lines. The black bars indicate the median normalized expression value for each score category. p values are calculated using Dunn’s test and adjusted for multiple comparisons according to Benjamini-Hochberg.
produced in one line (Fig. 5). This suggests that the PiACWP proteins are involved in germ tube and appressorium formation and thus, silencing them directly impacts the ability of *P. infestans* to initiate infection.

**CONCLUSIONS**

We applied quantitative proteomics to identify proteins in *P. infestans* that are potentially important in preinfection life stages. These included several proteins involved in cell wall formation. Nine of these proteins are also likely to play a significant role in early infection since the corresponding transcripts were highly abundant during appressorium formation on leaves and in the early stages of infection. By RNA interference we verified one group of proteins to be important for successful infection, being involved in proper formation of germinated cysts and appressoria. Our data contributes further insight into the molecular and cellular processes underlying the development of infection structures in the oomycete *P. infestans*, a devastating pathogen that causes enormous losses in potato and tomato culture. The increased awareness of the effects of fungicides and the phasing out of various effective compounds makes it important to better understand the infection process to provide suitable targets for the development of novel compounds. With our approach, we show that shotgun proteomics combined with validation of the identified proteins is instrumental to accelerate the process of target discovery.

**Fig. 5. Morphology of germinated cysts and appressoria.** A, Examples of normal and abnormal germinated cysts and normal and abnormal appressoria, respectively. B, The fraction of abnormal germinated cysts (expressed as percentage of all germinated cysts) in GFP dsRNA-treated lines (C1-C8) and *Piacwp1–3* dsRNA-treated lines (1–14). C, The fraction of abnormal appressoria (expressed as percentage of all appressoria) in GFP dsRNA-treated lines (C1-C8) and *Piacwp1–3* dsRNA-treated lines (1–14).
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