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To cite this version:

Agnès Attard, Edouard Evangelisti, Naïma Minet, Franck Panabières, Emeline Deleury, et al.. Transcriptome dynamics of Arabidopsis thaliana root penetration by the oomycete pathogen Phytophthora parasitica. BMC Genomics, BioMed Central, 2014, 15, 10.1186/1471-2164-15-538. hal-02635797

HAL Id: hal-02635797
https://hal.inrae.fr/hal-02635797
Submitted on 27 May 2020
Transcriptome dynamics of *Arabidopsis thaliana* root penetration by the oomycete pathogen *Phytophthora parasitica*
Transcriptome dynamics of *Arabidopsis thaliana* root penetration by the oomycete pathogen *Phytophthora parasitica*

Agnès Attard¹, Edouard Evangelisti², Naïma Kebdani-Minet¹, Franck Panabières¹, Emeline Deleury¹, Cindy Maggio¹, Michel Ponchet¹ and Mathieu Gourgues¹*

**Abstract**

**Background:** Oomycetes are a group of filamentous microorganisms that includes both animal and plant pathogens and causes major agricultural losses. *Phytophthora* species can infect most crops and plants from natural ecosystems. Despite their tremendous economic and ecologic importance, few effective methods exist for limiting the damage caused by these species. New solutions are required, and their development will require improvements in our understanding of the molecular events governing infection by these pathogens. In this study, we characterized the genetic program activated during penetration of the plant by the soil-borne pathogen *Phytophthora parasitica*.

**Results:** Using all the *P. parasitica* sequences available in public databases, we generated a custom oligo-array and performed a transcriptomic analysis of the early events of *Arabidopsis thaliana* infection. We characterized biological stages, ranging from the appressorium-mediated penetration of the pathogen into the roots to the occurrence of first dead cells in the plant. We identified a series of sequences that were transiently modulated during host penetration. Surprisingly, we observed an overall down regulation of genes encoding proteins involved in lipid and sugar metabolism, and an upregulation of functions controlling the transport of amino acids. We also showed that different groups of genes were expressed by *P. parasitica* during host penetration and the subsequent necrotrophic phase. Differential expression patterns were particularly marked for cell wall-degrading enzymes and other proteins involved in pathogenicity, including RXLR effectors. By transforming *P. parasitica* with a transcriptional fusion with GFP, we showed that an RXLR-encoding gene was expressed in the appressorium and infectious hyphae during infection of the first plant cell.

**Conclusion:** We have characterized the genetic program activated during the initial invasion of plant cells by *P. parasitica*. We showed that a specific set of proteins, including effectors, was mobilized for penetration and to facilitate infection. Our detection of the expression of an RXLR encoding gene by the appressorium and infection hyphae highlights a role of this structure in the manipulation of the host cells.

**Keywords:** *Phytophthora, Arabidopsis thaliana, Appressorium, Transcriptome, Root infection*

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Background

Plant pathogenic oomycetes have a particular physiology and are known for their devastating effects on agricultural crops and natural ecosystems. A small number of oomycetes, including downy mildews and Phytophthora and Pythium species, which are pathogenic on virtually all dicots and on some cereals [1], have a major impact on agriculture worldwide. Oomycete control strategies are currently very limited, because very few chemicals are effective against these microorganisms. Indeed, most of the molecules used to reduce the incidence of plant diseases caused by filamentous pathogens target fungal functions that are absent or dispensable in oomycetes, such as melanin, sterol or chitin biosynthesis. These organisms are phylogenetically related to brown algae and diatoms within the Stramenopiles and they have unusual biological, genetic and physiological features [2,3]. The selection of resistant plant genotypes remains an efficient anti-oomycete strategy, but this approach is costly and time-consuming and is, thus, restricted to crops with a high added value. The development of new methods to combat oomycetes thus requires improvements in our knowledge of the physiology and infection strategies of these pathogens. In particular, early events in infection, including the mechanisms underlying penetration and the modulation of plant responses to ensure successful infection are poorly documented. The modification of these processes through the development of new chemicals or plant engineering would reduce the incidence of diseases caused by oomycetes.

Characterizations of plant penetration by oomycetes at the cellular level have focused mostly on Phytophthora spp. Entry into the host is mediated principally by a specialized cellular structure called the appressorium [4-8]. Poor nutrient content, surface hydrophobicity and topology have been shown to induce appressorium differentiation in Phytophthora infestans [9]. This process also requires calcium [10]. Little is known about the molecular events governing the differentiation and functioning of appressoria. Gene inactivation strategies have clearly demonstrated a requirement for four proteins for the penetration process. The RNAi-mediated silencing of a family of four cellulose synthase genes from P. infestans revealed that cell wall organization is important for appressorium differentiation and plant infection [11]. The PiHMP1 gene encodes a membrane protein that accumulates in appressoria and haustoria and is required for early infection [12]. The PiBZP1 transcription factor from P. infestans is required for appressorium differentiation [13]. Finally, the silencing of the P. sojae mitogen-activated protein kinase PsSAK1 greatly decreases appressorium differentiation, providing a first clue to the signaling pathways involved in the control of this process [14].

Beyond candidate gene silencing strategies, a few medium- to large-scale analyses have been performed to decipher the molecular mechanisms governing appressorium differentiation in Phytophthora spp. Kramer and coworkers first detected, by 2D-SDS-gel electrophoresis, stage-specific peptides in P. infestans appressorium-like structures differentiated on artificial surfaces [15]. A comparative analysis then showed an accumulation of transcripts and proteins involved in amino-acid synthesis during the formation of appressorium-like structures in vitro [16]. These findings were confirmed by a second analysis of protein extracts from appressorium-like structures [17]. This study highlighted the accumulation of proteins involved in protein synthesis and energy metabolism, together with putative pathogenicity factors, such as the Crinkling and Necrosis protein CRN2 and proteins involved in protection against reactive oxygen species. Grenville-Briggs and coworkers recently made use of a coupled liquid chromatography/MS-MS system to identify membrane and cell wall-associated proteins [18]. These proteins included a transglutaminase, a glycosyl hydrolase and a group of three previously unknown but related proteins containing two repeats that accumulated in P. infestans appressoria. Numerous proteins previously characterized as PAMPs (pathogen-associated molecular patterns), such as CBEL-like proteins or elicins, and crinkler-like proteins accumulated in the cell walls of germinating cysts, suggesting that all these proteins accumulate before infection. Recent transcriptome studies, based on full genome data for P. infestans, P. sojae and P. capsici, have highlighted the upregulation of transcripts encoding proteins involved in gene expression and translation, primary metabolism, protein kinases, cell wall-degrading enzymes (CWDE) and various proteins used to manipulate plant cells (effectors) during in vitro appressorium differentiation [19-21]. Similar observations have been obtained with an expressed sequence tag (EST) approach applied to the broad-host range pathogen P. parasitica. An investigation of the genes expressed during appressorium formation identified sequences encoding numerous cell wall-degrading enzymes and pathogenicity-related proteins [8].

Taken together, these analyses provide interesting clues to the developmental program occurring during appressorium differentiation. However, all the studies investigating early events in plant-oomycete interactions have made use of artificial surfaces to induce appressorium differentiation or were performed on aerial parts of plants [16,18-20]. Artificial hydrophobic surfaces cannot be pierced and only the events from zoospore encystment to the differentiation of appressorium-like structures can be analyzed. It is not possible to characterize the steps from appressorium maturation to early plant penetration with such systems. Furthermore, most pathogenic oomycetes infect plant roots and the molecular
events described on aerial tissues may not reflect those governing root infection. Information is therefore required concerning the molecular events occurring during the appressorium-mediated penetration of the host root system by oomycetes.

We addressed this question, by analyzing early events in *P. parasitica* infection by a transcriptome analysis. This species infects the roots of a wide range of plants and is emerging as a model species [22]. We made use of the *P. parasitica/Arabidopsis thaliana* pathosystem to analyze the events occurring during the first few hours after the inoculation of roots with motile zoospores [23]. We hybridized a custom oligoarray containing almost one quarter of the *P. parasitica* genome with samples recovered from a time-course of infection ranging from 10.5, 30 and 96 hours post inoculation; 5 inoculated plants for each sample) as already reported [23].

As a rule, independent samples were used for Array hybridizations and RT-qPCR analyzes with an exception for purified appressoria RNA that are hardly obtained. RNA extraction from inoculated plant tissues was performed as described by [26]. *P. parasitica* RNA was extracted using Trizol reagent (Invitrogen, France).

**Methods**

**P. parasitica** and plant culture conditions

*Phytophthora parasitica* INRA-310 strain, selected as the reference for the *P. parasitica* genome sequencing project was mainly used for this study. Cultures were performed on V8 Medium and zoospore production was induced as previously described [24]. A *P. parasitica* race 0 strain was kindly provided by Elodie Gaulin (Toulouse III University) and was used for transformation experiments.

*Arabidopsis thaliana* plantlets were grown, inoculated and observed as previously described [23]. *A. thaliana* Col-O ecotype was used. An *A. thaliana* transgenic line expressing the mCherry plasma membrane marker PM-RB was kindly supplied by Professor Torii from Washington University and was used for the cytological analysis of the expression profile of a *P. parasitica* appressorium specific gene [25].

Appressorium differentiation was induced on onion epidermis as previously described [8].

**Sample preparation and RNA extractions**

For hybridization and subsequent validations of expression patterns by RT-qPCR, a series of two biological replicates each corresponding to RNA extractions of the following biological conditions were used: 1-Vegetative mycelium (recovered from two samples of 4 day-old cultures in liquid V8 medium at 24°C), 2- Motile zoospores (recovered from 8 independent cultures), 3-Appressorium differentiated on onion epidermis (epidermis from 20 onion bulbs inoculated with zoospores collected from 8 independent Petri dishes); appressoria collected 3 hours after inoculation (24°C), 4- Infection of *A. thaliana* roots by *P. parasitica* zoospores (samples recovered at 2.5, 6, 10.5, 30 and 96 hours post inoculation; 5 inoculated plants for each sample) as already reported [23].

As a rule, independent samples were used for Array hybridizations and RT-qPCR analyzes with an exception for purified appressoria RNA that are hardly obtained. RNA extraction from inoculated plant tissues was performed as described by [26]. *P. parasitica* RNA was extracted using Trizol reagent (Invitrogen, France).

**P. parasitica** oligoarray design and hybridizations

An unisequence set was obtained by clustering all *P. parasitica* EST sequences available in dbEST (Additional file 1: Figure S1). They originated from vegetative mycelium [27], zoospores and germinating cysts [28,29], appressoria [8] and *P. parasitica*-infected tomato plantlets, displaying symptoms of the necrotic step of the invasion [30]. Clustering was performed using TGICL package (http://compbio.dfci.harvard.edu/tgi/software/) and default parameters (Additional file 1: Figure S1). Unisequence composition is detailed in the supporting information (Additional file 2: Table S1). Ascribing sequences to plant or *Phytophthora* was initially done as already described [8]. This was supported by a subsequent BLASTN search against the recently released *P. parasitica* genome V2.0 (www.broadinstitute.org). Functional annotation was performed using blastx against the NCBI non redundant protein database (E value < 1E-05), searches against the Interpro database [31] and using Blast2GO annotation tool [32]. The *P. parasitica* oligoarray manufactured by NimbleGen systems (NimbleGen Systems, Reykjavik, Iceland) contained 11 independent 60-mer probes per unisequence with 4 technical replicates (Additional file 1: Figure S1). This array is fully described in the platform GPL17781 stored in the Gene Expression Omnibus (GEO) at NCBI (http://www.ncbi.nlm.nih.gov/geo).

CDNA synthesis, sample labeling, hybridization procedures, data acquisition and normalization were performed at the NimbleGen facilities (NimbleGen Systems, Reykjavik, Iceland). The complete expression dataset is available as series accession number GSE51252 in the GEO at NCBI. Average expression levels were calculated for each gene from the independent probes on array and were used for further analysis. Genes were considered as not expressed in a sample (background level) if the normalized value was less than 0.995th percentile of random probes found on the array. Data were subjected to the Anaïs statistical framework to identify differentially expressed sequences (Pvalue <0.05) [33]. Data were mean-centered and log-2 transformed using Epclust (http://www.bioinf.ub.edu/EP/EP/EPCLUST/). Hierarchical clustering (Pierson correlations, average linkage) and K-mean clustering (default parameters) were performed using Genesis program [34].
Real time RT-PCR analyses
Total RNA was treated with DNase I (Ambion, Austin, USA) and reverse transcribed (1 μg) to cDNA using i-Script cDNA synthesis (Biorad, Hercules, USA). Real Time PCR experiments were performed using 5 μl of 1:50 dilution of first strand cDNA and SYBRGreen (Eurogentec SA, Seraing, Belgium) using the Opticon 3 (Biorad, Hercules, USA). All assays were carried out in triplicates. Gene-specific oligonucleotides were designed using primer3 (http://frodo.wi.mit.edu) and their specificity was validated by the analysis of dissociation curves using genomic DNA as a template. The genes encoding ubiquitin conjugating enzyme (Ubc, CK859493), the 40S ribosomal protein S3A (WS21, CF891675), and the P. parasitica homolog (PpGAM26e01: BlastN, 91% identity) of the P. infestans Mago-Nashi protein (Pi000681) described to be stably expressed were selected as constitutive internal controls [19,35]. Quantification of gene expression was performed using delta CT method [36].

GO enrichment analysis
Full Gene Ontology files were downloaded at http://www.geneontology.org/GO.downloads.ontology.shtml. After reconstitution of GO pathways, occurrences of each GO term and their parent terms were numbered for each cluster and for the P. parasitica array dataset. Proportion of GO terms in each cluster was then compared to the proportion observed on the P. parasitica array. Terms with significant enrichment were identified using Fisher Exacts test with a p-value cut off at 0.05. To facilitate the analysis, only GO parent terms with level higher than 4 were considered.

P. parasitica transformation
A Gateway vector was obtained to accelerate gene expression analyses in Phytophthora spp. using transcriptional and translational fusions. The reporter system, corresponding to a fusion between Green Fluorescent protein (GFP) and Escherichia coli β-glucuronidase (GUS), was derived from the pKGWSF7 vector dedicated to plant transformation (from University of Gent, http://gateway.psb.ugent.be/; [37]). It was modified to be suitable for Phytophthora transformation. A genetin resistance cassette was obtained as a ClaI (blunt ends with DNA pol I)/Sacl fragment from the pTeGHNH vector [30]. It contains the P. parasitica translation elongation factor 1 (Tef1) promoter, the NPTII coding sequence conferring genetin resistance and the Ham34 terminator from Bremia lactucae. This fragment was cloned into a vector fragment corresponding to an AflII (blunt)/Sacl fragment from the pKGWF57 Gateway cloning vector, replacing the kanamycin resistance cassette used for plant selection. The resulting plasmid was named pIPO-1.

To obtain a translational fusion between the promoter sequence of a RXLR encoding gene (CL380) with the GFP-GUS reporter system (pCL380::GFP-GUS), a 1144 bp fragment upstream of CL380 sequence start codon was amplified on genomic DNA from P. parasitica PP-INRA310 and cloned into the pIPO-1 vector. P. parasitica race 0 protoplast transformation was performed as previously described [30].

Confocal microscopy
A Zeiss LSM 510 META confocal microscope was used (Carl Zeiss GmbH, Jena, Germany). GFP excitation was obtained at 488 nm. Penetration structures were observed two to thirty hours after inoculation of 5×10⁵ zoospores on onion epidermis. Infection of A. thaliana roots was observed eight and thirty hours after inoculation of 5×10⁵ zoospores. For imaging of penetration and invasion on onion epidermis, acquisition parameters were calibrated at two hours post inoculation and unchanged at the following time points to enable comparison.

Results
Design of the P. parasitica oligoarray
We retrieved all the P. parasitica EST sequences available from public databases. These sequences were obtained at various developmental stages and from cell types involved in plant infection: mycelium, zoospores, appressoria differentiated on onion and tomato necrotrophic stage of infection [8,27-30]. In total, 12632 ESTs were assembled into 1572 contigs and 3879 singletons, giving a final 5451-unisequence set (Additional file 2: Table S1). A NimbleGen custom oligo-array was designed (Additional file 1: Figure S1). Approximately 97% of the unisequences were represented by more than five 60-bp probes. Only 24 sequences did not fit the NimbleGen criteria for probe design and were discarded from the analysis. Following this design step, 56524 probes corresponding to 5427 sequences were spotted onto the array. We attributed 4398 sequences (81%) to P. parasitica and 729 sequences (13%) to the plant (Additional file 1: Figure S1). The origin of 324 sequences (6%) remained unknown (Additional file 3: Table S2). In total, 4700 probe sets corresponded to sequences from P. parasitica or of unknown origin. The remaining probe sets matched sequences of plant origin. Finally, 163000 random probes were added to complete the array, as controls.

Blastx searches of sequences attributed to P. parasitica against a protein set based on the recently released genome annotation gave 3806 hits with identity levels of more than 95%. Blastx searches (e-value = 1E-20) using the 4398 P. parasitica sequences as queries also revealed that 320 sequences (7%) had no ortholog in the genomes of P. infestans, P. ramorum and P. sojae and therefore constituted probable species-specific sequences. Functional
annotation of the 4398 and 324 sequences attributed to *P. parasitica* and of unknown origin, respectively, was performed following blastx searches (E-value cut off: 1E-05) against the NCBI non-redundant (NR) protein database, with the blast2GO tool [32]. A GO term was associated with 3516 sequences (65%), whereas the function of 1935 sequences remained unknown (Additional file 3: Table S2).

**Analysis of gene expression during early steps of the *P. parasitica/A. thaliana* interaction**

The custom oligo-array was used to identify the modulations of the *P. parasitica* transcriptome during the onset of a compatible interaction. The samples analyzed corresponded to vegetative mycelium, motile zoospores, purified appressoria and a time course of early infection in *A. thaliana*. The *A. thaliana* samples were collected 2.5, 6, 10.5 and 30 hours after inoculation (hai), from roots inoculated with *P. parasitica* motile zoospores, thus reflecting the natural mode of infection. We selected stages ranging from appressorium-mediated penetration (2.5 hai) to the first occurrence of dead cells, indicative of the switch to necrotrophy (30 hai), as previously described [23]. There were two biological replicates. Each replicate corresponded to RNA extracts from pooled independent biological samples for each condition, to ensure the robustness of our results.

Only hybridization data relevant to *P. parasitica* or sequences of unknown origin were retained for analysis. In total, 4194 sequences (89% of the 4700 probes corresponding to *P. parasitica* sequences or sequences of unknown origin) gave a hybridization signal stronger than the background in at least one condition and were considered to correspond to expressed genes. The oligo-array contained 314 of the 320 putative *P. parasitica*-specific sequences. Significant expression in at least one biological sample was detected for 266 of these sequences, confirming that they corresponded to *P. parasitica*-specific expressed genes. Interestingly, 2864 sequences (61%) and 2987 sequences (64%) were expressed in purified appressoria and during the appressorium-mediated penetration of *A. thaliana* roots (2.5 hai), respectively. Thus, with the inoculation method used for this analysis, a high proportion of transcripts can be detected even at the earliest stages of the interaction.

The Anaïs statistical framework was used to identify differentially expressed sequences [33]. Using a *P*-value threshold of 0.05, 3783 sequences were considered to display differential patterns of accumulation between at least two sets of conditions. Maximum fold-change, defined as the ratio of the maximum and minimum hybridization signal values, was calculated for each sequence. In total, 3471 sequences had a maximum fold-change of more than 2 and 1806 sequences had a maximum fold-change of at least 4.

**Cluster analysis of gene expression patterns**

The expression patterns of the 1806 sequences displaying at least a four-fold modulation of expression were analyzed. We performed a hierarchical clustering of the expression patterns (Figure 1A) and genes with coordinated expression were grouped into 12 major clusters (K-mean clustering, Figure 1B, Additional file 4: Table S3).

Clusters I (124 sequences), II (154 sequences), III (144 sequences), IV (388 sequences), V (94 sequences), VI (101 sequences) and XX (92 sequences) contained sequences for which expression was modulated during the onset of infection (Figure 1B). Clusters I, II, V, VI and XX contained sequences with a specific modulation of expression (induced or repressed) during early contact with plant cells, as they were detected in purified appressoria and during the first few hours of *A. thaliana* infection (2.5, 6 and 10.5 hai). By contrast, clusters III and IV contained sequences whose accumulation or repression begins in motile zoospores, corresponding to the pre-infection stage.

Sequences from cluster VII (366 sequences) were specifically modulated in zoospores. Interestingly, clusters VIII (70 sequences) and IX (130 sequences) contained sequences with similar patterns of expression in zoospores and in *A. thaliana* roots collected 2.5 hours after inoculation (Figure 1B). This may be due to the contamination of *A. thaliana* root tissues by zoospores, either motile or initiating cyst formation, in the first sample collected for the interaction. This finding is consistent with previous reports indicating that plant infection by motile *P. parasitica* zoospores is an asynchronous process. It thus highlights the advantages of the simplified system previously used to obtain purified appressoria [8].

Cluster XI (74 sequences) grouped together sequences that accumulated preferentially in mycelium (K-mean clustering, Figure 1B). However, most of the sequences found in this cluster also accumulated 2.5 hours after *A. thaliana* inoculation. This may be due to cysts failing to penetrate and the plant and instead initiating mycelial growth around *A. thaliana* roots, as reported during the initial steps of tomato root infection by *P. parasitica* [30].

Finally, transcripts from cluster XII (69 sequences) accumulated throughout the development of plant infection (Figure 1B). This accumulation increased during infection, reaching a maximum at the last stage studied (30 hai).

**Validation of the transcriptome data by RT-qPCR**

The expression patterns observed on hybridization of the *P. parasitica* oligo array were validated by quantitative RT-PCR. We selected 58 sequences from the six clusters containing sequences with expression modulated during the appressorium-mediated penetration of the host (clusters I, II, III, IV, V and VI; red on Figure 1).
A biological sample corresponding to the *A. thaliana/P. parasitica* interaction was added, to monitor expression of the selected sequences during the necrotrophic stage. This sample corresponded to inoculated *A. thaliana* root tissues collected four days after inoculation with zoospores. Forty-four of the 58 genes assessed (75%) displayed a modulation of expression during the onset of the interaction, consistent with the results of array hybridization (Figure 2 and Additional file 5: Table S4). Nine of the 14 sequences for which expression profiling

![Figure 1](https://www.biomedcentral.com/1471-2164/15/538)
results were not validated by RT-qPCR were from cluster V (Additional file 5: Table S4).

This cluster probably included sequences with artifactual hybridization signals. After we had discarded data for the members of this cluster, the expression patterns for 43 of 48 (90%) sequences were validated by RT-qPCR, demonstrating the robustness of our transcriptome data.

We also used the RT-PCR results to improve our clustering analysis. The grouping of sequences in cluster I, which contained transcripts transiently accumulating
during the onset of infection was validated, because eight of the nine sequences had expression patterns consistent with that deduced from oligoarray hybridization (Figure 2, Additional file 5: Table S4). The transcripts of these genes accumulated in smaller amounts during the necrotrophic phase of the *P. parasitica/A. thaliana* interaction (4 days after inoculation). This cluster included sequences that accumulated during early infection of the host. The content of clusters III and IV was also validated with all and 8 of the 10 sequences, respectively, displaying the predicted expression pattern (Figure 2, Additional file 5: Table S4). The use of the sample corresponding to necrotrophy also confirmed that the sequences grouped in these clusters accumulated or were repressed transiently in zoospores and during the penetration process. Sequences from cluster II had lower accumulation during early infection than during vegetative mycelial growth and the necrotrophic phase (4 days after inoculation). Nevertheless, seven of these nine sequences also accumulated to low levels in zoospores (Figure 2, Additional file 5: Table S4). This suggests that cluster II could be grouped with cluster IV for a single analysis. Cluster VI contained sequences specifically induced during the penetration of onion epidermis (purified appressoria, Figure 1). This cluster was poorly validated because five of the 10 sequences accumulated during the penetration of both *A. thaliana* roots and onion (Additional file 5: Table S4). This confirms that the results obtained with our simplified inoculation protocol reflect the situation occurring in natural conditions of plant penetration [8]. On the basis of this RT-qPCR analysis, clusters I and VI were grouped together for subsequent analyses.

**Functions of the sequences accumulating during appressorium-mediated plant penetration**

We focused our analysis on transcripts modulated during the onset of a compatible interaction (clusters I-VI). A functional annotation of the 20 genes displaying the highest fold-change in each cluster is presented in Table 1. We performed a GO enrichment analysis of the clusters, for annotation purposes. Sequences specifically accumulating during the appressorium-mediated penetration of the host plant (purified appressoria, *A. thaliana* 2.5 hai, 6 hai and 10.5 hai, clusters I and VI) were highly enriched in cell wall-degrading enzymes (CWDEs, Additional file 6: Table S5_I and Additional file 7: Table S5_VI). There were 64 sequences encoding CWDE on the array: 14 and 8 had expression patterns characteristic of clusters I (grouping 124 sequences) and VI (grouping 101 sequences), respectively (Additional file 4: Table S3). Figure 3A shows the expression patterns of all the sequences associated with GO terms relating to cell wall degradation. Thirty CWDEs were found to be preferentially expressed during the first few hours of infection. The other 34 enzymes were expressed in zoospores, mycelium or both mycelium and at the necrotrophic stage of interaction with *A. thaliana*. GO terms associated with the modulation of plant defenses were also overrepresented during the early stages of infection (Additional file 6: Table S5_I). Hence, six of the 14 sequences encoding RXLR effectors present on the oligoarray were present in cluster I. One of these six sequences corresponded to the PSE1 effector, which has recently been demonstrated to contribute to *P. parasitica* pathogenicity [38]. The expression patterns of sequences encoding cytoplasmic (RXLR and CRN) effectors are presented in Figure 3B. Twelve of the RXLR effectors are induced during the penetration process, whereas only a few CRN effector transcripts accumulated at this stage. Other functions relevant to pathogenicity or the elicitation of plant defense responses were also identified in clusters I (2 elicitin-like, 2 NPP1-like and 1 M81 elicitor-like) and VI (a putative protease inhibitor) (Table 1 and Additional file 4: Table S3). GO annotations relating to ribosomes were also enriched in cluster VI, providing evidence for the activation of the translation machinery during early infection (Additional file 7: Table S5_VI).

An analysis of clusters I and VI showed these clusters to contain sequences involved in protein and amino-acid metabolism, including four proteases and four putative amino-acid transporters. Similarly, eight and three sequences encoding functions involved in the detoxification of plant toxic metabolites (ABC transporters and major facilitator superfamily members) were identified in clusters I and VI, respectively (Additional file 4: Table S3). Interestingly, sequences relating to sugar metabolism were poorly represented in these clusters. Cluster I included a sequence corresponding to mannose 6P isomerase, a pyruvate kinase and a NAD-dependent malic enzyme. A single sequence encoding a malate dehydrogenase was identified in cluster VI (Additional file 4: Table S3). A similar situation was observed for sequences relating to fatty-acid catabolism. Only one sequence encoding a long-chain fatty acid CoA ligase and one sequence encoding an acyl-CoA dehydrogenase were identified in clusters I and VI, respectively (Table 1 and Additional file 4: Table S3).

The prominent functions identified for sequences from cluster III (sequences accumulating in zoospores and during host penetration, 144 sequences) were mostly similar to those identified in clusters I and VI. GO enrichment analysis highlighted the accumulation of sequences with functions relating to ribosome biogenesis (Additional file 8: Table S5_III). However, a detailed analysis of this cluster revealed the occurrence of three additional cell wall-degrading enzymes and numerous infection-associated sequences, including five M81-like sequences, two OPEL-like sequences, one EPIC-like sequence and one elicitin
Table 1: Functional annotation of the sequences from clusters I-VI

| CL    | Sequence       | Myc | Zoo | App | 2,5h | 6h  | 10,5h | 30h | FC | ID               | Description                                      | E value |
|-------|----------------|-----|-----|-----|------|-----|-------|-----|----|------------------|--------------------------------------------------|---------|
| I     | CL1533Contig1  | 102 | 749 | 95  | 10813| 1022| 712   | 139 | 114| no hit           | elicitin-like protein INF4 [P. infestans]        | 3E-10   |
|       | pptgam36a11r.1 | 127 | 131 | 336 | 1972 | 3482| 13284 | 713 | 105| XP_002895059.1   | secreted RxLR effector peptide protein, [P. infestans] | 2E-13   |
| I     | ppo3h07a24t.1  | 97  | 104 | 9105| 1711 | 4473| 1226  | 450 | 94 | XP_002998010.1   | Amino Acid/Auxin Permease (AAAP) Family [P. infestans] | 1E-134  |
| I     | ppogam36a11r.1 | 127 | 131 | 336 | 1972 | 3482| 13284 | 713 | 105| XP_002895059.1   | secreted RxLR effector peptide protein, [P. infestans] | 2E-13   |
| I     | ppo3h07a24t.1  | 97  | 104 | 9105| 1711 | 4473| 1226  | 450 | 94 | XP_002998010.1   | Amino Acid/Auxin Permease (AAAP) Family [P. infestans] | 1E-134  |
| I     | CL1408Contig1  | 120 | 178 | 10915| 1767 | 3707| 5999  | 740 | 91 | XP_002896645.1   | carboxyhydrate-binding protein, [P. infestans] | 5E-19   |
| I     | ppo3h08i21t.1  | 122 | 128 | 6892 | 5236 | 3381| 818   | 288 | 57 | XP_002901148.1   | carbohydrate-binding protein, [P. infestans] | 2E-13   |
| I     | ppt4j31g10r.1  | 126 | 120 | 6982 | 5236 | 3381| 818   | 288 | 57 | XP_002901148.1   | carbohydrate-binding protein, [P. infestans] | 2E-13   |
| I     | ppo3h08i21t.1  | 122 | 128 | 6892 | 5236 | 3381| 818   | 288 | 57 | XP_002901148.1   | carbohydrate-binding protein, [P. infestans] | 2E-13   |
| I     | ppt4j31g10r.1  | 126 | 120 | 6982 | 5236 | 3381| 818   | 288 | 57 | XP_002901148.1   | carbohydrate-binding protein, [P. infestans] | 2E-13   |
| I     | ppo3h08i21t.1  | 122 | 128 | 6892 | 5236 | 3381| 818   | 288 | 57 | XP_002901148.1   | carbohydrate-binding protein, [P. infestans] | 2E-13   |
| I     | ppo3h08i21t.1  | 122 | 128 | 6892 | 5236 | 3381| 818   | 288 | 57 | XP_002901148.1   | carbohydrate-binding protein, [P. infestans] | 2E-13   |
| I     | ppo3h08i21t.1  | 122 | 128 | 6892 | 5236 | 3381| 818   | 288 | 57 | XP_002901148.1   | carbohydrate-binding protein, [P. infestans] | 2E-13   |
| I     | ppo3h08i21t.1  | 122 | 128 | 6892 | 5236 | 3381| 818   | 288 | 57 | XP_002901148.1   | carbohydrate-binding protein, [P. infestans] | 2E-13   |
| I     | ppo3h08i21t.1  | 122 | 128 | 6892 | 5236 | 3381| 818   | 288 | 57 | XP_002901148.1   | carbohydrate-binding protein, [P. infestans] | 2E-13   |
| I     | ppo3h08i21t.1  | 122 | 128 | 6892 | 5236 | 3381| 818   | 288 | 57 | XP_002901148.1   | carbohydrate-binding protein, [P. infestans] | 2E-13   |
| I     | ppo3h08i21t.1  | 122 | 128 | 6892 | 5236 | 3381| 818   | 288 | 57 | XP_002901148.1   | carbohydrate-binding protein, [P. infestans] | 2E-13   |
| I     | ppo3h08i21t.1  | 122 | 128 | 6892 | 5236 | 3381| 818   | 288 | 57 | XP_002901148.1   | carbohydrate-binding protein, [P. infestans] | 2E-13   |
| I     | ppo3h08i21t.1  | 122 | 128 | 6892 | 5236 | 3381| 818   | 288 | 57 | XP_002901148.1   | carbohydrate-binding protein, [P. infestans] | 2E-13   |
| II    | CL1024Contig1  | 451 | 116 | 119  | 6434 | 1077 | 814   | 167 | 57 | XP_001339148.2   | PREDICTED: polymerase polyprotein-like [Danio rerio] | 3E-53   |
| II    | ppt4j51g10r.1  | 125 | 144 | 900  | 1436 | 3606 | 2879  | 845 | 29 | XP_002901054.1   | secreted RxLR effector peptide protein, [P. infestans] | 2E-15   |
| II    | ppt4j51g10r.1  | 125 | 144 | 900  | 1436 | 3606 | 2879  | 845 | 29 | XP_002901054.1   | secreted RxLR effector peptide protein, [P. infestans] | 2E-15   |
| II    | ppt4j51g10r.1  | 125 | 144 | 900  | 1436 | 3606 | 2879  | 845 | 29 | XP_002901054.1   | secreted RxLR effector peptide protein, [P. infestans] | 2E-15   |
| II    | ppt4j51g10r.1  | 125 | 144 | 900  | 1436 | 3606 | 2879  | 845 | 29 | XP_002901054.1   | secreted RxLR effector peptide protein, [P. infestans] | 2E-15   |
| II    | ppt4j51g10r.1  | 125 | 144 | 900  | 1436 | 3606 | 2879  | 845 | 29 | XP_002901054.1   | secreted RxLR effector peptide protein, [P. infestans] | 2E-15   |
| II    | ppt4j51g10r.1  | 125 | 144 | 900  | 1436 | 3606 | 2879  | 845 | 29 | XP_002901054.1   | secreted RxLR effector peptide protein, [P. infestans] | 2E-15   |
| II | ppgam04e11r.1 | 649 | 2816 | 231 | 262 | 328 | 349 | 557 | 12 | no hit | conserved hypothetical protein [P. infestans] | 7E-76 |
| II | CL1478Contig1 | 771 | 2708 | 235 | 250 | 347 | 341 | 514 | 12 | XP_002998017.1 | transmembrane protein, [P. infestans] | 7E-71 |
| II | CL471Contig1 | 5487 | 4101 | 582 | 848 | 477 | 756 | 1567 | 12 | XP_002896830.1 | conserved hypothetical protein [P. infestans] | 1E-100 |
| II | CL1485Contig1 | 1608 | 838 | 158 | 143 | 186 | 180 | 347 | 11 | XP_002897750.1 | short/branched chain specific acyl-CoA dehydrogenase, [P. infestans] | 1E-125 |
| II | CL471Contig1 | 5487 | 4101 | 582 | 848 | 477 | 756 | 1567 | 12 | XP_002896830.1 | conserved hypothetical protein [P. infestans] | 1E-100 |
| II | ppt4j38b11r.1 | 681 | 2149 | 332 | 246 | 202 | 432 | 11 | XP_002896848.1 | conserved hypothetical protein [P. infestans] | 1E-108 |
| III | ppo3h14g02t.1 | 142 | 11120 | 10795 | 18486 | 7479 | 2452 | 503 | 130 | XP_002997786.1 | glycoside hydrolase, [P. infestans] | 1E-43 |
| III | ppo3h14f01t.1 | 126 | 5446 | 7895 | 14463 | 5042 | 1485 | 437 | 115 | no hit |  |  |
| III | CD051442.1.1 | 218 | 15744 | 14847 | 23012 | 17903 | 9763 | 5659 | 105 | no hit |  |  |
| III | CD051500.1.1 | 157 | 15890 | 5422 | 7601 | 5856 | 5131 | 1149 | 101 | no hit |  |  |
| III | CL306Contig1 | 148 | 14591 | 3542 | 5108 | 3343 | 3089 | 710 | 99 | XP_002898718.1 | conserved hypothetical protein [P. infestans] | 1E-146 |
| III | CL804Contig1 | 130 | 9313 | 6270 | 2748 | 2823 | 2240 | 1429 | 72 | XP_002904112.1 | sulfatase-like protein [P. infestans] | 1E-132 |
| III | CL515Contig1 | 131 | 7782 | 2639 | 1063 | 1153 | 1103 | 519 | 59 | XP_002904421.1 | conserved hypothetical protein [P. infestans] | 0 |
| III | CD051686.1.1 | 135 | 6845 | 2453 | 1660 | 868 | 844 | 290 | 51 | no hit |  |  |
| III | CL1187Contig1 | 181 | 9106 | 1049 | 3912 | 5197 | 6878 | 5043 | 50 | XP_002896478.1 | poly [ADP-ribose] polymerase, [P. infestans] | 1E-174 |
| III | CF891673.1.1 | 157 | 5465 | 5871 | 2105 | 4405 | 2699 | 1995 | 37 | no hit |  |  |
| III | CL232Contig1 | 200 | 7317 | 3970 | 3044 | 4343 | 2281 | 3612 | 37 | XP_002904949.1 | conserved hypothetical protein [P. infestans] | 1E-168 |
| III | CL1195Contig1 | 135 | 4941 | 2491 | 3037 | 2412 | 1683 | 724 | 37 | XP_002895053.1 | conserved hypothetical protein [P. infestans] | 1E-129 |
| III | CL181Contig1 | 276 | 7495 | 9615 | 1909 | 1423 | 1212 | 1047 | 35 | XP_002900508.1 | conserved hypothetical protein [P. infestans] | 1E-119 |
| III | ppo3h09b05t.1 | 172 | 5371 | 703 | 1742 | 819 | 937 | 463 | 31 | ABG80552.1 | cell 5A endo-1,4-beta-glucanase [P. ramorum] | 2E-97 |
| III | ppo3h07h24t.1 | 112 | 3238 | 1398 | 543 | 840 | 680 | 469 | 29 | XP_002906516.1 | transmembrane protein, [P. infestans] | 1E-117 |
| III | ppo3h13d10t.1 | 115 | 3128 | 1867 | 885 | 647 | 518 | 171 | 27 | XP_002998505.1 | GPI-anchored serine-rich elicitin INL3b-like protein [P. infestans] | 1E-38 |
| III | CL449Contig1 | 125 | 3223 | 2186 | 1589 | 1510 | 843 | 410 | 26 | XP_002898095.1 | conserved hypothetical protein [P. infestans] | 7E-96 |
| III | ppt4j29b11r.1 | 158 | 3765 | 2184 | 1048 | 557 | 849 | 399 | 24 | XP_002902649.1 | Drug/Metabolite Transporter (DMT) Superfamily [P. infestans] | 1E-108 |
| III | CL330Contig1 | 1867 | 23600 | 43890 | 40265 | 40618 | 35214 | 14295 | 24 | XP_002904340.1 | conserved hypothetical protein [P. infestans] | 1E-102 |
| IV | CL8Contig1 | 44525 | 163 | 325 | 452 | 825 | 9662 | 7144 | 274 | XP_002898441.1 | conserved hypothetical protein [P. infestans] | 1E-152 |
| IV | CL639Contig1 | 22961 | 606 | 806 | 221 | 192 | 166 | 413 | 139 | ABG23233.1 | unknown [Hyaloperonospora parasitica] | 1E-36 |
| Cluster | ID            | Start | Length | GC Content | Identity | Similarity | E-value | Description                                |
|---------|---------------|-------|--------|------------|----------|------------|---------|--------------------------------------------|
| IV      | ppo3h02e04t.1 | 19582 | 157    | 3139       | 1151     | 2639       | 7487    | 7743 124 XP_002900352.1 Proton-dependent Oligopeptide Transporter (POT) Family [P. infestans] 8E-98 |
| IV      | ppgam01h01r.1 | 17201 | 173    | 339        | 204      | 164        | 142     | 881 121 XP_002901432.1 conserved hypothetical protein [P. infestans] 6E-33 |
| IV      | CL90Contig1   | 13934 | 167    | 1552       | 489      | 7712       | 8560    | 18029 108 XP_002999240.1 pyrophosphate vacuolar membrane proton pump [P. infestans] 0 |
| IV      | CL32Contig1   | 14467 | 189    | 275        | 148      | 258        | 429     | 4440 97 AAM18483.1 AF494014_1 exo-1,3-beta-glucanase [P. infestans] 0 |
| IV      | ppgam24d04r.1 | 13873 | 149    | 200        | 667      | 295        | 697     | 973 93 XP_002907089.1 protein kinase [P. infestans] 1E-127 |
| IV      | CL210Contig1  | 12224 | 133    | 178        | 647      | 362        | 905     | 11665 92 XP_002909387.1 conserved hypothetical protein [P. infestans] 7E-69 |
| IV      | ppgam36c10r.1 | 16676 | 187    | 4612       | 1365     | 566        | 1463    | 8770 89 XP_002904661.1 Major Facilitator Superfamily (MFS) [P. infestans] 1E-126 |
| IV      | CL119Contig1  | 10489 | 126    | 163        | 144      | 246        | 461     | 494 84 XP_002900778.1 Annexin (Annexin) Family [P. infestans] 1E-166 |
| IV      | ppgam37e05r.1 | 15664 | 342    | 205        | 390      | 190        | 188     | 2589 83 XP_002907653.1 oxidoreductase [P. infestans] 4E-85 |
| IV      | CL71Contig1   | 10898 | 137    | 1568       | 339      | 1504       | 4481    | 9186 79 ABH11757.1 elicitin-like protein 6 precursor [P. nicotianae] 4E-44 |
| IV      | ppo3h07a23t.1 | 9263  | 118    | 124        | 161      | 162        | 175     | 1752 78 XP_002907297.1 P-type ATPase (P-ATPase) Superfamily [P. infestans] 1E-120 |
| IV      | CL92Contig1   | 12589 | 213    | 871        | 357      | 5799       | 12282   | 16142 76 XP_002988388.1 carbohydrate-binding protein, [P. infestans] 1E-127 |
| IV      | ppgam07a10r.1 | 8342  | 249    | 671        | 214      | 8582       | 1544    | 1290 50 XP_002903354.1 mucin-like protein [P. infestans] 8E-38 |
| IV      | ppgam02h08r.1 | 4632  | 136    | 625        | 454      | 3462       | 10250   | 8890 75 XP_002986569.1 glucosylceramidase, [P. infestans] 1E-109 |
| IV      | ppt4j09a01r.1 | 8222  | 115    | 199        | 259      | 220        | 399     | 8676 75 XP_002909387.1 conserved hypothetical protein [P. infestans] 2E-46 |
| V       | ppt4j18e03r.1 | 15537 | 715    | 353        | 538      | 227        | 207     | 2704 75 XP_002902980.1 acyl-CoA synthetase short-chain family member, [P. infestans] 1E-124 |
| V       | CL758Contig1  | 12392 | 169    | 311        | 676      | 908        | 2489    | 3671 73 XP_002904197.1 maltose O-acetyltransferase, [P. infestans] 1E-156 |
| V       | ppo3h06f02t.1 | 409   | 777    | 214        | 8582     | 1544       | 1200    | 509 40 XP_002997773.1 conserved hypothetical protein [P. infestans] 7E-78 |
| V       | ppgam12h06r.1 | 781   | 868    | 171        | 5385     | 359        | 274     | 138 39 no hit |
| V       | ppgam31f01r.1 | 119   | 197    | 159        | 2339     | 520        | 353     | 164 20 no hit |
| V       | ppt4j25b05r.1 | 97    | 176    | 126        | 1823     | 424        | 369     | 127 19 AAR152176.1 heat shock protein 70 [P. nicotianae] 2E-20 |
| V       | CL1505Contig1 | 480   | 596    | 349        | 5757     | 3617       | 1585    | 1274 17 XP_002895485.1 ATP-binding Cassette (ABC) superfamily [P. infestans] 1E-136 |
| V       | ppt4j21a06r.1 | 445   | 118    | 199        | 1866     | 303        | 357     | 311 16 XP_002895988.1 maltose O-acetyltransferase, [P. infestans] 5E-86 |
| V       | ppgam36f04r.1 | 108   | 116    | 171        | 1707     | 275        | 240     | 140 16 no hit |
| V       | ppgam17d07r.1 | 159   | 458    | 362        | 2083     | 608        | 514     | 333 13 XP_002906389.1 conserved hypothetical protein [P. infestans] 2E-65 |
| V       | CL344Contig1  | 138   | 179    | 483        | 1684     | 534        | 446     | 257 12 no hit |
| V       | ppgam04f05r.1 | 128   | 167    | 462        | 1554     | 521        | 297     | 166 12 no hit |
| V       | ppo3h02b24t.1 | 112   | 407    | 356        | 1344     | 495        | 796     | 671 12 XP_002895175.1 conserved hypothetical protein [P. infestans] 5E-51 |
| V       | ppo3h06n03t.1 | 129   | 135    | 526        | 1544     | 284        | 204     | 175 12 NP_001063268.1 Os09g0438100 [Oryza sativa Japonica Group] dbj 0,000009 |
| V       | ppt4j07f03r.1 | 106   | 122    | 165        | 1257     | 236        | 187     | 145 12 XP_002903318.1 conserved hypothetical protein [P. infestans] 1E-15 |
| V       | ppo3h03i13t.1 | 161   | 608    | 253        | 1692     | 729        | 454     | 216 11 XP_002904470.1 conserved hypothetical protein [P. infestans] 5E-97 |
Three sequences encoding functions involved in the evasion of toxic molecules (ABC transporters and major facilitator superfamily members) were observed. Finally, this cluster also contained four proteases and one amino acid/auxin permease, confirming the importance of amino-acid uptake during early infection. Only two glycolytic enzymes (glucokinase and a glyceraldehyde 3P dehydrogenase) and a fatty-acid metabolism-related short-chain dehydrogenase were identified in this cluster.

Cluster V, which contained 94 sequences specifically expressed during the penetration of *A. thaliana* roots,

| Cluster | Sequence Name | E Value | Description |
|---------|---------------|---------|-------------|
| V       | Xp_002903425.1| P. infestans |
| V       | XP_002909505.1| mannitol dehydrogenase, [P. infestans] |
| V       | XP_002904550.1|folate-Biopterin Transporter (FBT) family [P. infestans] |
| V       | XP_002904736.1|conserved hypothetical protein [P. infestans] |
| V       | XP_002905279.1|cleavage induced hypothetical protein [P. infestans] |
| V       | XP_002905279.1|cell 12A endoglucanase [P. sojae] |
| V       | XP_002904437.1|conserved hypothetical protein [P. infestans] |
| V       | XP_002909523.1|Amino Acid-Polyamine-Organocation (APC) Family [P. infestans] |
| V       | XP_002901038.1|carbohydrate-binding protein, [P. infestans] |
| V       | XP_002902444.1|conserved hypothetical protein [P. infestans] |
| V       | XP_002901373.1|conserved hypothetical protein [P. infestans] |
| V       | XP_002904844.1|ATP-binding Cassette (ABC) Superfamily [P. infestans] |
| V       | XP_002901401.1|conserved hypothetical protein [P. infestans] |
| V       | XP_00293232.1|N-acetyltransferase-like protein [Hyaloperonospora parasitica] |
| V       | XP_002900313.1|cutinase, [P. infestans] |
| V       | XP_002903927.1|pectin lyase, [P. infestans] |
| V       | XP_002909405.1|conserved hypothetical protein [P. infestans] |
| V       | XP_002518625.1|conserved hypothetical protein [Ricinus communis] |
Figure 3 Hierarchical clustering of the genes encoding cell wall degrading enzymes and cytoplasmic effectors. Based on GO annotations, sequences corresponding to cell wall degrading enzymes (A) and sequences corresponding to RXLR and Crinkler effectors (B) are presented. Pearson correlation method with average linkage on conditions was used. Gene expression level is indicated as a Log2 transformation of relative value calculated on gene average signal value. Green and red were used for down-regulated and up-regulated conditions respectively. Myc, mycelium; Zoo, zoospores, App, Appressorium; 2.5 h, A. thaliana infected roots recovered 2.5 hours after inoculation; 6 h, A. thaliana infected roots recovered 6 hours after inoculation; 10.5 h, A. thaliana infected roots recovered 10.5 hours after inoculation; 30 h, A. thaliana infected roots recovered 30 hours after inoculation.
but not onion epidermis, was not supported by RT-qPCR experiments, suggesting that this cluster should be considered with caution. Moreover, most of the sequences in this cluster lacked robust annotation. Nevertheless, like clusters I and VI, this group contained various pathogenicity-related sequences, including a range of apoplastic and cytoplasmic effectors: four RXLR effectors, two CRNs, one elicitin-like sequence, one IPI-like sequence, one NPP1-like sequence and one protease (Table 1, Additional file 9: Table S5_V and Additional file 4: Table S3).

**Functions of sequences repressed during appressorium mediated penetration of the host**

An analysis of the sequences of cluster II (154 sequences specifically repressed during appressorium-mediated penetration) revealed enrichment for functions involved in signaling (Additional file 10: Table S5_B). A more detailed analysis identified 15 potential protein kinases, including three phosphatidylinositol kinases and two MAP-kinases (Table 1 and Additional file 4: Table S3). Sequences relating to lipid metabolism (2 acyl-CoA dehydrogenases, 2 acyl-CoA ligases) and ubiquitin-mediated protein degradation (2 E3 ubiquitin ligases and one ubiquitin-like protein) were also identified in this cluster.

For sequences repressed both in zoospores and during the penetration process (cluster IV, 388 sequences), most of the associated GO terms identified related to metabolism (Additional file 11: Table S5_IV). Primary metabolism was globally downregulated, as indicated by the numbers of sequences relating to glycolysis/neoglucogenesis, the pentose phosphate pathway, lipid metabolism, and their overall repression level (Table 1 and Additional file 4: Table S3). Similarly, the ubiquitin-proteasome pathway (12 proteasome components and 9 sequences associated with the proteolytic process) was repressed during early infection (Table 1 and Additional file 4: Table S3).

Surprisingly, an analysis of cluster IV led to the identification of functions otherwise overrepresented during the penetration process. The sequences of this cluster included sequences encoding enzymes involved in cell wall degradation (15 sequences), proteases (13 sequences), elicitin-like proteins (4 sequences) and potential ABC transporters (8 sequences, Table 1 and Additional file 4: Table S3). These findings suggest that multiple functions are achieved by specific proteins during the penetration process, with these proteins being downregulated during the other steps of the *P. parasitica* life cycle.

**An RXLR effector is expressed in the appressorium and infectious hyphae**

A significant number of transcripts encoding RXLR effectors were found to accumulate during host penetration. These proteins are generally thought to be delivered into the plant cytoplasm via haustoria [39]. We analyzed the expression pattern of an RXLR sequence from cluster I. This gene, represented by the unisequence CL380, was chosen for study because it was strongly expressed during the penetration process (Table 1). *P. parasitica* transformants expressing a pCL380::GFP-GUS transcriptional fusion were obtained and used in the onion epidermis-based simplified penetration assay, for a precise analysis of CL380 expression during penetration. Two independent transformants gave similar GFP expression patterns. Expression was detected in germinated cysts (2 hai) and a faint GFP signal was observed in the cyst and germ tube (Figure 4A, 2 h). Fluorescence remained weak in germinated cysts differentiating into appressoria (Figure 4A, 2 h). The GFP signal increased 3 hours after inoculation, at the onset of penetration, and peaked six hours after inoculation (Figure 4A, 3 h and 6 h). The GFP signal was localized in infectious hyphae, and the cysts and appressoria appeared to have no cytoplasm at this stage (Figure 4A, 6 h). The GFP signal decreased 10 hours after inoculation, when the infectious hyphae grew into the cells and became weakly detectable, 30 hours after inoculation, in heavily colonized tissues. As GFP is stable for almost 24 h, the decrease in fluorescence intensity is accounted for by both transcription arrest and the dilution of the existing GFP in the developing infectious hyphae. This result confirmed that the CL380 effector accumulated transiently during the penetration process. Zoospores from transformants expressing the pCL380::GFP-GUS transcriptional fusion were then used to inoculate *A. thaliana* plantlets. A transgenic line accumulating a plasma membrane-targeted mCherry fluorescent protein was used to visualize the boundaries of the plant cell cytoplasm. We observed mCherry fluorescence around the infectious hyphae, suggesting that *P. parasitica* invades plant cells by growing between the plasma membrane and the cell wall (Figure 4B). As in onion epidermis, GFP fluorescence was observed just after penetration, in the infectious hyphae of the transgenic *P. parasitica* strain, whereas no fluorescence was detected with the wild-type strain (8 hours post inoculation for the image presented, Figure 4B). This result confirms that this RXLR-encoding gene is expressed during penetration of the first plant cell. The fluorescence subsequently decreased but continued to be observed in some areas of the infected roots at the late biotrophy stage (24 hours post inoculation for the image presented, Figure 4B). This may be due, in part, to the stability of GFP, which delays the decrease in fluorescence. Moreover, as *P. parasitica* infection is an asynchronous process, the infection events observed 30 hours after the inoculation of *A. thaliana* may correspond to different biological stages. Some parts of the roots may undergo early biotrophic *P. parasitica* colonization, even 30 hours after inoculation. It was more difficult to detect mCherry fluorescence at this stage, which suggests that plant cells are already affected by *P. parasitica* colonization. Taken
**Figure 4** *P. Parasitica* appressorium transiently express a RXLR encoding effector during the penetration process. (A and B) Expression pattern of the CL380 RXLR encoding gene using a pCL380::GFP-GUS fusion. (A) Kinetic of early infection steps using a simplified penetration assay based on onion epidermis. GFP fluorescence from a *P. parasitica* strain carrying the pCL380::GFP-GUS construct was monitored 2 to 30 hours after inoculation of zoospores on onion epidermis. (B) Analysis of the CL380 expression pattern during early *A. thaliana* infection. *A. thaliana* plantlets where inoculated with zoospores from a *P. parasitica* strain carrying the pCL380::GFP-GUS construct and fluorescence was monitored during infection of the first cell (6 hours after inoculation for the presented image) and late biotrophic phase (24 hours after inoculation for the proposed image). Plant cell membranes (red) are visualized with the mCherry plasma membrane marker pm-RB. A representative image for each stage is presented. GFP and mCherry fluorescence was visualized using a confocal laser scanning microscope. Bars: 10 μM; Arrows: appressoria; Stars: Infectious hyphae.
together, these results confirm that *P. parasitica* transiently expresses RXLR effectors during the penetration process.

**Discussion**

We developed a custom oligoarray, using all the *P. parasitica* sequences available at the start of this project. These sequences were obtained in a range of biological conditions, including plant infection. A set of 5451 unisequences was generated by EST clustering and assembly and oligonucleotides were designed for all but 24 of these sequences. Overall, 87% of these sequences were attributed to *P. parasitica* or were of unknown origin. Based on the recently released complete *P. parasitica* genome sequence, we determined that the array made it possible to study the expression of about 20% of the predicted genes of this species. The oligoarray was used to monitor gene expression during the onset of infection. The objective was to characterize the genetic program activated during appressorium differentiation and the penetration of the first host cells. RNA recovered from vegetative cultures, pre-infection structures (motile zoospores and cysts differentiating into appressoria on onion) and samples collected from the plant (corresponding to *A. thaliana* tissues collected from 2.5 to 30 hours after infection) was used to characterize the initial steps of infection. We detected 89% of the sequences in at least one set of conditions. This high rate of detection is slightly higher than the 69%, 79% and 75% reported during analyses of the early infection process in *P. infestans*, *P. sojae* and *P. capsici*, respectively [19-21]. This result is unsurprising, because EST sequences were used for the design of the array, rather than genome-based predicted open reading frames. In particular, 64% of the sequences were detected 2.5 hours after the inoculation of *A. thaliana* roots, which is not surprising because 20% of the ESTs used for the assembly of sequences analyzed with the array were obtained from germinated cysts with appressoria [8]. Thus, our methods were effective for detecting sequences expressed at early stages of infection. A large proportion of the sequences were differentially expressed, with 74% and 38% displaying modulations of at least two-fold and at least four-fold, respectively, between at least two sets of conditions. This proportion is higher than the proportion of genes displaying a two-fold modulation of expression (46%) described by Judelson and coworkers, who analyzed the events from sporangium cleavage to the *in vitro* differentiation of appressoria [19]. This difference may reflect the large proportion of genes with expression modulated during plant penetration, a biological condition not tested in the transcriptome analysis for *P. infestans*. Other studies on fungi have generated findings consistent with this conclusion. Only 29% of *Magnaporthe grisea* genes have been shown to display a two-fold modulation of expression during *in vitro* appressorium differentiation, whereas up to 44% of *Colletotrichum higginsianum* genes were found to display a four-fold modulation of expression in a transcriptome analysis of samples corresponding to plant infection [40,41].

A clustering analysis identified five highly validated clusters containing sequences modulated during appressorium-mediated penetration of the host. Sequences accumulating transiently during the penetration of the first plant cells, some of which were already accumulating in zoospores before infection, were identified. Similarly, sequences displaying transient downregulation upon host penetration were also identified. Such sequences, displaying specific downregulation in appressoria, were not identified in *P. infestans* germinated cysts with appressoria obtained *in vitro* [19]. The perception of environmental cues at the plant surface may contribute to this downregulation.

An analysis of the predicted function of the genes transiently expressed or repressed provided insight into the genetic program activated during early infection. The cluster grouping together sequences specifically repressed during the first few hours of infection (cluster II) contained a large number of genes relating to lipid and sugar metabolism. Consistent with this observation, only a few sequences relating to sugar or lipid degradation were identified in cluster I, which contained sequences transiently accumulating during the penetration process. By contrast, many sequences relating to protein degradation and amino-acid uptake were identified among the sequences accumulating at this stage. Taken together, these results suggest that *P. parasitica* may use amino-acid uptake from the plant as a carbon source, as soon as penetration occurs. By contrast, sugar and lipid metabolism may be repressed at early stages of infection. Jupe and coworkers recently reported an enrichment in functions relating to gene expression and metabolism during the biotrophic phase of tomato infection by *P. capsici* [21]. They suggested that amino-acid uptake from the plant would not be favored by *P. capsici* at early stages of infection. This study provided no information about sugar and lipid metabolism but, as we obtained conflicting results, additional studies are required to determine which carbon sources are used by *Phytophthora* species during early stages of infection.

A large number of sequences relating to signaling were identified in the cluster grouping together sequences transiently repressed during penetration, within which protein kinases were particularly abundant. In addition, few, if any, signaling-related sequences were identified among the cellular functions for sequences accumulating during penetration of the host plant by *P. parasitica*. As calcium is known to be required for appressorium differentiation in *P. infestans*, related signaling pathways should have been detected during penetration [10]. The absence of these functions among the penetration-specific genes may
be due to the corresponding transcripts and/or proteins accumulating in zoospores before infection, in avoid the need for de novo expression in appressoria and young infectious hyphae. Indeed, the penetration process occurs very rapidly after zoospore germination in P. parasitica [8,23]. Consistent with this hypothesis, several protein kinases, including a calcium-dependent kinase, and transcription factors were observed among the cluster II sequences transiently expressed in zoospores. Thus, the zoospore, in addition to its role in dissemination, may be considered to be a pre-infection stage expressing important functions required for the penetration process.

An analysis of the sequences transiently accumulating during penetration highlighted functions that have been reported to influence the behavior of the interaction. The proteins concerned included a set of proteins triggering the necrosis of plant tissues and including elicitors, Nlp-like proteins and proteins homologous to M81 elicitors. Sequences encoding protease inhibitors and transporters putatively involved in the efflux of toxic plant molecules were also observed. Such sequences have been reported to accumulate in P. infestans germinated cysts and probably constitute general weapons in the arsenal of Phytophthora spp. [18,19]. Cell wall-degrading enzymes were particularly abundant among the transcripts transiently accumulating during the first few hours of infection. Judelson and coworkers also detected numerous CWDEs in P. infestans germinated cysts [19]. We also noticed that CWDEs displayed very unusual expression patterns. Some enzymes were specific to penetration, whereas others were expressed in the mycelium or during the necrotrophic phase of the interaction. This result confirms our previous hypothesis that a specific set of P. parasitica CWDEs may be mobilized to soften the plant cell wall and facilitate penetration [8]. The finding that other CDWE genes are expressed in vegetative cultures or during the necrotrophic phase of the interaction suggests that necroptrophy is related to saprophytic growth and that this second set of enzymes is used to obtain sugars from the walls of dead cells.

Finally, most of the RXLR effectors represented on the array displayed transient expression during penetration. This finding was not unexpected, because most of these sequences originated from appressorium-derived cDNAs [8]. Nevertheless, this result indicates that, as observed for CWDEs, a specific set of effectors is activated during penetration. No such expression pattern was not observed for transcripts encoding CRN proteins, suggesting different roles for these two classes of cytoplasmic effectors. Previous studies reported the detection of transcripts encoding secreted effectors in appressoria from Phytophthora species such as P. infestans, P. sojae, and P. capsici and in appressoria from ascomycetes, such as Magnaporthe grisea and Colletotrichum higginsianum [19,21,40,41]. However, the secretion of Phytophthora cytoplasmic effectors has been documented only in hau-storia to date. By using a transcriptional fusion with the GFP reporter gene, we were able to detect the expression of an effector in the appressorium and infectious hyphae during the penetration process. Our work thus suggests that appressoria and infectious hyphae are not only involved in plant penetration, they may also act as secretory organs for the transfer of cytoplasmic effectors into the host. These penetration-specific effectors may be involved in manipulating plant cells to facilitate establishment of the pathogen. Wang and coworkers described successive waves of effector expression during plant infection by P. sojae [42]. These authors suggested a relay between these successive waves, interfering with plant immunity at various levels. We have shown that the PSE1 effector, which is transiently expressed during penetration, can interfere with auxin physiology to facilitate plant infection [38]. Additional functional analyses of effectors transiently expressed during the onset of infection are required to determine whether this specific set of proteins plays a particular role in modulating plant development.

This study provides new insight into the process by which Phytophthora species penetrate their hosts. Based on our results for about one fifth of the gene content of this species, we propose several hypotheses concerning the biology of the infection process of P. parasitica. Future projects, making use of the full genome sequence, which is now available, will complete this analysis and should make it possible to test our hypotheses.

Conclusion
By using precisely calibrated interaction systems, we characterized the genetic program activated by P. parasitica during the initial infection of A. thaliana cells. Expression of a series of genes is transiently modulated during the penetration of the host. These modulations account for a specific developmental program occurring at the penetration stage. During penetration, genes encoding proteins involved in lipid and sugar metabolism are down-regulated whereas genes encoding functions controlling the transport of amino acids seemed favored. Similarly, cell wall degrading enzymes and other proteins involved in pathogenicity including RXLR effectors are highly modulated. Interestingly, P. parasitica uses distinct genes from these families during penetration and subsequent necrotrophic phase. We reconsidered the respective roles of the different developmental stages occurring during the infection cycle in this study. We suggest that the appressorium is involved in manipulating host cells to facilitate infection.
Additional files

Additional file 1: Figure S1. Pipeline used for sequence clustering, sequence origin determination and oligoarray design.

Additional file 2: Table S1. Clustering of P. parasitica EST sequences. Sequence composition of each contig as obtained with TGIGL package is presented. Each contig was named ClxContigx, with x corresponding to the cluster and contig number.

Additional file 3: Table S2. Functional annotation of the sequences. Predicted origin of the sequences is indicated together with BlastX result (E value < 1E-05) against the P. parasitica protein database (Broad Institute) and BlastX result (E value < 1E-05) against NCBI non-redundant (NR) protein database are presented together with GO annotations as proposed by blast2GO tool.

Additional file 4: Table S3. Clustering of the expression patterns. Sequences contained in each expression profile clusters as obtained after K-means clustering are presented. For all the sequences, normalized hybridization signal in each condition (mean of the two biological replicates), maximum fold change (max FC) observed between the different biological samples and BlastX best hit result (E value < 1E-05) against NCBI non-redundant (NR) protein database are indicated. For oligoarray hybridizations results, samples corresponded to mycelium grown in V8 medium (Myc), swimming zoospores (Zoo), samples enriched for appressoria differentiated on onion epidermis (App), and A. thaliana plantlets inoculated with P. parasitica zoospores and recovered at different time points of the infection: 2.5 h (appressorium-mediated penetration), 6 h (apressorium-mediated penetration), 6 h (biotrophic growth, two to three cells invaded), 10 h (invasive growth along the stele) and 30 h (switch to necrotrophy).

Additional file 5: Table S4. Validation of the transcriptome results and clustering analysis. mRNAs corresponding to sequences from clusters A to F were quantified by quantitative RT-PCR in samples corresponding to mycelium grown in V8 medium (Myc), swimming zoospores (Zoo), samples enriched for appressoria differentiated on onion epidermis (App), and A. thaliana plantlets inoculated with P. parasitica zoospores at different time points of the infection: A. thaliana 2.5 hours after inoculation (apressorium-mediated penetration), A. thaliana 6 hours after inoculation (biotrophic growth, two to three cells invaded), A. thaliana 10 hours after inoculation (invasive growth along the stele), A. thaliana 30 hours after inoculation (switch to necrotrophy) and A. thaliana 4 days after inoculation (necrotrophy). Biological replicates are indicated as rep1 and rep2. Data are presented as expression ratios relative to UBC, WS21 and Mago Nashi reference genes (2−Δ ΔCT), nd: not detected. Column “array validation” indicates if the expression values obtained following quantitative RT-PCR show the same variation as that observed following oligoarray hybridization. Yes (except XXX) is indicated if all but one condition are in agreement with oligoarray hybridization results. Columns “cluster validation”: indicates if the observed variation corresponds to the predicted variation within each cluster (k-means clustering, Genesis program). → Cluster X indicates if, based on the qRT-PCR expression analysis, the sequence must be analyzed with a different cluster.

Additional file 6: Table S5, I. GO enrichment analysis of cluster. We estimated the differences between the observed number of sequences associated to GO terms in each cluster with expected number if randomly distributed. For each GO term, the number of sequences associated to this term in the cluster (column “occurrence in cluster”) and the number of sequences associated to this term represented on the array (column “occurrence on array”) are indicated. The total number of sequence represented in the cluster and on the oligoarray that were used for calculations of the frequencies, are mentioned (columns “sequences in cluster” and “sequences on array”). Fisher Exact test was used to identify significant enrichment for individual GO terms. P-value of the test is indicated for each GO term. Column “sequences” indicated the names of each sequence with the corresponding GO term in the cluster.

Additional file 7: Table S5, VI. GO enrichment analysis of cluster VI.

Additional file 8: Table S5, III. GO enrichment analysis of cluster III.

Additional file 9: Table S5, V. GO enrichment analysis of cluster V.

Additional file 10: Table S5, II. GO enrichment analysis of cluster II.

Additional file 11: Table S5, IV. GO enrichment analysis of cluster IV.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

AA participated to the design of the oligoarray, obtained the biological samples, analyzed the transcriptome data and edited the manuscript. EE performed the gene enrichment analysis of the clusters, obtained the transgenic strains and performed the confocal analyses. NMW obtained the biological samples, performed the q-RT-PCR validations and obtained the transgenic strains. FP edited the manuscript. ED obtained the EST assembly and participated to the design of the oligoarray. CM performed the q-RT-PCR validations. MP obtained the biological samples. MG participated to the design of the oligoarray, obtained the biological samples, analyzed the transcriptome data, obtained the transgenic strains, performed the confocal analyses and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was funded by grants from INRA, Direction scientifique ‘Plante et Produits du végétal’ and Department ‘Santé des Plantes et Environnement’. We thank Prof. Torii (Washington USA) for providing the A. thaliana transgenic line expressing the PM-RB marker.

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Received: 4 October 2013 Accepted: 3 June 2014 Published: 29 June 2014

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