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Brief Report

Proteomics of the Oomycete *Phytophthora parasitica* Strain INRA 310

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Abstract: The phytopathogen *Phytophthora parasitica*, from the Oomycetes class, known to be the tobacco black shank agent, can induce devastating diseases in various crop, plant and forest ecosystems. The genus *Phytophthora* has been studied at the cellular level, suggesting that different developmental steps are induced by the expression of some specific genes. However, these studies have only been carried out on certain species, such as *Phytophthora infestans* and *Phytophthora cactorum*. As for *Phytophthora parasitica*, which can be considered as one of the top ten oomycete pathogens due to the economic impact and effect it has on food security, even less functional analyses and transcriptomics data are available. To date, little is known about the protein expression of *Phytophthora parasitica*, information that is essential for achieving a better understanding of this species. In this study, we aimed to gain insight into the proteomics of the mycelium of the *Phytophthora parasitica* strain INRA 310 by addressing the following questions: (i) how many predicted proteins can be detected on the mycelium of *P. parasitica* INRA 310, and (ii) what proteins can be detected? The proteomics experiments were performed on the mycelium of the strain *Phytophthora parasitica* INRA310, using the nanoliquid chromatography-MS/MS technique. A total of 219 proteins were identified, including ten unknown proteins and 209 proteins involved in lipid, carbohydrate, nucleotide, energy production and other metabolic pathways. This proteomics study is, to our knowledge, the first to be performed on the mycelium of *Phytophthora parasitica* INRA 310. It gives a brief first insight into its in vitro-expressed proteins. This work may be the first step before further, more comprehensive studies are undertaken with the aim of better understanding the biology of this species and its pathogenicity.

Keywords: *Phytophthora parasitica*; proteomics; oomycetes

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1. Introduction

The *Phytophthora* genus, which encompasses filamentous, fungus-like eukaryotes, is related to brown algae and currently comprises more than 180 species, some of which cause severe diseases in many plant species in both agricultural and forest environments [1].

Elucidating the mechanisms of the interactions between hosts and oomycetes, as well as the biology of oomycetes, is fundamental to implementing disease control measures. Recently, several genomes of certain *Phytophthora* species have been sequenced, expanding our understanding of their pathogenicity and evolution. Moreover, several investigations have been performed on the proteomics and transcriptomics of different *Phytophthora* species, including *Phytophthora cactorum* and *Phytophthora infestans* [2–7], documenting novel insights into their metabolism and host–pathogen interactions.

A proteomics analysis based on liquid chromatography–tandem mass spectrometry (LC-MS/MS) revealed 283 and 272 extracellular proteins, including elicitins [8] and cell-wall-degrading enzymes, for *Phytophthora infestans* and *Phytophthora plurivora*, respectively [9].

It has been shown that most *Phytophthora* species secrete hundreds of pathogenicity factors, which contribute towards the development of infection. Two main types of pathogenicity factors have been studied: apoplastically active proteins and translocated effector proteins [10]. Apoplastic effectors are secreted through conventional secretion into the apoplastic space, while translocated effectors act on the host cell [10]. Elicitins, small cysteine-rich secreted proteins, protease inhibitors, cell-wall-degrading/modifying enzymes and phospholipases are among the most studied apoplastically active proteins [10]. They can act by triggering a hypersensitive response in a plant, leading to cell death, or by protecting *Phytophthora* from plant-defensive reactive oxygen species. However, at least for some of them, their mechanisms are still not fully understood. As for translocated effector proteins, analyses of the genomic sequences of *Phytophthora* species have made it possible to identify the RXLR–EER motif, conserved in hundreds of small, secreted proteins [11]. RXLR effectors act through numerous pathways on host proteins, including relocating and destabilising target host proteins [12,13].

To take one example, the *Phytophthora sojae* effector PsAvh52 can relocate a host acetyltransferase into the nucleus, which acetylates histones, leading to the upregulation of genes involved in susceptibility [14]. As for the *Phytophthora sojae* effector PsAvh238, it acts by interacting with and destabilising the host enzyme, GmACS1, which is involved in ethylene biosynthesis [12].

The variety of effectors remains far from being exhaustively described, illustrating a need for more genomics, transcriptomics and proteomics studies. Moreover, studies on some *Phytophthora* species have been overlooked.

Little functional analyses and transcriptomics data are available regarding the soil-borne pathogen *Phytophthora parasitica*, which can be considered as one of the top ten oomycete pathogens due to the impact it has on economics and food security [15–18]. *Phytophthora parasitica* has a wide range of hosts, including *Nicotiana tabacum* and *Arabidopsis thaliana*, which have been developed as pathosystem models for the study of interactions between plants and *Phytophthora parasitica*. *Phytophthora parasitica*, for example, is the causative agent of tobacco black shank disease. It attacks all stages of growth, usually targeting the roots, but also the stems and leaves. The disease becomes visible through an extending darkening of plants cultivated both in greenhouses and those grown in fields, until they dampen off, sometimes resulting in significant economic losses. This has led to the roll-out of several management programmes in an attempt to fight black shank disease, including the use of cultivars resistant to all strains of *Phytophthora parasitica*. The soil-borne pathogen *Phytophthora parasitica*, which affects a wide range of plant species, offers a unique opportunity to investigate virulence and general pathogenicity mechanisms, as well as the relationships with host specificity. *Phytophthora parasitica* is able to produce toxins and enzymes that can damage and digest plant cells. It can spread through water, soil and insects. It causes symptoms such as black spots on leaves, damaged flowers and fruit, soft roots and stems and leaves that yellow and fall off. It grows at temperatures between 5 °C and 30 °C and at a pH of between four and eight. It is, moreover, characterised by its susceptibility to metalaxyl treatments and copper-based fungicides [19–21].

Recently, an improved genome sequence of the *Phytophthora parasitica* strain INRA 310 was generated, making it possible to identify several classes of repetitive sequences, including two families that displayed features of satellite DNA with distinct characteristics [22].

Available data on this species are far from sufficient to fully understand its biology and, no proteomics study has, to our knowledge, been performed on this species. With the aim of better characterising its biology, we carried out a brief comprehensive proteomics study on the mycelium of *Phytophthora parasitica* INRA 310.

2. Materials and Methods

For this proteomics study, we chose to use the *P. parasitica* strain INRA 310. Many isolates of *Phytophthora parasitica* have a host preference and differential virulence profiles identified against a range of hosts [19]. Some isolates, such as the *P. parasitica* strain INRA

310, infect many plant hosts, including the model plant *Arabidopsis thaliana*, allowing for molecular studies of species interacting in concert [23–26].

Thus, the *P. parasitica* strain INRA 310 is particularly adapted for studies on the generic mechanisms of pathogenicity and those governing host specificity. Initially, this strain was the *P*. *parasitica* Dastur isolate 310, which was collected in Australia from a tobacco plant, and then stored at INRA, Sophia Antipolis, France, in the local strain collection [27].

Phytophthora was grown on commercial V8 juice [28]. This juice, used as nutritional support, is composed of 86% tomato juice and 13% juice from other vegetables (carrots, beets, parsley, lettuce, watercress and spinach), salt and spice extracts. The vegetable juice culture medium was first clarified by mixing it with 15% (*w*/*v*) of CaCO3 and centrifuging at 2000× *g* for 10 min, as previously described [29]. Agar was then added (1.5% (*w*/*v*)) to the supernatant diluted in sterile distilled water (1 vol/4 vol). The two liquid and agar media were then used for experiments after autoclaving for 30 min at 120 °C. Cultures were observed each day. After 10 days, bacterial contamination was checked by seeding mycelial cultures on COS agar plates incubated at 37°C for 24 hours.

After 11 days, when growth was sufficient, the mycelial proteins were extracted following a protocol based on the paramagnetic bead method, using single-pot solid-phase-enhanced sample preparation technology (SP3) [30], which consisted of capturing proteins on the hydrophilic surface of carboxylated magnetic beads. We chose this method for processing the protein samples due to its rapidity, robustness and efficiency, as previously described [30–33].

Briefly, the mycelium was ground to a fine powder with a mortar and pestle under liquid nitrogen. It was then incubated for five minutes at 95 °C with a lysis buffer (2% SDS 100 mM dithiothreitol (DTT) 100 mM Tris-Hcl buffer at pH 8.0) before being sonicated. In total, 25 µg of the total soluble proteins obtained was combined with one volume of Sera-Mag Speed Beads (GE Life Sciences (Chicago, IL, USA) CAT# 24152105050250, CAT# 44152105050250). The proteins were bonded to the beads with one volume of 100% ethanol and shaken; then, they were rinsed three times, followed by reduction and alkylation. At each step, the supernatant with the common contaminants was discarded using a magnetic rack (MagneSphere®, Promega (Madison, WI, USA), CAT#Z5342), allowing only the proteins bonded to the beads to be preserved. The final step was the protein digestion, performed by adding 1 µg of a sequencing-grade trypsin solution (Promega, Charbonnières, France) for 20 h at 37 °C. The peptides could then be efficiently recovered for further processing. The SP3 approach has already been shown to be rapid and efficient at processing proteins and peptides, independent of the quantity of the sample and solubilisation agents used, and has also shown high sensitivity both in shotgun and quantitative proteomic analyses. It has several other advantages, including its compatibility with numerous lysis methods and quantitative labelling reagents, and is highly cost-effective.

The digested sample was checked with 12% SDS-PAGE. The proteomics experiments were performed using a NanoAcquity UPLC System coupled to a Synapt-G2 Si HDMS with a Travelling-Wave-Ion-Mobility Mass Spectrometry instrument (Waters, Saint-Quentin-en-Yvelines, France).

The digested samples were desalted, first by using C18 Spin Columns (ThermoFisher Scientific, Illkirch, France) following the manufacturer's protocol. Concentrated peptides were then eluted onto a trapping column (nanoAcquity UPLC 2G-V/M Trap 5mm Symmetry C18, Waters) for concentration and desalting, before being eluted on a C18 column (nanoAcquity UPLC 1.7 mm CSH C18, Waters, Milford, MA, USA) and separated using a 100 min gradient (300 nL/min, 5–40% acetonitrile and 0.1% formic acid).

Data-dependent MS/MS monitoring was performed in positive mode. The GFP lockmass correction was applied to the spectra. Raw MS data were processed using PEAKS Studio 6.0 software. The database used to identify proteins contained the online protein sequences of the *Phytophthora parasitica* strain INRA-310 (TrembL and Swiss-Prot, 20 May 2020, 26,438 sequences). We considered them as identified when the proteins presented with one or more peptides.

3. Results

A total of 219 proteins were identified in the mycelium of the *P. parasitica* INRA 310 (Table S1).

BLASTP analyses against the NCBI nr database revealed that 209/219 proteins had an annotated ortholog in other species of *Phytophthora*, with a percentage of identity varying between 96% and 100%. Two hundred and eleven proteins were 100% identical with their closest orthologs, and the best BLAST hits were found in *Phytophthora nicotianae*, *Phytophthora infestans* T30-4, *Phtophthora parasitica* P1569, *Phytophthora parasitica* P10297, *Phytophthora kernoviae*, *Nothophytophthora* sp. Chile5 and *Peronospora effusa*.

BLAST analyses against COG databases revealed 210 proteins that were classified into 19 COG categories, including 63 assigned to translation, structure and ribosome biogenesis, 29 to energy production and conversion, 22 to carbohydrate transport and metabolism, 19 to post-translational modification, protein turnover, chaperones and ten to amino acid transport and metabolism (Figure 1).

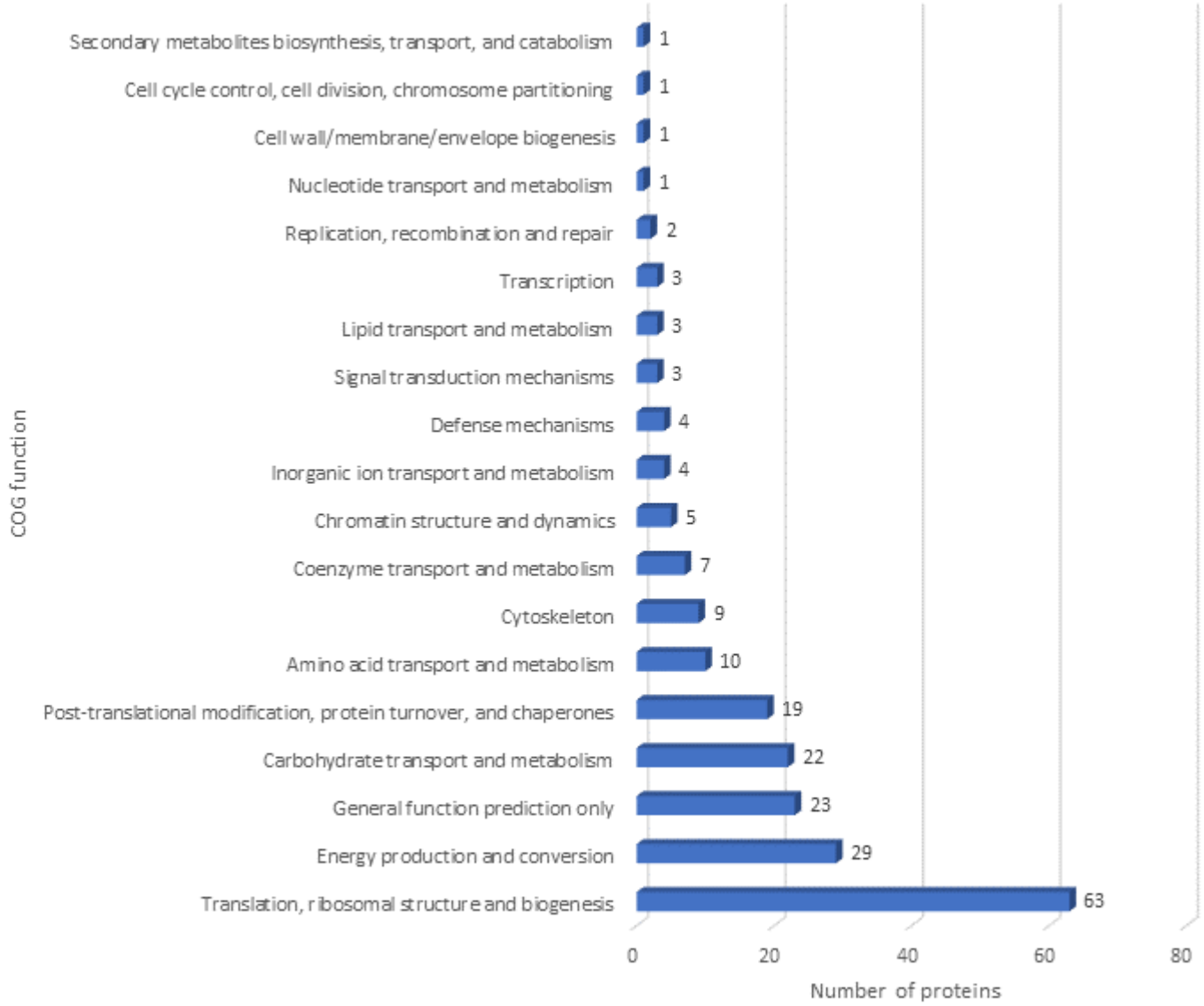

Figure 1. Functional categorisation of the *P. parasitica* INRA 310 proteins identified in our study.

In our study, one elicitin was detected (Orf PPTG_09075). The best BLAST hits included *Phytophthora infestans* T30-4, *Phytophthora boehmeriae*, *Phytophthora hibernalis*, *Phytophthora megasperma* and *Phytophthora megakarya*, with identities ranging from 86.7% to 96%.

Other proteins of interest detected included a transposase (PPTG_03149), for which the ten best BLAST hits included hits from different strains of *Phytophthora parasitica*, with an identity percentage ranging from 46.465% to 100%. A total of three ATP binding cassette (ABC) transporters (PPTG_19943, PPTG_02247 and PPTG_18883) were also detected. Their closest orthologs belonged to other strains of *Phytophtora parasitica*, but also to *Phytophtora nicotianae*, *Phytophtora aleatoria* and *Phytophtora cactorum*, sharing between 46.774% and 100% identity, with coverage ranging from 97% to 100%.

A total of ten hypothetical proteins (Table S1) with no homologs in the public nr database or in other *Phytophthora* species were also detected.

4. Discussions

In this study, 219 proteins were identified in the mycelium of *Phytophthora parasitica*. This was significantly lower than in previous studies performed on other species of *Phytophthora*. For example, one large-scale quantitative proteomics study identified a total of 2061 proteins in *P. infestans*, of which 618 were allocated to different profiles of abundance according to the stage of life [34]. It should be noted that the *P. infestans* genome is more than twice as long as that of *P. parasitica*, but is predicted to encode 20,172 proteins, slightly fewer than the 23,240 predicted proteins of the *P. parasitica* INRA 310. Another comparative proteomics study on *Phytophthora chlamydospora*, *Phytophthora gonapodyides* and *Phytophthora pseudosyringae* identified approximately 3000 expressed genes for each species, while their genomes were predicted to encode between 17,749 proteins (for *P. pseudosyringae*) and 23,348 proteins (for *P. gonapodyides*) [35]. These significant differences may be explained by the fact that in the latter study, McGowan et al. used the Ultimate 3000RSLC Dionex and a ThermoFischer Scientific Q Exactive mass spectrometer. For the proteomic analysis of *P. infestans*, Resjo et al. used HPLC-MS/MS on the Eksigent nanoLC2D HPLC system coupled to an LTQ Orbitrap XL ETD. This Orbitrap technology, used in both mass spectrometry instruments, is much more efficient than the DDA method used with our mass spectrometer for our analyses.

While in our study only one elicitin was detected, another transcriptomics study of the mycelium of another strain of *Phytophthora parasitica* (strain 149) grown in synthetic medium identified more than 3500 ESTs that were further assembled. The most prominent sequences belonged to the elicitin family, or were involved in the metabolism of lipids [36]. These proteins are a conserved class of apoplastically active effectors, which elicit a defence response from plants [15,37] and play a role in the induction of the host hypersensitivity response that combines the programmed death of immediately stimulated cells and nonspecific systemic resistance to various pathogens [38,39]. The elicitins are recognised by host defence receptors as microbe-associated molecular patterns (MAMPs). Elicitins have already been described in several *Phytophthora* strains and as a component of the *Phytopthora ramorum* cell wall [40–42]. Although the elicitin INF4 of *Phytophthora infestans* does not cause host cell death, its expression in *Nicotiana benthamiana* leads to the development of pathogen colonisation, suggesting that the elicitin INF4 may be a virulence factor. Experiments on transgenic *Phytophthora infestans* expressing INF4-mRFP have shown that this elicitin is secreted during infection from haustoria [43].

The three ABC transporters identified may play a role in the virulence, toxic efflux or resistance to chemicals, as previously described [44,45]. ABC transporters actively pump toxic substances out of oomycete cells, thus, reducing sensitivity to the chemical. This is, notably, the basis of the resistance mechanism to metalaxyl [46] and mefenoxam. More interestingly, this kind of resistance mechanism through detoxification may develop after the treatment of an initially sensitive strain [47], which has been demonstrated for *Phytophthora infestans*. Inversely, in the absence of the chemical, the oomycete can, after several generations, lose the acquired resistance [48]. This is why it is recommended to regularly change fungicides in order to not develop *Phytophthora infestans* resistance to metalaxyl or mefenoxam.

More interestingly, the ten effectively expressed hypothetical proteins detected may represent novel proteins involved in specific developmental processes or the pathogenicity of *Phytophthora* spp.

5. Conclusions

Recently published genomic data on *Phytophthora parasitica* have given us a better understanding of the fundamental issues regarding the biology and pathogenicity of this species and how it interacts with its hosts. The results of our brief proteomic analysis on the mycelium of *Phytophthora parasitica*, which represents the first such analysis carried out to date, are, undoubtedly, only preliminary.

The roles of the proteins detected herein are not fully understood. Further studies on the various stages of infection, combining transcriptomics and proteomics, are needed to accelerate the understanding of these questions, with the ultimate aim of developing novel disease control strategies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/crops3020011/s1, Table S1: the results of proteomics analyses provided from the mycelium of *Phytophthora parasitica* INRA 310. The "DDA_01" to "DDA_06" sheets contain the results of the six replicates. The "Compilation" sheet summarises the results of the six replicates. For each sheet, the data include the number of the replicates for the best hits listed in the description column, the accession number in the database used, the validation parameters, such as the confidence score (-10lgP), the coverage of the peptides on the protein (coverage (%)), the number of high-confidence peptides assigned to the protein (#Peptides), the number of unique high-confidence peptides, i.e., those not found in other proteins (#Unique), the presence of post-translational modifications (PTM) and the mass of the identified protein (mass). The last column contains the functional annotation of the protein assigned (description).

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