Profiling the Secretome and Extracellular Proteome of the Potato Late Blight Pathogen *Phytophthora infestans*

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Oomycetes are filamentous organisms that cause notorious diseases, several of which have a high economic impact. Well known is *Phytophthora infestans*, the causal agent of potato late blight. Previously, *in silico* analyses of the genome and transcriptome of *P. infestans* resulted in the annotation of a large number of genes encoding proteins with an N-terminal signal peptide. This set is collectively referred to as the secretome and comprises proteins involved in, for example, cell wall growth and modification, proteolytic processes, and the promotion of successful invasion of plant cells. So far, proteomic profiling in oomycetes was primarily focused on subcellular, intracellular or cell wall fractions; the extracellular proteome has not been studied systematically. Here we present the first comprehensive characterization of the *in vivo* secretome and extracellular proteome of *P. infestans*. We have used mass spectrometry to analyze *P. infestans* proteins present in seven different growth media with mycelial cultures and this resulted in the consistent identification of over two hundred proteins. Gene ontology classification pinpointed proteins involved in cell wall modifications, pathogenesis, defense responses, and proteolytic processes. Moreover, we found members of the RXLR and CRN effector families as well as several proteins lacking an obvious signal peptide. The latter were confirmed to be *bona fide* extracellular proteins and this suggests that, similar to other organisms, oomycetes exploit non-conventional secretion mechanisms to transfer certain proteins to the extracellular environment. *Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.035873, 2101–2113, 2014.*

Phytophthora infestans, the causal agent of tomato and potato late blight, is one of the most notorious plant pathogens in modern history. It was responsible for the Irish Potato Famine in the mid-19th century and recurrent outbreaks have been reported ever since. The *Phytophthora* genus comprises over hundred plant pathogenic species and belongs to the oomycetes, a lineage with filamentous organisms that morphologically resemble fungi but are more closely related to brown algae and diatoms (1, 2).

To facilitate growth, cell wall assembly, cell wall modification, and acquisition of nutrients, organisms require extracellular proteins. Prominent extracellular proteins are hydrolytic enzymes such as proteases, lipases, and glycosyl hydrolases, which digest complex substrates into small units that act as nutritional sources. Pathogen derived proteins facilitate host tissue degradation resulting in colonization or invasion, and they are considered to act as pathogenicity factors (3). Microbial pathogens also need an extensive set of proteins that play a role in host-pathogen interplay. For plant pathogens, these proteins are required during penetration and colonization of the plant tissue and are frequently referred to as effector proteins (3). The genomes of *Phytophthora* spp. encode hundreds of such putative effector proteins (4, 5). Two groups of effectors, apoplastic and cytosolic, are discerned dependent on the site of action. Among apoplastic effectors are protein inhibitors, secreted to counteract apoplastic host plant derived proteins, and hydrolytic enzymes such as proteases. Other apoplastic effectors interfere with the host membrane-cell wall integrity and can trigger host cell death (3, 6). Cytoplasmic effectors translocate into the plant cell, targeting various subcellular compartments where they modulate plant cell signaling, suppress immunity, and metabolic processes in the plant cytosol and nucleus for the pathogens benefit (7). In *P. infestans* these predicted host-translocated effectors encompass the RXLR (short for the four amino acids that form the motif, Arginine, Any, Leucine, and Arginine) and CRN (crinkling and necrosis inducing)[1] effectors.

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[1] The abbreviations used are: CRN, crinkling and necrosis producing; PLO, phospholipase D.

These are large and complex protein families, with around 560 RXLRs and 200 CRNs members encoded in the genome (4). Apoplastic and cytosolic effector classes are mostly small modular proteins that contain an N-terminal signal peptide to facilitate secretion. Their C-terminal part comprises additional effector modules including host targeting signals, as is the case for RXLRs and CRNs, and a functional domain exerting its function (8). Both RXLR and CRN′s were originally identified as inducers of plant cell death and defense-related gene expression during *in planta* expression (3, 9) although not all CRNs promote infection (10). Effector genes frequently have distinct patterns of expression during various life stages and colonization of host plants (4).

Phytophthora research in the last decade benefitted largely from high-throughput bioinformatics tools. EST mining resulted in the identification of various putative extracellular proteins (9). With the elucidation of various *Phytophthora* and other oomycete genomes, a wealth of information was retrieved from genome sequences by *in silico* gene annotation (4, 5, 11–14). Genome mining resulted in the identification of many novel genes and a large repertoire of potential virulence factors (4, 5, 15, 16). In the *P. infestans* genome, a genome-wide inventory of genes encoding proteins with a signal peptide resulted in the initial identification of 2228 candidates, later refined to 1415 secretome proteins, many of which are potential pathogenicity factors (17). The *in silico* refinement was based on the archetypal secretion pathway, and, thus, it consisted in scoring for presence or absence of a signal peptide in combination with cellular compartment prediction and presence of transmembrane domains. There are several limitations in this *in silico* approach. Firstly, accurate gene annotation is essential. N-terminal inaccuracies result in signal peptide detection failures whereas other erroneous predictions can result in the misinterpretation of transmembrane domains or targeting sequences, which would lead to including or excluding them from the predicted secretome. Secondly, signal peptide sequences are extremely heterogeneous and weakly predicted ones were excluded. In addition, the term “secretome” is frequently misinterpreted as the extracellular proteome whereas it is limited to the collection of signal peptide containing proteins that are handled via the endoplasmic reticulum and Golgi apparatus before secretion (18). Many proteins identified in the plant cell apoplast belong to leaderless secretory proteins (LSP) (19, 20) and similar findings have been reported for fungi and animals (21, 22). Meanwhile, several unconventional protein secretion systems have been described including self-sustained protein translocation, ABC-transporter based secretion, exosome/autophagosome mediated secretion, and microvesicle shedding/blebbing (19, 22–26). It can be therefore anticipated that similar mechanisms exist in oomycetes.

One of the most powerful methods to evaluate the final outcome of gene expression is the identification of the resulting proteins using proteomics. This approach has frequently been applied in fungi to elucidate the proteome and secretome under various conditions including plant-pathogen interactions (27, 28). Using this technology, however, only limited information has been gained in *Phytophthora.* In *P. palmivora* three actin isoforms were identified by proteomics (29). Four enzymes, involved in amino acid biosynthesis were retrieved from *P. infestans* (30). Thirteen proteins with a life stage specific expression pattern were identified by 2D-gel electrophoresis, including CRN2 (31). For *P. sojae* and *P. ramorum,* a global proteomic approach was used to detect proteomic differences between life stages (32, 33), and a recent large-scale phosphoproteome analysis revealed the phosphorylation status of thousands of proteins and provided novel information on life stage specific phosphorylation events in *P. infestans* (34). Despite their importance, proteomic studies on *Phytophthora* extracellular proteins are even more limited. The identification of individual extracellular protein components in culture filtrates was described for elicitins (35–38), CBELs (39), and glucanase inhibitor proteins (GIPs; (40, 41)). Proteomic analysis of secreted proteins of *P. infestans* cultures grown on a synthetic medium resulted in the unambiguous identification of nine signal peptide containing proteins (9). Studies on cell wall located proteins of *P. ramorum* and *P. infestans* revealed the presence of effector proteins or pathogen-associated molecular pattern molecules, either as part of the incorporated or immobilized moiety. In addition, proteins both with and without predicted signal peptide were identified (42, 43). Despite these efforts, a more comprehensive overview of the *in vivo* extracellular proteome of *P. infestans*, or any other oomycete, is currently lacking.

Here we describe the *in vivo* repertoire of secreted and extracellular proteins from *P. infestans*. In an attempt to mimic various natural environments, mycelium was grown in liquid media varying in composition. We recently described that *P. infestans* secretes an enzyme with phospholipase D (PLD) activity (44). Here, this PLD activity was used as an extracellular marker to monitor the effect of media composition. We initially used (LC)-MS/MS to identify the proteins present in the extracellular medium based on the predicted secretome (17), but the search was further extended to identify additional proteins present in the medium that either do not have a signal peptide or were not predicted as secreted proteins. Our proteomics results did not only lead to the identification of many extracellular proteins that can now be considered either valid secretome proteins or LSP extracellular proteins, but it also led to the correction of many ORFs annotations in the *P. infestans* genome. This work provides, therefore, a comprehensive characterization of the *in vivo* secretome and extracellular proteome of *P. infestans* and it additionally supplies the data essential for future research.

EXPERIMENTAL PROCEDURES

Phytophthora infestans Culture Conditions and Sampling—*P. infestans* strains T30–4 and NL-88069 were routinely cultured at 18 °C in the dark on Rye agar medium supplemented with 2% sucrose (45). Mycelial plugs (Ø 0.5 cm) obtained from the edge of the growing colony were used to inoculate varying liquid cultures of 15 ml ranging from nutrient rich to nutrient poor media. The media used were V8 (nonclarified), V8 clarified (V8Cl), various dilutions ($V8_{1/2}$ and $V8_{1/4}$), Plich medium (PL, ± yeast), and Henniger medium (Hen) (46). The extracellular medium was harvested either after 10 days of sustained growth, or after overnight incubation with fresh medium, as described in previous studies (9, 38, 47). Medium was recovered or replaced by tilting of the Petri dish to such an angle that the mycelial mat remained undisturbed and the fluid congregated and could be retrieved by pipetting. Replacement of the growth medium involved rinsing the mycelial mat with growth medium. Upon collection of the samples the extracellular medium was immediately centrifuged for 2 min at 10,000 × *g* and the supernatant was collected and filtered through 0.2 μM filters. Viability staining revealed no significant damage of hyphae during the extracellular medium retrieval. Squeezing of mycelia was performed by folding mycelia into a stack and by pressing until all fluid was expelled. The mycelial mat was then refolded and allowed to rehydrate for ~1 min. This process was repeated three times.

Phospholipid Analysis—Extracellular PLD activity was determined as described previously (44). Radio-labeling of *P. infestans* was performed by overnight incubation of mycelial plugs (grown in a 96 wells plate in V8), with 100 μCi carrier-free $^{32}PO4^{3-}$ (GE Healthcare, Diegem, Belgium) in a volume of 200 μl. Upon addition of propanol (2% final concentration) the mycelial plugs were either incubated at room temperature or frozen in liquid nitrogen for 5 min and left to defrost for 15 min. Incubations were terminated by addition of 20 μl perchloric acid (50%, v/v) and the lipids isolated as described before (44). Phospholipids were separated using the alkaline solvent system (48). Radiolabeled phospholipids were visualized by phosphoimaging (Storm, Molecular Dynamics; Sunnyvale, CA, USA).

Protein Sample Preparation—All sample media were concentrated by ultrafiltration with a MWCO membrane (Vivaspin 15R, 2000 MW; Sartorius, Gottingen, Germany) and protein content was determined using the BCA Protein Quantification Kit (Thermo Fisher Scientific, San Jose, CA). 100 μg of sample were precipitated by the addition of six volumes of acetone (overnight, 4 °C). Precipitated proteins were then dissolved in 100 μl of 6 M Urea plus 200 mM NH_4HCO_3. Samples were reduced with dithiothreitol (15.5 μM, 1 h, 37 °C) and alkylated in the dark with iodoacetamide (3.1 μM, 30 min, 25 °C). The resulting protein extract was then diluted with 200 mM NH_4HCO_3 (dil. 1/6) and digested with 10 μg of trypsin (Promega, Madison, Wi; cat # V5113) (overnight, 37 °C). Finally, the peptide mix was acidified with formic acid and desalted with homemade Empore C18 column (3 M Inc.) prior to LC-MS/MS analysis (49).

Chromatographic and Mass Spectrometric Analysis—The peptide mixes were analyzed using a LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) coupled to an EasyLC (Thermo Fisher Scientific (Proxeon), Odense, Denmark). Peptides were loaded directly onto the analytical column at a flow rate of 1.5–2 μl/min using a wash-volume of four times the injection volume, and were separated by reversed-phase chromatography using a 12-cm column with an inner diameter of 75 μm, packed with 5 μm C18 particles (Nikkyo Technos Co., Ltd. Japan). Chromatographic gradients started at 97% buffer A and 3% buffer B with a flow rate of 300 nl/min, and gradually increased to 93% buffer A and 7% buffer B in 1 min, and to 65% buffer A and 35% buffer B in 120 min. After each analysis, the column was washed for 10 min with 10% buffer A and 90% buffer B. Buffer A: 0.1% formic acid in water. Buffer B: 0.1% formic acid in acetonitrile.

The mass spectrometer was operated in positive ionization mode with nanospray voltage set at 2.2 kV and source temperature at 275 °C. Ultramark 1621 for the FT mass analyzer was used for external calibration prior the analyses. Internal calibration was performed using the background polysiloxane ion signal at *m/z* 445.1200. The instrument was operated in data dependent mode in which a survey scan was followed by the sequential fragmentation of the ten most intense precursors. Full MS scans were acquired with 2 microscans at resolution of 30,000, and a mass range of *m/z* 350–2000. Auto gain control (AGC) was set to 1e6, dynamic exclusion to 60 s, and charge state filtering was set to disqualify singly charged peptides. Normalized collision energy of 35% was used. Fragment ion spectra produced via high-energy collision dissociation (HCD) were acquired in the Orbitrap mass analyzer with a resolution of 7500. AGC was set to 5e4, isolation window to 2.0 *m/z*, and activation time to 0.1 ms. A maximum injection time of 100 ms was used during data acquisition. All data were acquired with Xcalibur software v2.1.

Data Analysis—The Proteome Discoverer software suite (v1.4.0.288 Thermo Fisher Scientific) and the Mascot search engine (v2.3.01, Matrix Science LTD, London, UK, (50)) were used for peptide identification. Data were initially searched against an in-house generated database based on the *in silico* predicted secretome of *P. infestans* as described by Raffaele *et al.* (17) (supplementary Table S1). A precursor ion mass tolerance of 7 ppm at the MS1 level was used, and up to three miscleavages for trypsin were allowed. The fragment ion mass tolerance was set to 20 mmu. Oxidation of methionine and N-terminal protein acetylation were defined as variable modification, whereas carbamidomethylation on cysteines was set as fixed modification. In all cases, false discovery rate (FDR) in peptide identification was evaluated by using a decoy database and it was set to a maximum of 1%. Identified proteins were grouped in protein groups using the algorithm implemented in Proteome Discoverer software suite (v1.4.0.288 Thermo Fisher Scientific), and only peptides uniquely mapping to a protein group were taken into consideration for the identification of protein groups and protein group members.

Unassigned spectra from the *P. infestans* secretome sample were searched against a spectral library created with all PSMs belonging to control media samples using Spectrast tool (51), and matching spectra were excluded from further analyses. The remaining unassigned spectra were analyzed with PEAKS v6.0 using a *de novo* sequencing strategy associated with database search (52). A subset of NCBInr including only *P. infestans* sequences (January 2012, nearly 37350 sequences) was used and FDR was set to a maximum of 5%. The identified proteins were annotated by comparison with other close organisms using Blast2GO (53). Newly identified proteins were manually curated and those related to secretion were added to the predicted secretome (17), which was used for a second database search (Mascot v2.4, Proteome Discoverer v1.4.0.288) using the same parameters of the initial database search. All used proteins (final versions used for analysis) are listed in supplementary Table S2. The acquired data in this study is publicly available in the ProteomeXchange repository with the accession number PXD000802.

Bioinformatic Analysis—Signal peptide prediction was performed using a combination of SignalP (version 3.0 and 4.1), TargetP (version 1.1), and TMHMM (version 2.0) with default settings (54–56). Proteins were considered secreted, if both the neural-network as well as the hidden-markov model in both SignalP versions identified a signal peptide. Moreover, to select a candidate as a secreted protein, we also required TargetP to predict the proteins to enter the secretory pathway. To prevent false positive assignments, we subsequently scanned the proteins for transmembrane domains using TMHMM. If

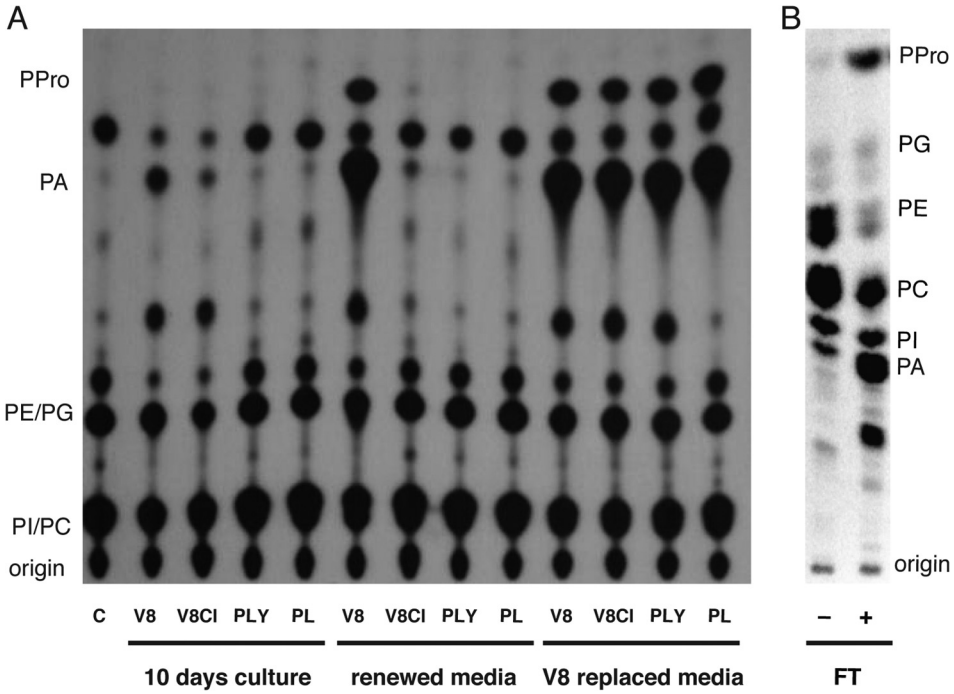


FIG. 1. Extracellular PLD activity depends on secretion rather than hyphal rupture and varies depending on the nutritional value of the growth media. *A*, Metabolically labeled phospholipids were isolated and vesicles generated as described before (44). Vesicles were incubated in the presence of 2% propanol with buffer only (*C*) or with extracellular medium derived from 10 days culture with indicated media, with overnight incubated renewed medium or overnight incubated with V8 medium. Lipids were visualized by phosphoimaging after extraction and separation by ethyl acetate TLC (44). The experiment was repeated twice with similar results. *B*, *P. infestans* mycelial plugs were metabolically labeled with ^{32}P and left untreated, or snapfrozen and thawed (FT) for 15 min in the presence of 2% propanol. Phospholipids were extracted, separated by alkaline TLC (7474) and analyzed by phosphoimaging. The origin, phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidic acid (PA), and phosphatidylpropanol (PPro) are indicated. A representative experiment is shown.

no transmembrane or only a single transmembrane domain with significantly overlap (≥10 amino acid; start position of transmembrane domain within the first 35 amino acids) with the predicted signal peptide, was detected, we retained the protein. If more than a single transmembrane domain was predicted, the protein was discarded.

RESULTS

Phospholipase D Activity Release is Dependent on Media Composition—We previously showed that PLD activity was present in extracellular medium of *P. infestans* strains, as demonstrated by the production of phosphatidic acid (PA) and the PLD specific marker phosphatidylalcohol (PPro), when cultured on V8-agar or RS-agar plates flooded with V8 juice medium (44). *P. infestans* is capable of growing in various media ranging from vegetable based, nutritiously rich extracts (*e.g.* V8 juice or Rye sucrose; RS) to minimal media with poor nutritional content (*e.g.* Plich medium). It was deduced that PLD activity in the extracellular medium could act as an enzymatic marker to monitor *P. infestans* response to changing nutritional conditions. *P. infestans* strain NL-88069, was tested for extracellular PLD activity after 10 days of growth in V8 medium (V8), Clarified V8 (V8Cl), Plich with (PL+Y) and without yeast extract (PL-Y). Our results show that PLD activity correlated to the amount of nutritious elements present in the medium (Fig. 1*A*, 10 days culture). The highest activity, indicated by the production of PA and PPro, was detected in nutritious V8 medium whereas PL-Y extract lacked PLD activity. We anticipated that in all growth media, the mycelium is capable to secrete PLD activity but required a nutritional trigger. To test this hypothesis, we renewed the media 1 day before sampling. No PLD activity increase was detected under low nutritional conditions whereas PLD activity was detected in V8 based extracellular media. Surprisingly, the amount of PLD activity tremendously increased under renewed medium (Fig. 1*A*). This implies that renewal of V8 medium results in a quantitative release of active PLD enzyme. This point was strengthened by substituting all growth media with V8 juice 1 day before sampling which resulted in the detection of PLD activity for all used media at similar high levels (Fig. 1*A*, V8 replaced media). To discard the possibility that the detected PLD activity might be caused by hyphal rupture, we squeezed the mycelial mat repeatedly in the presence of sample medium, which resulted in only a minor increase in PLD activity. This increase was negligible when compared both to the activity release upon medium renewal and to the PLD activity obtained after snap-freezing the mycelial tissue (Fig. 1*B*). The latter treatment resulted in a major nonspecific breakdown of structural phospholipids such as phosphatidylcholine (PtdCho, −70%) and phosphatidyletha-

nolamine (PtdEtn, −90%) ($n = 3$). Altogether, our results show that extracellular active PLD release is mediated by the growth media nutrient content.

Extracellular Proteome Covers a Significant Part of the In Silico Predicted Secretome—The medium-dependent PLD release suggests that the extracellular proteome composition is highly dependent on the environmental conditions and can be rapidly altered upon its modification or replacement. Therefore, to achieve optimal characterization of the secretome and extracellular proteome, we collected extracellular medium from *P. infestans* mycelium cultivated in various media with nutrient value differences. Extracellular medium was harvested in triplicates at 10 days after inoculation with a hyphal plug of strain T30−4. Proteins present both in the extracellular medium and in fresh growth media (controls) were analyzed per triplicate by LC-MS/MS (supplementary Table S3). Initially, we used the *in silico* secretome database described by Raffaele *et al.* (17) to corroborate the presence in extracellular media, and thus, validate these predicted secreted proteins as part of the secretome of *P. infestans*. During the data analysis, only unique peptides per protein group (defining a protein group as a set of undistinguishable proteins given the identified peptides), that were present in at least two replicates and absent in the control fresh medium were considered for protein identification. This analysis resulted in the identification of an initial set of 149 different protein groups that corresponded to 254 proteins. This made up for 18% of the original predicted secretome (17).

Based on the high number of not-annotated high-quality spectra present in the initial secretome analysis, we anticipated that there were many more proteins present in the extracellular medium that could be missed either because of genome annotation errors or to the lack of signal peptide (non-conventional secretion pathway). In order to identify additional extracellular proteins, we proceeded to re-annotate unassigned spectra from the initial database search using a *de novo* peptide sequencing strategy assisted with database search (Fig. 2*A*).

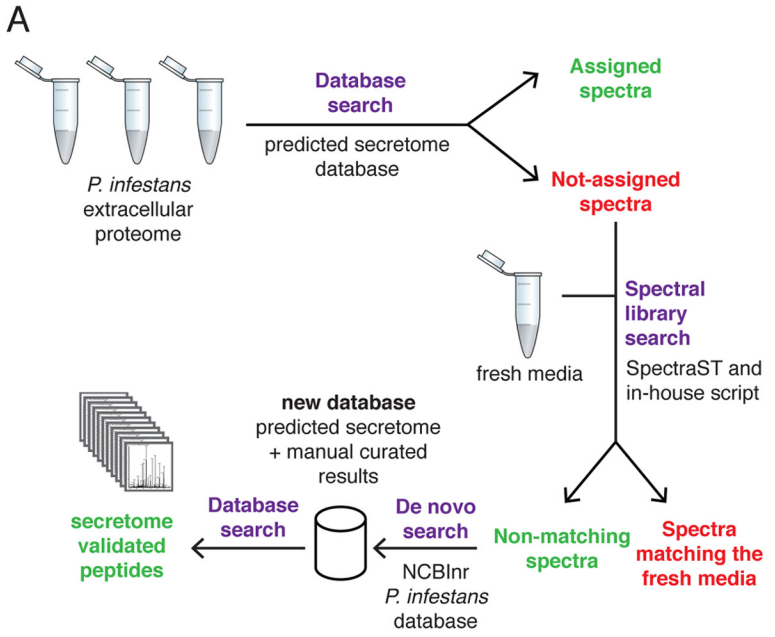
The *de novo* proteome analysis assisted with a complete *P. infestans* database (NCBI, *P. infestans*) resulted in the identification of 1105 individual proteins based on unique peptides (supplementary Table S4), with the highest number of proteins obtained in *P. infestans* cultures grown in V8 medium (649 proteins) and the lowest amount of identified proteins in PL-Y medium (222 proteins). Among the identified proteins, there were 316 proteins containing a signal peptide of which 212 had already been predicted to be secreted proteins (17). Identified proteins by *de novo* sequencing were manually curated to search for additional evidences supporting potential secreted proteins. Putative secreted proteins were assessed based on automatic annotation by sequence homology with other phyla such as the oomycetes *P. sojae* and *P. ramorum* (53), and on the prediction of a signal peptide (54). For example, several peptides were identified for the C-terminal part of protein PITG_19649, which was not included in the initial secretome list (17), but which had been annotated as putative "endopolygalacturonase" based on the presence of a glycosyl hydrolase domain (4). This piece of evidence urged us to revise the 5′-end of the gene model resulting in a signal peptide bearing protein explaining its detection in the extracellular medium (Fig. 2*B*). Nearly 150 reference gene models were re-annotated in a similar manner when necessary (see supplemental Table S5). Whenever gene models were updated, the encoded proteins were re-assessed for the presence of signal peptides and compared with proteins encoded in close phyla. Forty-four proteins now encode a signal peptide that had not been considered in the reference annotation (17).

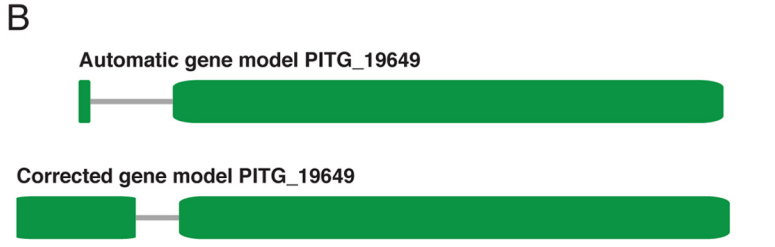
During the *de novo* analyses of the extracellular proteome we detected many transmembrane-containing proteins. Transmembrane containing proteins were previously identified from the oomycete cell walls (42, 43). In total, 31 proteins were identified by *de novo* proteome analysis that contained a signal peptide and a single transmembrane domain. The location of the transmembrane location was determined for each protein. This revealed that with only few exceptions all transmembrane domains were located in the C-terminal extreme of the protein (Fig. 2*C*).

Finally, a new database was built containing the *in silico* predicted secretome (17) and the manually curated proteins that were identified by *de novo* peptide sequencing (supplementary Table S2). A Mascot database search was performed on the new database, containing a total of 2253 entries from *P. infestans*, to validate the new peptide and protein identifications. Only unique peptides per protein group that were present in at least two replicates and absent in the control fresh medium (*blank*) were considered for protein identification. In total, 200 protein groups (283 proteins) corresponding to the secretome and extracellular proteome of *P. infestans* were identified, from which 201 proteins had already been predicted as secreted proteins (supplementary Tables S1, S6, and S7). Moreover, 227 proteins identified in this study contained a signal peptide, from which 14 proteins were not considered in the original list reported by Raffaele *et al.* (17) as they result from manual gene model corrections.

The number of identified proteins ranged from 63 protein groups (75 proteins) in the minimal PL-Y to 159 protein groups (220 proteins) in $V8_{1/4}$ medium thus correlating with the nutritional value of the media and their PLD activity. The complete distribution of hits per medium is shown in Fig. 3. About half of the identified extracellular proteins were detected in three or more growth media (Fig. 3*C*), and an important protein overlap was observed between cultures grown on the V8 family growth media and the rest of growth media, with almost no new proteins identified in the PL+Y, PL-Y and Hen media (Fig. 3*D* and 3*E*).

Incubation with Fresh Medium Results in Minor Changes in the In Vivo Secreted Proteome—After identifying the proteins





>PITG_19649
MKVLASTLAILALVAAIANGDDVDQSEVAQQFQGNQDYEVGQVSPASQVGQDSPYQVDQDD
TCTLTGTYINGTDVSQCSTIIIDSLSVPAGVMLDLTNVTDGANIK**FQGTSTFGTELWAGPLIK**LT
GNDLTVTGPGTLDGQGSWYWPQGQNITRPVFFRLSRVANSK**LSGFTIQNMPFR**TFSILSSNY
TTISELTIDSRAGNGSAK**NTDGFDLSR*NNHVTISNNR***VYNQDDCLAMQSSSNTIFSNNYCSG
SHGISIGSLGGPEQNVNTTVSGLLVK**DNTIVNSTNGIR**IKTIIGLK**GLVTNVTYTNNR**LVNVKNA
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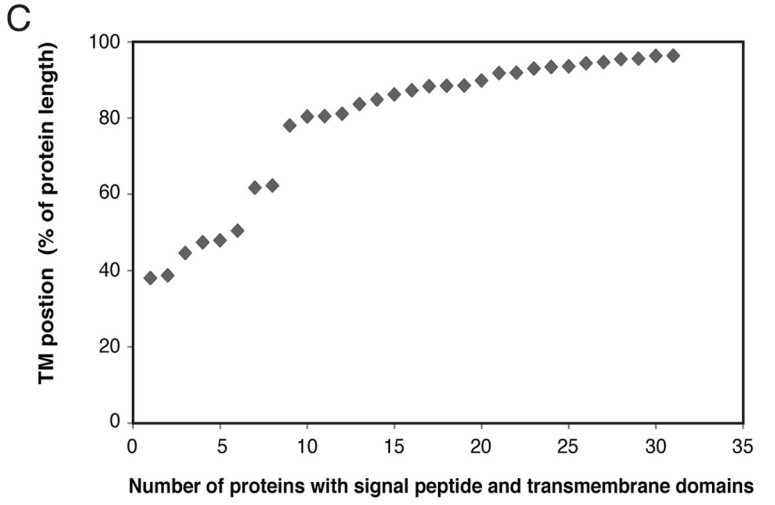

FIG. 2. *A,* General mass spectrometric and data analysis workflow used in this study. *B*, Manual annotation of gene model PITG_19649 encoding an endo-polygalacturonase. Top: automatic gene model (www.broadinstitute.org), numbers indicate protein length in AA; Middle: corrected gene model; Bottom: final protein prediction in AA. Signal peptide (SignalPv3.0: 1.000; SignalPv4.0: 0.862) is underlined. All amino acids retrieved in peptides by mass spec analysis are indicated in bold and adjacent peptides are differentiated by (non-)italics. *C*, Proteins identified from the workflow shown in *A*, were analyzed for the presence of a signal peptide (SignalP; version 3) and a single transmembrane domain (TMHMM). The transmembrane (TM) location is plotted as relative distance from the protein start for all 31 identified proteins.

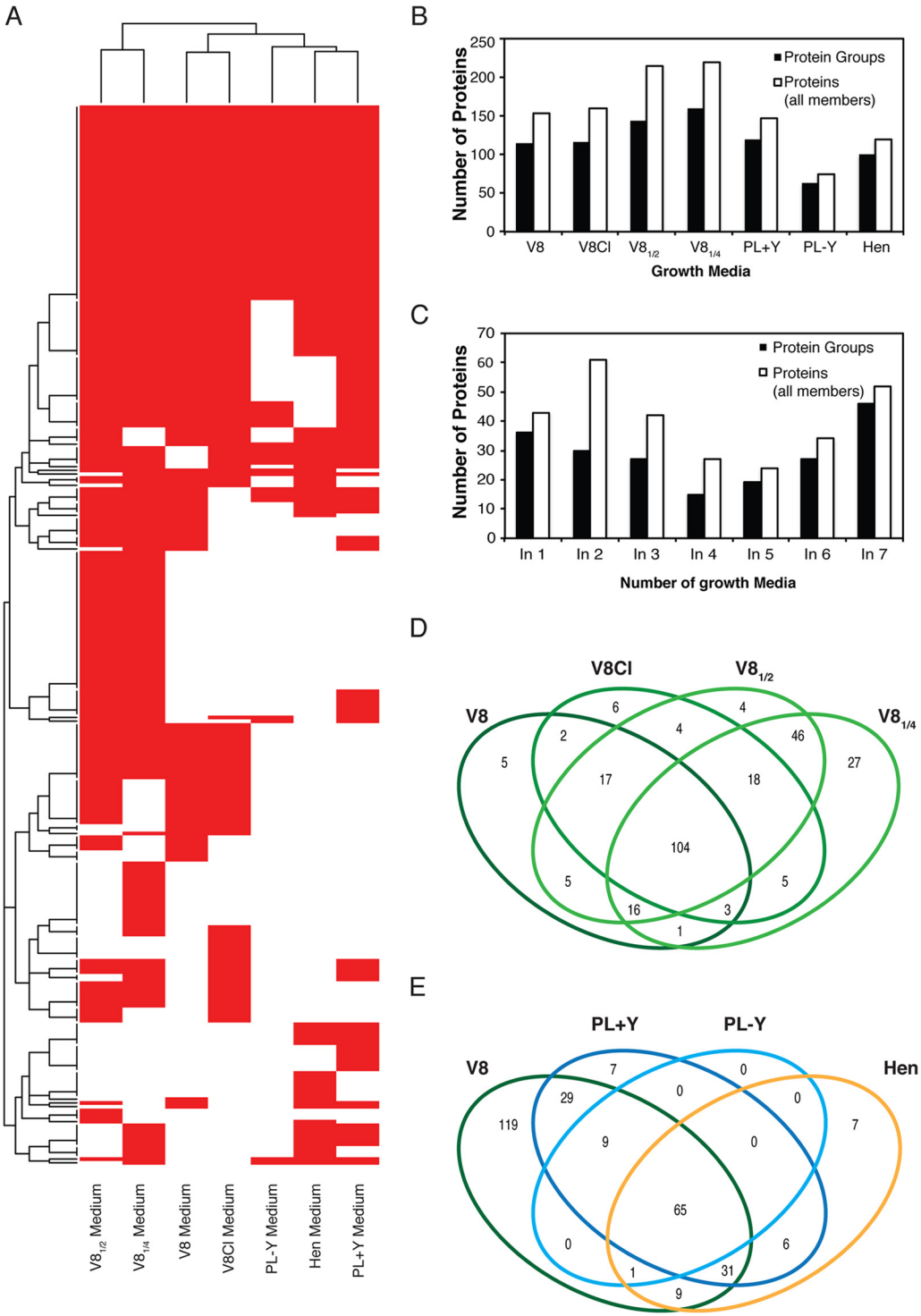

FIG. 3. *A*, Heatmap representing the presence (red) and absence (white) of the identified proteins after a 10-day culture of *P. infestans*. *B*, Number of extracellular proteins and protein groups from *P. infestans* identified in different media. *C*, Number of extracellular identified protein groups and all protein group members (*Proteins*) and from *P. infestans* identified in at least *n* different media. *D* and *E*, Venn diagrams representing the overlap among all protein group members from *P. infestans* identified in different media. In all cases, *P. infestans* was cultured for 10 days, and protein identification was based on an in-house compiled database containing both the predicted secreted protein entries and the manually curated proteins identified by *de novo* strategy.

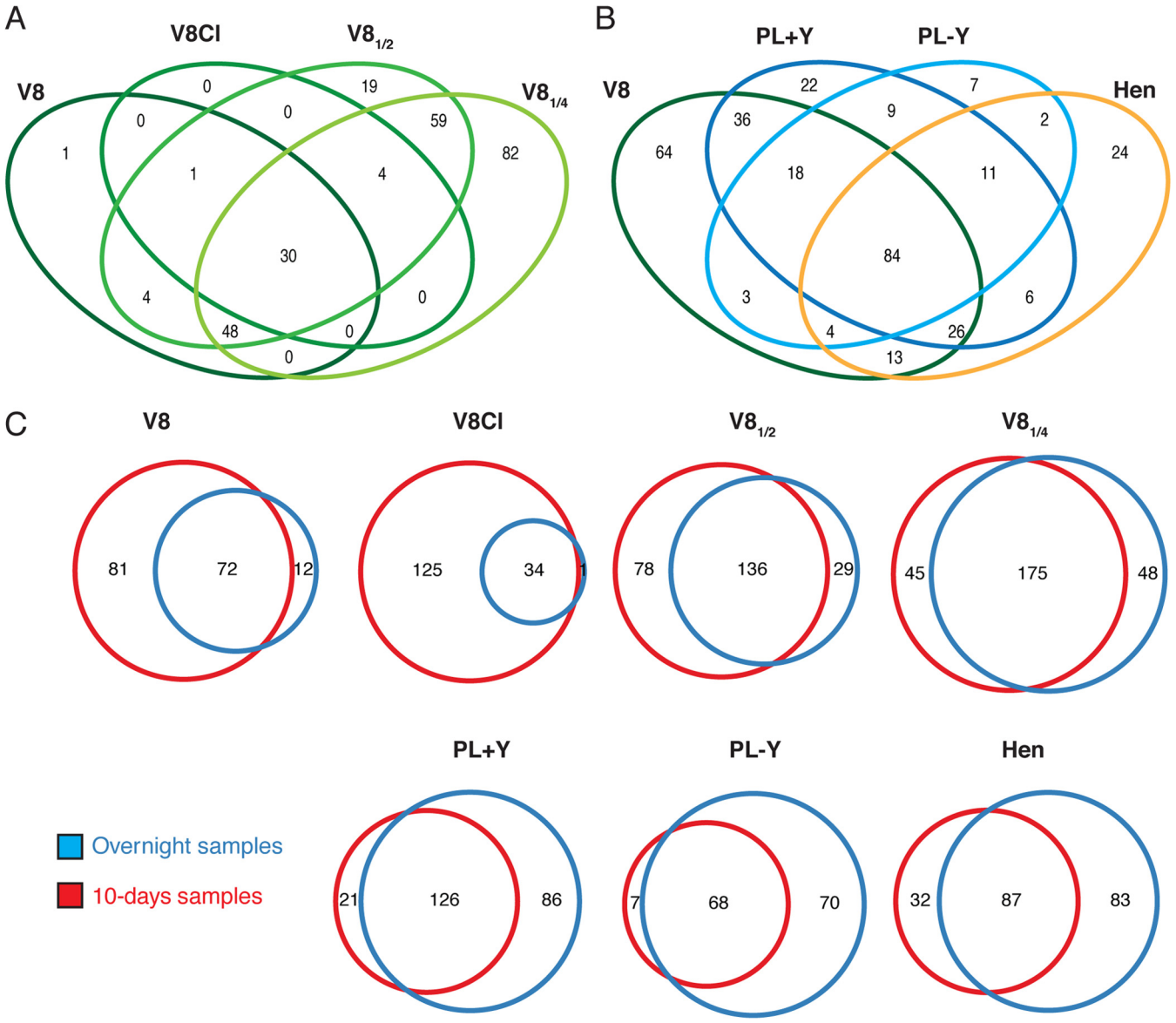

FIG. 4. *A*, Venn diagrams representing the overlap among all protein group members from *P. infestans* identified in different media after an overnight culture. *B*, Venn diagrams comparing all the protein group members identified from *P. infestans* in different media after a 10-day culture and an overnight culture. In all cases, protein identification was based on an in-house compiled database containing both the predicted secreted protein entries and the manually curated proteins identified by *de novo* strategy.

present in the secretome of the mycelial mat after 10 days of growth, we aimed to determine whether fresh media could trigger the release of new secreted proteins similarly to the observed induced release of active PLD. For this purpose, the 10-day mycelial mat was repeatedly washed and incubated overnight with fresh medium. Overnight extracellular media were processed as described above and subjected to LC-MS/MS analysis. Acquired data was analyzed using a Mascot database search with the same database and parameters as the ones used for the 10-day cultures (FDR $\leq$ 1%, peptide level). In total 330 proteins were detected of which 244 had previously been detected in the 10-day cultures. Because equivalent amounts per protein were retrieved per volume of extracellular medium, we conclude that there is a significant *de novo* protein secretion upon overnight incubation with fresh medium. However, the types and quantities of the proteins identified in these new samples were over 80% similar to the 10-day samples suggesting that the extracellular proteome composition does not respond majorly to the application of fresh medium (Fig. 4). The sequences of all the peptides identified by LC-MS/MS fragmentation are given in supplementary Table S8.

DISCUSSION

The main goal of this study was to characterize the *in vivo* secretome and extracellular proteome of *P. infestans* during

mycelial growth. Because purification of extracellular proteins from intracellular fluids of potato layers is technically challenging because of plant tissue collapse, we mimicked such natural hyphal growth conditions by using various media that differ in nutrient content. Fresh extracellular medium was also analyzed for protein composition but only a very limited number of proteins were identified, even in the full strength, non-clarified V8 media showing that original proteins coming from the fresh medium are highly diluted and degraded. The identification of secreted and extracellular proteins rendered a global *P. infestans* extracellular proteome that validates the existing predicted secretomes, which was highly based on *in silico* analysis.

Evaluation of PLD Activity in Different Media—Initially, we inferred that protein secretion activity is dependent on the growth conditions, as shown by the presence of the PLD activity in the different media. The release of extracellular PLD activity during an overnight incubation with fresh nutrient rich medium points to the rapid release of quantitative amounts of proteins suggesting the existence of highly regulated mechanisms. The mechanism that triggers protein secretion in *Phytophthora* remains to be determined but, as reported for fungi, nutrient sensing G protein-coupled receptors (GPCRs) could play a crucial role in this process (57). Indeed, the *Phytophthora* genomes harbor around 60 genes encoding putative GPCRs, among which are novel classes that might be involved in direct downstream signaling and play roles in chemotaxis as well as in developmental aspects (58, 59).

Initial Predicted Secretome and Definition of the Extracellular Proteome by De Novo Protein Identification—Once PLD activity was assessed, we used our experimentally generated dataset to search the *in silico* secretome database described by Raffaele *et al.* (17) and thus validate the predicted secreted proteins as part of the *P. infestans* secretome. A first analysis resulted in the identification of an initial set of 149 different protein groups that corresponded to 254 proteins, which represents the validation of around 18% of the original predicted secretome (17). However, because of the high number of high-quality spectra that were not annotated in this initial secretome analysis, we proceeded to re-annotate unassigned spectra from the initial database search. We performed a *de novo* peptide identification strategy assisted with database search in order to identify additional extracellular proteins that were not included in the predicted secretome.

Over one thousand individual protein groups were identified in these analyses and although several of the extracellular proteins identified were bearing a secretion signal peptide, many did not. Although hyphal lysis may occur, there are evidences that the presence of intracellular proteins in the extracellular medium—such as enolases, ribonucleases, and related proteins—might act as virulence factors and be involved in a variety of extracellular functions (22, 60, 61). Therefore, proteins identified by *de novo* sequencing were annotated by sequence homology with other oomycetes to search for additional evidence supporting their identification. Over a hundred reference gene models were re-annotated to confirm the presence of a signal peptide. Finally, the comparison of our *de novo* results, with previous studies in *P. infestans*, showed a considerable overlap among the identified proteins in the extracellular medium (9, 42), and similar results were obtained when comparing our dataset to the extracellular proteome of *C. albicans* (62).

Validation of the Predicted Secretome—To validate the *in silico* predicted secretome and control the false-discovery rate, a new database was build containing both the original *in silico* predicted secretome (17) and the manually curated proteins that were identified by *de novo* peptide sequencing (supplementary Table S2). In total, 200 protein groups (283 proteins; supplementary Table S7) corresponding to the secretome of *P. infestans* were identified, from which 201 proteins had already been predicted as secreted proteins in previous studies. Our study does not only validate a fraction of the *in silico* predicted secretome, but it greatly extends this validated subset (*i.e.* by over 40% as it adds 82 proteins to the 201 protein subset).

Among the proteins that were identified as part of the secretome of *P. infestans* are those that play a role in defense to oxidative stress such as catalases, peroxidases, and thioredoxin proteins (Table I). We could also identify five out of the seven berberine-like proteins encoded in the genome. These proteins that are involved in alkaloid biosynthesis and in the production of hydrogen peroxide through the oxidation of numerous metabolites (63), and they are thought to be important virulence factors induced during plant infection (17, 64). Alternatively, these proteins might also protect *Phytophthora* from plant counter defenses. Our dataset cannot shed light on the function of these proteins, but it clearly shows that berberine-like proteins are widely secreted by *Phytophthora* during hyphal growth.

Localized secretion of most effectors is known to take place at the haustorium, a specialized structure originating from the hyphae, which is not penetrating the plant cell but invigilates living host plant cells. In the fungus *Magnaporthe oryzae*, a differentiation in secretion systems was recently described. Apoplastic effectors are secreted from invasive hyphae whereas cytoplasmic effectors were delivered via the biotrophic interfacial complex (65). Although our data does not yet support such a distinguishing mechanism in *Phytophthora*, we detected a large quantity of predicted cytosolic effectors RXLRs (20 identified) and CRNs (13 identified) being released from hyphae in the absence of haustoria. In the case of CRNs, it remains challenging to identify the individual gene products because most peptides are shared among the different CRNs because of high conservation levels, only a single unique peptide was derived.

Moreover, we detected 11 out of the 40 elicitins encoded in the genome (4). Elicitins are oomycete specific proteins belonging to the pathogen associated molecular patterns

TABLE I
Main protein families detected as part of the secretome of P. infestans

Protein families	Identifiers
Defense to oxidative stress	
Thioredoxines	PITG_10972, PITG_14917
Catalases	PITG_05579
Peroxidases	PITG_05579, PITG_18316, PITG_05254, PITG_04900
berberine-like proteins	PITG_02928, PITG_02930, PITG_06585, PITG_06591, PITG_02935
Pathogen associated proteins and cytosolic effectors	
RXLRs	PITG_19617, PITG_15972,PITG_08278, PITG_08943, PITG_20303, PITG_20301, PITG_04090, PITG_04085, PITG_20300, PITG_18683, PITG_14371, PITG_12706, PITG_16275, PITG_23131, PITG_23129, PITG_10654, PITG_15278, PITG_12710, PITG_09216, PITG_09218
CRNs	PITG_14309, PITG_22536, PITG_17199, PITG_17185, PITG_05043, PITG_05049, PITG_18847, PITG_19377, PITG_19509, PITG_18503, PITG_18571, PITG_18497, PITG_19373
Elicitins	PITG_12551, PITG_12561, PITG_21410, PITG_12562, PITG_16907, PITG_19604, PITG_01024
NPPs (Necrosis inducing *Phytophthora Proteins*)	PITG_04208, PITG_16866, PITG_19938, PITG_22053, PITG_22734
Trans-membrane domain-containing proteins and sheddases	
Single transmembrane proteins	PITG_06662, PITG_14156, PITG_04568, PITG_06481, PITG_07210, PITG_00646, PITG_19270, PITG_20079, PITG_07536, PITG_05561, PITG_10064, PITG_10543, PITG_09768, PITG_02058, PITG_07537, PITG_07523, PITG_06215, PITG_07521, PITG_00894, PITG_17411, PITG_06867, PITG_15746, PITG_11524, PITG_01397, PITG_01395, PITG_14917, PITG_09798, PITG_07774, PITG_11993, PITG_06170, PITG_09760
Aspartic protease	PITG_09387
Cysteine protease	PITG_02423, PITG_03020, PITG_12041, PITG_16276
Metalloproteases	PITG_00056, PITG_09851
SCP-like proteins	PITG_10409, PITG_10408, PITG_15746, PITG_11060, PITG_10410

(PAMPs) that trigger a hypersentive response in plants (66). Among those, INF1 was detected at high levels in all media and was thus the most prominently present elicitin together with INF2A-like, INF4, INF5A, and INF7 (38).

The analysis of the validated secretome also revealed a total of 31 proteins that contain a signal peptide in combination with a single transmembrane domain, which divides the mature proteins in an extracellular and a cytoplasmic domain. With few exceptions, nearly all single transmembrane domains were C-terminally located (Fig. 2*C*) and all detected peptides corresponded to the extracellular domain, upstream of the transmembrane region. Although unspecific protein shearing or degradation cannot be discarded, the presence of these proteins in the extracellular medium might also reflect ectodomain shedding. Shedding is the proteolysis of ectodomains of membrane proteins by a sheddase and it has been well established in various organisms in which both the extracellular and the cytosolic remnants might act as functional components (67–69). In fungi, membrane proteins, *e.g.* Pral, Msb2, have been described to contain a single transmembrane domain, which is shed by proteolytic cleavage by proteases, (68, 69). Therefore, the identification of transmembrane proteins as secreted proteins suggests the existence of a specific protein shedding activity in *P. infestans*. Although the concrete shedding enzymes in *P. infestans* remain to be identified, aspartic proteases, metalloproteases, cysteine proteases, and sperm-coating-like proteins could play a role in this process. Indeed, we identified one aspartic protease, two metalloproteases and four cysteine proteases among the secreted proteins in *Phytophthora*, as well as five SCP-like (Sperm-coating proteins) proteins (Table I). The SCP-like domain family is found in eukaryotes, prokaryotes and archaea and it includes mammalian cysteine-rich secretory proteins (CRISPS) involved in the reproductive system (70), and plant pathogenesis related proteins (PR-1s) (71–73). So far, no SCP-like protein of *Phytophthora* has directly been linked to pathogenesis or proteolytic activity although their identification among the secreted proteome could anticipate their role in protein shedding activity.

CONCLUSIONS

Living organisms interact with their environment by sensing cues and responding to them by different physiological adaptations. Our results suggest that *P. infestans* is capable to sample its environment for cues of nutrient composition, and while doing so, it surrounds itself with a set of extracellular proteins that prepares it for encountering and infection of the host plant. Here we provide a comprehensive characterization

of the *in vivo* secretome and extracellular proteome of *P. infestans* and it additionally supplies the data files that will be essential for future research. Our proteomics results do not only lead to the identification of many extracellular proteins that can now be considered valid secretome proteins, but they also lead to the correction of many ORFs annotations in the *P. infestans* genome. The generated dataset demonstrates the validity and shortcoming of *in silico* analysis at the same time. *In silico* analysis obviously lacks sensitivity toward oomycete protein transport peculiarities, strengthened by the used cut-offs affecting the subcellular localization prediction. Strikingly, many proteins do not belong to the usual suspects such as those lacking a signal peptide, or those encoding valid transmembrane domain(s). Our approach considerably benefitted from manual gene model re-annotation, something unfeasible for full genome-based proteome analysis. However, it illustrates the potential scale of currently unconsidered proteins encoded in the genome.

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S This article contains supplemental Tables S1 to S8.

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