



**Expression patterns of
pathogenicity genes during
Phytophthora pisi infection of pea roots**

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Expression patterns of pathogenicity genes during *Phytophthora pisi* infection of pea roots

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ABSTRACT:

The genus *Phytophthora* (phylum Oomycota), contains destructive plant pathogens that cause enormous economic damage to many important crop species. These fungus-like microorganisms employ diverse mechanisms to break down plant defences. Understanding of the strategies by which these pathogens colonize their host is essential to establish improved methods for controlling *Phytophthora* infection. *P. pisi* is a new species of *Phytophthora* that causes root rot in pea and is a putatively devastating pathogen for cultivation of pea in many parts of the world. Furthermore, like other species of oomycetes such as *P. infestans* and *P. sojae* this species can be used as a model in molecular plant-microbe interactions research. This study was planned to provide an insight into the transcriptome of *P. pisi* during the infection process of pea roots and also on necrotic material. Thus, pea roots were inoculated with *P. pisi* and then harvested at six different time points. Colonization levels of *P. pisi* in pea roots as well as the expression of genes possibly involved in the infection process were monitored using different DNA-based methods (quantitative PCR and (RT)-PCR). Ten candidate pathogenicity genes were identified and sequenced for *P. pisi* and their expression was measured during the infection process using quantitative reverse transcription (RT)-PCR. The putative pathogenicity genes cysteine protease (*Pro1*), putative endo 1,3; 1,4 beta glucanase (*Glu1*), phosphoenolpyruvate carboxykinase (*Pck1*), enzyme inhibitors (*Gip1*) and (*Epi1*), crinkler-like protein (*Crn1*) and putative ABC-transporter (*Pdr1*), were expressed during infection. Transcript levels of *Pro1*, *Glu1*, *Pdr1* and *Crn1* increased over the experimental period, while transcript profiles of *Gip1* and *Epi1* showed high expression during the first 2-6 hours following inoculation. No expression was detected for pyruvate dehydrogenase (*Pdh1*), or the putative effector genes *Avr1b-1* or *Nip1*. In a follow-up experiment, *Pro1* expression was higher during *P. pisi* growth on dead pea roots at 20 hours post-inoculation when compared with growth on living roots. *Epi1* was only expressed on live host material. Domain structure analysis revealed that *P. pisi Pro1* protease contains three conserved domains, a peptidase domain, an inhibitor domain and a lipid binding domain. Phylogenetic analysis of *P. pisi* protease revealed that this protein is most closely related to a homologue from *P. sojae* and likely belongs to a cathepsin-L-like group from the C1A subfamily of peptidases.

POPULAR SCIENCE:

Phytophthora pisi is a new agricultural pathogen, causing root rot in pea and is a putative devastating pathogen for this cultivation. It belongs to the phylum Oomycota, the fungus-like microorganisms that include some of the most destructive plant pathogens. They employ diverse strategies to break down plant defences. To establish improved control methods for oomycete infection, it is important to understand the strategies by which these pathogens colonize their host. This study was planned to identify putative pathogenicity factors of *P. pisi* during the infection process of pea roots. Hence, pea roots were inoculated with *P. pisi* and then harvested at six different time points. Colonization levels of *P. pisi* in pea roots as well as the expression of genes possibly involved in the infection process were monitored using different DNA-based methods (quantitative PCR and (RT)-PCR). Ten candidate pathogenicity genes were identified and sequenced for *P. pisi* and their expression was measured during the infection process. Expression of genes associated with hydrolytic enzymes including protease (*Pro1*) and glucanase (*Glu1*) as well as genes involved in transport of toxins (*Pdr1*) and host tissue necrosis (*Crn1*) increased during the infection, whereas genes encoding proteins which inhibit plant enzymes (*Gip1* and *Epi1*) were expressed highly during the first 2-6 hours following inoculation. No expression was detected for the gene involved in glycolysis (*Pdh1*), or for the putative effectors *Avr1-b1* and *Nip1*. Domain structure and phylogenetic analysis was carried out for a *P. pisi* protease.

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INTRODUCTION

Oomycetes:

Oomycetes, also known as water molds, are among the organisms that cause many serious plant diseases and large economic losses in agriculture. They are a diverse group of eukaryotic organisms that can be found in many ecological niches, including both saprophytes and parasites of plants, animals, insects and various microorganisms (Thines et al, 2010). More than 60% of the known species of oomycetes are parasitic on plants (Beakes and Sekimoto, 2009; Kamoun et al., 2003).

The major plant-pathogenic genera of oomycetes are *Albugo*, *Bremia*, *Peronospora*, *Phytophthora*, *Plasmopara* and *Pythium* (Kamoun et al., 2003). Among these genera, species of the genus *Phytophthora* are arguably the most destructive pathogens of dicotyledonous plants such as potato, pepper, tomato, soybean, pea and alfalfa (Kamoun et al., 2003; Tyler et al., 2006). This genus belongs to the order *Peronosporales* and has more than 80 species of hemibiotrophic pathogens (Kamoun et al., 2003; Tyler, 2007). The most notable pathogenic oomycete is *P. infestans*, the casual agent of late blight in potato, which caused the Irish potato famine in the mid-nineteenth century. *P. sojae* is another pathogen that causes stem and root rot of soybean, with an annual cost worldwide of \$1-2 billion (Tyler, 2007).

Unique biological features of oomycetes:

Oomycetes was for long time classified to the fungal kingdom, due to their filamentous growth habit. However, modern molecular analyses show that they are more closely related to brown algae (heterokonts) in the stramenopile kingdom (Kamoun et al., 2003; Thines et al, 2010). Both fungi and oomycetes form mycelia and spores during asexual and sexual reproduction and have filamentous growth in the vegetative stage. However, there are several important morphological and physiological differences between these two groups. The cell wall of most oomycetes consists mainly of cellulose and beta-glucans rather than chitin, as in fungi. Another distinction is that the vegetative stage of fungi is haploid, whereas oomycetes are diploid. Oomycetes and fungi also synthesize the amino acid lysine by different metabolic pathways. The asexual spores of most oomycetes (sporangia) produce zoospores with two flagella from which one is whiplash and the other is tinsel type. In contrast, only a few true fungi produce motile spores with only one whiplash flagellum (Kamoun, 2003).

Mechanism of *Phytophthora* infection and disease spread:

All *Phytophthora* spp. grow primarily as aseptate hyphae. Three kinds of asexual spores can be produced, sporangia, zoospores and chlamydospores. Sporangia can differentiate to produce 10-30 zoospores or in airborne species they can germinate directly to form hyphae. The biflagellate zoospores break through the vesicle wall and actively swim away. They are generally short-lived and quickly form adhesive cysts, which can germinate to produce hyphae or a secondary zoospore. In many species such as *P. sojae* the zoospores are the most important agent of dispersal and infection of roots. Chlamydospores are thick-walled resting spores, which are produced in some species and can be found on the dead plant material (Tyler, 2007). During sexual reproduction, the male differentiated organ called the antheridium fuses with the female differentiated organ called oogonium and a single haploid nucleus is transferred to the oogonium. The fertilized oogonium forms a long-lived oospore, which eventually can produce hyphae (Tyler, 2007).

Pathogenicity factors and effector molecules of *Phytophthora*:

Plant pathogens employ a variety of strategies to colonize and infect their hosts. After attaching to and penetration of the host surface, necrotrophic pathogens typically grow through plant tissues as hyphae while secreting a variety of enzymes and toxins that kill and degrade host tissues. Biotrophic pathogens on the other hand, rely on living host tissues for their nutrient acquisition. Typically, biotrophic pathogens penetrate the host cell wall but not the plasma membrane, developing specialized feeding structures. Many oomycete pathogens can be characterized as hemibiotrophs where an initial biotrophic phase eventually is followed by a necrotrophic phase.

In order to establish a successful infection, pathogens need to modulate biochemical, morphological and physiological processes in their hosts. In oomycetes, this is typically achieved through secretion of proteins and other molecules collectively known as effectors (Hogenhout et al, 2009). In the current work, effectors are defined as all pathogen proteins and small molecules that manipulate host-cell structure and function, either by facilitating infection (toxins and virulence factors) or by activating defence response (elicitors and avirulence factors) (Kamoun, 2006). Oomycete effectors localize to different sites in their host plant tissue. Apoplastic effectors act in the extracellular space, where they interfere with

the plant proteins involved in defences. These effectors include inhibitors of plant glucanases and proteases (Rose et al., 2002; Tian et al., 2004). Other oomycete effectors, known as cytoplasmic effectors, are delivered into host cells. For instance, certain effectors with avirulence activities are translocated inside host cells where they are recognized by resistance (R) proteins (Hogenhout et al., 2009; Schornack et al., 2009). Several cytoplasmic effectors are modular proteins containing an N-terminal signal peptides for secretion as well as a C-terminal domain, which in cytoplasmic effectors carry the biochemical effector activity (Kamoun, 2006; Schornack et al., 2009). Many effector genes have distinct patterns of expression during colonization of host plants. Some effectors, such as apoplastic enzyme inhibitors, show a transcriptional up-regulation during the biotrophic phase of infection, whereas others, such as necrosis-inducing proteins, are expressed later during the necrotrophic phase (Schornack et al., 2009).

The pea pathogen *Phytophthora pisi*:

P. pisi causes root rot in pea, and is an emerging disease in Southern Sweden. *P. pisi* is putatively a novel species, although it is possibly identical to a taxon described in 1959 as *P. erythroseptica* var *pisi* (Bywater and Hickman., 1959). Based on a multigene molecular phylogenetic analysis, the most closely related species is *P. sojae*, an important root and stem pathogen of soy bean (Heyman et al in prep). The root symptoms caused by *P. pisi* are similar to those produced by *Aphanomyces euteiches*, the main casual agent of root rot in pea. Pea plants infected with *P. pisi* are stunted with soft rotted roots. The symptoms usually do not expand above the cotyledons. In addition to pea, this pathogen is able to infect a group of closely related legumes such as faba bean, vetch, chickpea, lentils, spring pea and sweet pea. *P. pisi* is homothallic and produces typical large, aplerotic oospores with thick walls and amphigynous antheridia in infected roots and on growth media. The asexual sporangia are variable in shape and size, up to 65 um in length (Heyman, F., pers. comm).

Candidate pathogenicity genes of *P. pisi*:

In this study a set of *P. pisi* genes for gene expression analysis was selected based on previous studies on different species of *Phytophthora*. Two genes are associated with metabolic pathways (glycolysis and gluconeogenesis), two genes encoding hydrolytic

enzymes (cystein protease and glucanase), one gene from the “crinkler” family, one gene encoding an RXLR effector, an ABC transporter gene involved in drug resistance, two genes encoding enzyme inhibitors (protease and glucanase inhibitors) and one gene encoding a necrosis- inducing protein (Nip). Some of these genes (such as crinklers and enzyme inhibitors) represent known important effector categories (Kamoun, 2006), and others (genes involved in glycolysis and gluconeogenesis) have been shown to be upregulated specifically during the infection phase in *Phytophthora* gene expression studies although their role in pathogenesis might still be unclear (Judelson et al., 2008; Torto-Alalibo et al., 2007).

OBJECTIVE

As an emerging disease, the future impact of *P. pisi* pea root rot in Sweden, and the rest of Europe, is hard to predict. The reason behind the current increase in *P. pisi* incidence is not known. In addition, future control measures must consider the implementation of integrated pest management in the European Union. Research on *P. pisi* infection biology and pea resistance will be beneficial for future decisions on efficient control strategies. However, as a putatively novel species, very limited information about laboratory protocols and gene sequences are available for *P. pisi*. Therefore, the first objective of the current study is to establish an *in vitro* infection system of *P. pisi* on pea. The second objective is to develop a quantitative PCR method to measure *P. pisi* and pea DNA, and to use this method to follow colonization patterns in infected material. The third objective is to identify putative pathogenicity factors in *P. pisi* by testing for differential gene expression during infection of pea.

MATERIALS AND METHODS

Plant and pathogen material:

Pea seeds (cv “Finulf”) were sterilized in sodium hypochlorite (10%) for 5 min, and washed in water. Then 15 seeds were put in a row along the edge of a wet paper towel, which was then rolled. The rolled towels with the seeds were then placed in beakers filled with water to 2cm depth, and incubated in darkness at 25°C for four days. Totally around 450 pea roots were germinated.

Strain 97603 of *P. pisi* was maintained on dilute Granini Juice agar (4% filtered juice and 2% Bacto Agar). For producing *P. pisi* zoospores, flasks containing 25 ml liquid Lima bean extract medium (Oardc, 1994) were inoculated with three pieces of oomycete mycelium. After incubation for 3 days in darkness at 25°C the mycelium was rinsed with autoclaved river water in 3 steps to produce zoospores. In the first step, the lima bean media was removed and the mycelium was rinsed twice with river water. The second and third steps were carried out 4 and 6 hours after the first step respectively and in each time the mycelium was rinsed once with river water. After the three rinsing steps, the mycelium was incubated overnight in darkness at 25°C.

Inoculation and harvesting of the pea roots:

Pea seedlings with approximately 4 cm roots were mounted on supporting racks made from the top of 96-tip pipette boxes, and placed in a glass jar in a way that approximately 2 cm of the roots were in contact with zoospore suspension (2.5×10^6 zoospores/ml) (Fig. 1). Six racks each carrying 40 peas were placed in zoospore suspension for 30 min followed by washing in autoclaved river water. Incubation was continued in long day light (16 h) at 27°C. The pea roots were harvested at 2, 6, 20, 27, 48 and 72 hours post-inoculation (hpi). For each time point, four harvesting replicates each consisting of ten pea roots were harvested. About 1 cm from the end of the pea roots were cut and ground to powder with a pestle in liquid nitrogen mortars and stored at -80°C. Zoospore attachment and infection was monitored microscopically (Leica DM5500 B) or using a stereomicroscope (Leica M165FC).

In a separate study involving dead plant material, 80 peas were germinated as described. After 4 days, 40 pea seedlings were incubated at 60°C for 3 hours and the rest were kept as the live material. Live and dead roots were subsequently inoculated as described above. The roots were harvested 20 hpi. Each treatment (dead and live host material) contained four replicates. Ten pea roots were harvested in each replicate.



Fig. 1. Inoculation system of pea roots with *P. pisi* zoospore. The racks carrying 40 pea seedlings placed in zoospore suspension.

RNA isolation, quality analysis and cDNA synthesis:

RNA was isolated from about 100 mg of ground, infected tissue and 72 hours old *P. pisi* mycelia using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Traces of DNA was removed by DNase I treatment; 3.4 µl DNase I buffer and 1 µl DNase I (Fermentas) were added to 30 µl of each RNA sample, incubated in room temperature for 20 min followed by adding 1 µl of EDTA and enzyme inactivation by incubation at 70°C for 10 min. RNA concentration was determined spectrophotometrically (NanoDrop, Saveen Werner). RNA quality was analysed by electrophoresis on a Agilent Bioanalyzer using the RNA 6000 Nano kit (Agilent Technologies) according to the manufacturer's instructions.

Reverse transcription of approximately 370 ng/µl of DNase-treated RNA was carried out in a 20 µl reaction volume, using iScript Reverse Transcription Kit (Bio-Rad) according to the manufacturer's instructions. The synthesized cDNA was diluted 10x into a final volume of 200 µl.

PCR primer design and PCR conditions:

PCR primers were designed to amplify approximately 500 bp amplicons from candidate *P. pisi* pathogenicity genes, based on conserved region of genes in the *P. sojae*, *P. infestans*, *P. ramorum* and *P. capsici* genome sequences (Table 1). Additional primers were designed to amplify and sequence the full-length sequence of *Pro1* (Table 1). Sequence alignments and PCR primer design were done using the MegAlign and Primer Select programs, respectively (DNASTAR).

PCR was run in total volume of 50 µl containing 1x Dream Taq Green Buffer (Fermentas), 200 nM dNTP, 0.75 mM MgCl₂, 0.02 U/µl Dream Taq DNA Polymerase (Fermentas), 1.5 µM primers and 15 ng of *P. pisi* genomic DNA. Amplification started by heating the samples to 94°C for 4 min followed by 35 amplification cycles, each cycle consisting of 30 sec at 94°C, 30 sec at annealing temperatures ranging from 58 to 72°C, and 1 min at 72°C. The optimal annealing temperature for each primer pair was determined in a preliminary run using a gradient thermocycler. The last cycle was followed by 5 min at 72°C and then 12°C. Amplified products were analysed by electrophoresis on 1% agarose gels. PCR products were purified with AMPure magnetic beads (Agencourt). The purified fragments were sequenced by the company Macrogen in both forward and reverse directions.

Table 1. PCR primers used for partial amplification of candidate genes in *Phytophthora pisi* genome.

Gene	Reference ^a	Forward primer 5'-3'	Reverse primer 5'-3'	Amplicon ^b
<i>AvrIb-1</i>	AY426744	GCTACGTCGTGACCT GCAACGC	CCTTCTTTGCCCACTTCTCG T	267
<i>Cystein protease</i>	CF844845	ACGAGTTCTCGGCCT GGATG	TCCCCACGAGTTCTTGACC TTC	815
		CCATTGAGGCCGACC AGA AG		(seq) ^c
		AGAAGACCAAGACCA TCA CTCTCT	AAGCTGACCGTCTCTATTC CA CTC	(seq) ^c
			CGCGCCTGTGGTGGAGA	(seq) ^c
<i>Putative endo 1,3; 1,4 beta glucanase</i>	CF840448	ACATCGGCGTGCTGA AGAAGG	TTACACGGGGCAGTTGGTC T	707
<i>Phosphoenolpyruvate carboxykinase</i>	CF853080	CTACGGCGGCGAGAT GAAGAAGG	GCGCGAGTTGGGCTGGTGG TG	430
<i>Pyruvate dehydrogenase</i>	CF848005	CCCTCCGTCGCGTCCT CAC	GGCGGCCTTCAGCAGACCA	560
<i>PsojNIP</i>	AF511649	CAAGCCTCAAATCCA CATCAGCAA	GCGTCGCCAAAGTCCGTGT C	516
<i>Crn2</i>	BE584012	ACGTGCTGGTGGTGG TTC	CTTCAATCAGCATCTTCAC TTCAG	495
<i>Gip1</i>	AF406607	CCTCACCTCTGCGCTC GTCG	CTCGCCAAACCGATGAGG ATGT	663
<i>Epi</i>	CF842223	TACAAGCGCATCTAC GGAAAGT	GACTCGCGCAGAATCGTGT	683
<i>Pleiotropic drug resistance transporter</i>	AAT85568	GTGCGCTTCGAGAAC CTGTCCTT	CGCTGTCCAGGCCCGTAGA G	625

^a GenBank accession number of homologue in *P. sojae*, ^b Length in base pairs, ^c Internal primers for sequencing

Sequence and phylogenetic analysis:

Sequence reads were edited using SeqMan and EditSeq programs (DNASTAR) and compared with sequences available in the GenBank database (Benson et al., 2008), using BLAST (Altschul et al., 1997). Conserved protein domains were identified through the Conserved Domain database (Marchler-Bauer et al., 2009). Additional cysteine protease sequences were retrieved from the *P. sojae* genome database (<http://genome.jgi-psf.org>), and from the MEROPS database (Rawlings et al., 2008). Sequences were aligned with Clustal W implemented in MEGA4 (Tamura et al., 2007). Phylogenetic analyses were performed using Neighbour-Joining implemented in MEGA4, using pair wise deletion of gaps and the Poisson

correction distance of substitution rates. Statistical support for phylogenetic grouping was estimated by 1000 bootstrap resamplings.

Quantitative PCR (qPCR) primer design and quantification of transcript levels:

Primers for qPCR were designed towards partial sequences of *P. pisi* candidate genes to amplify amplicons ranging from 80 to 150 bp using Primer Select software (DNASTAR) (Table 2). Primer annealing temperatures were evaluated by gradient-PCR, using *P. pisi* genomic DNA as template. Genomic DNA from pea was used as a negative control for the qPCR primers. Primer efficiency values were determined by amplification of serial dilutions of target gene PCR products. Purification of target gene PCR products were done by mixing with 1/10 volume of NaOAc (3M, pH 5.2) and 2.5 volume of 95% ETOH followed by 1 hour incubation at -20°C. The DNA was pelleted by centrifuging for 20 min at 13000 rpm. After removing the supernatant, the pellet was washed with 70% ethanol and redissolved in 20 µl water.

QPCR reactions were performed in an iQ5 multicolour Real-Time PCR Detection System (Bio-Rad) using the Maxima SYBR Green qPCR Master Mix Kit (Fermentas). Five µl of the cDNA solution described above was used as a template for each 20 µl PCR reaction. The primer concentration of all genes assayed was 150 nM. The reactions were performed using the following conditions: one cycle of 95°C for 5 min, 40 cycles of 95°C for 10 s, 60°C (some cases at 62 or 66°C) for 30 s and 72°C for 30 s. The threshold cycle (Ct) values were determined based on two technical replicates. To confirm that the signals were the result of a single amplified product, melting curve analysis was performed after the run. Transcript copy numbers were calculated from the Ct values based on standard curve amplification, and gene expression was calculated as the ratio between target gene transcript number and 1000 40S ribosomal gene (*S3a*) transcripts (Judelson et al., 2008) as the pathogen housekeeping gene.

Quantification of pathogen colonization:

Host and pathogen DNA levels in infected material were quantified with qPCR as described above. Five µl of RNA solution prior to DNase treatment was used as the template. *S3a* was used as reference gene for the pathogen while elongation factor alpha (*efa*) (Vicente die et al., 2009) was used as reference gene for the host. Colonization was calculated as the ratio

between pathogen *S3a* and host *efa* gene copy number in the residual DNA present in the crude RNA extracts. The ratio was based on the mean value of four biological replicates.

Table 2. qPCR primers used for monitoring *Phytophthora. pisi* transcripts during infection of pea roots.

Gene	Forward 5'-3'	Reverse 5'-3'	Amplicon (bp)	Annealing temp °C
<i>Avr1b-1</i>	AGATCTCGTCCGTCGCTCGCTTAG	CCCCCGCATCGTCCTCTTCAT	83	60
<i>Pro1</i>	TCAACCCGCAGGACGAGCAC	CACCCCGGACTTGTAGAAGTGA	107	60
<i>Glu1</i>	TGTTGGGCCACGTATCCTTCC	AGCAGCTGCGGCACCTTGAC	106	60
<i>Pck1</i>	GTGGTGCGGGATGTGGTAGAT	AACGAGCCGGACATTTTCAAC	147	60
<i>Pdh1</i>	GAAGGGCGAAAACAAGCAGTAAT	CTCGGCCGCCAAGCAGTTCTA	164	60
<i>Nip1</i>	GGTACCTGCCCAAAGACGACAC	GCGTGCTGCCACTGAAG	176	60
<i>Crn1</i>	CGCGACCACGGACTGAACA	ATGGCCTAATCCGTCGTGCTC	161	60
<i>Gip1</i>	CGGCGGTGCTCTTATCAGTCCT	CCGCTCGCCGTCCTTAC	156	62
<i>Epi1</i>	AGATGCGCTTGTACTCGTCCAGAA	GCCGTGTACCAGCCCGTTATC	118	66
<i>Pdr1</i>	GCCACCGACCAGCACCTCAC	GCCGGCCGGAAGACCAAC	107	62
<i>S3a</i>	TCACCAAGAAGCGCCCAAACCA	CCAATGATCTCCGGCACGAACT	157	60
<i>Efa</i>	AAGCTAGGAGGTTGACAAG	ACTGTGCAGTAGTACTTGGTG		60

Statistical analyses:

Temporal expression data was analysed using ANOVA (Statistica ver. 9.1, StatSoft) to test for effect of time on gene expression. Pairwise comparisons were made using the Fisher LSD method at the 95% significance level. Gene expression data from 20 hpi growth on live and dead roots was analysed by Student's *t*-test.

RESULTS

In vitro infection system:

Zoospores attached primarily to the tip of the pea roots, and had germinated already at 2 hpi. Approximately 20 hours hpi, most pea root tips showed soft rot that is the typical symptom of *P. pisi* infection, showing that the exposure to the zoospore suspension was sufficient to initiate successful infection (Fig. 2). By 72 hpi, infected root tissue contained oospores as well as mature sporangia releasing zoospores.

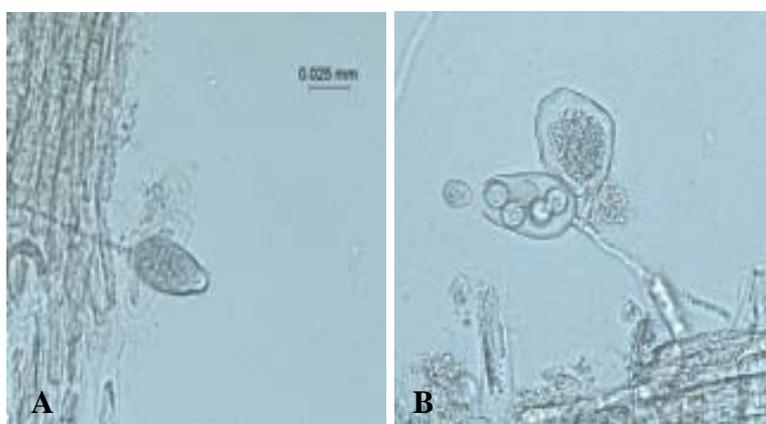


Fig. 2. Asexual structure of *P. pisi* (sporangium) (A). Release of zoospores from sporangium approximately 72 hours after pea root- inoculation (B).

Colonization of pea roots by *P. pisi*:

Colonization of pea roots by *P. pisi* was measured as the ratio between the amounts of pathogen and host DNA in samples, based on the genes *S3a* (*P. pisi*) and *efa* (pea) respectively. The *P. pisi* / pea DNA ratio showed a significant increase at 48 hpi in comparison to early sampling time points ($P = 0.011$) (Fig. 3).

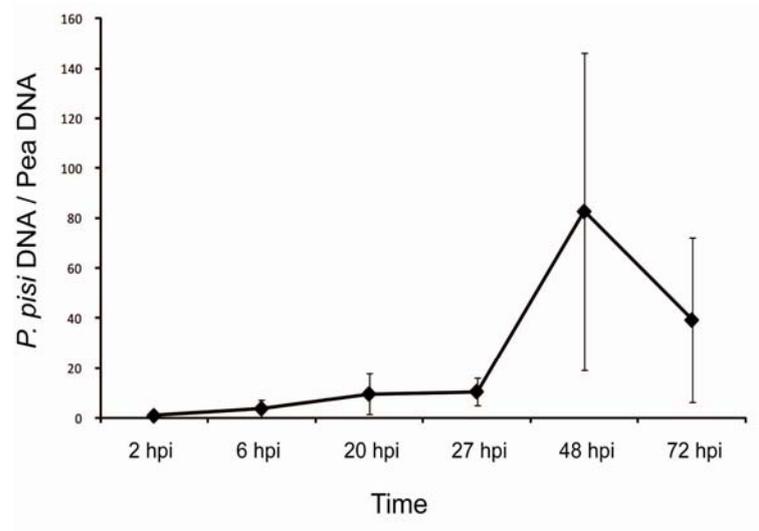


Fig. 3. Temporal colonization profile of *P. pisi* in pea seedling roots. *P. pisi* colonization is expressed as the ratio between *P. pisi S3a* gene copy number and pea *efa* gene copy number. Error bars represent the standard deviation of four biological replicates.

Gene expression analysis:

Analysis of the DNase treated RNA using the Agilent Bioanalyser assay revealed that all samples contained RNA of sufficiently high quality for qPCR based gene expression analysis (Fig. 4).

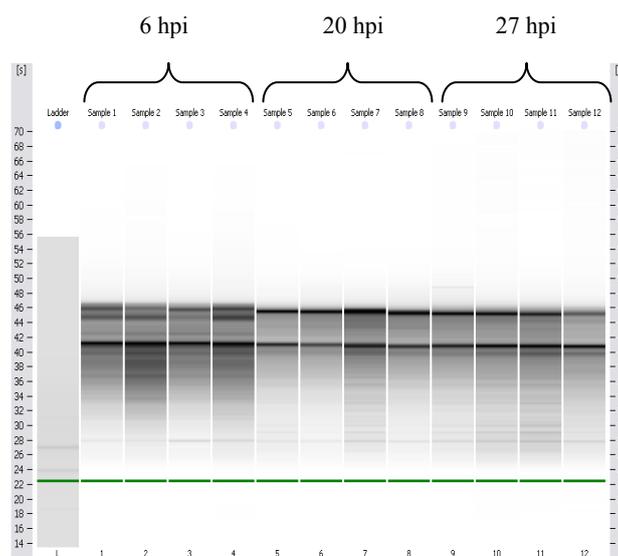


Fig. 4. Virtual gel image of total RNA from infected pea roots at 6, 20 and 27 hpi produced using automated gel electrophoresis in an Agilent bioanalyzer. One ul of DNase I-treated total RNA was analyzed for each sample.

All qPCR primer pairs designed for the pathogen gene expression amplified a single product with the expected size, and no amplification was observed in the controls containing genomic pea DNA (Fig. 5). In addition, results from melt curve analyses in the qPCR assays confirmed a single amplification product for every primer pair (data not shown).

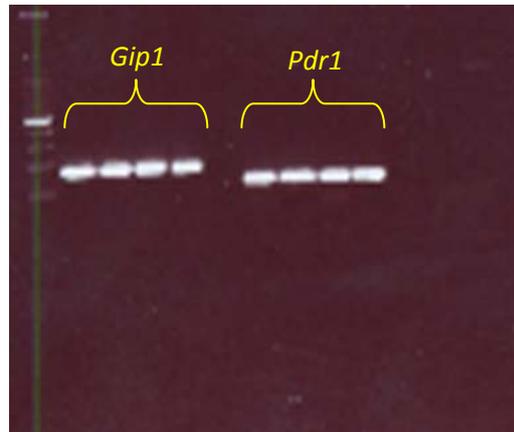


Fig. 5. Amplification of *Gip1* (130bp) and *Abc1* (179 bp) in *P. pisi* genome at annealing temperatures ranging from 60°C to 70°C, using relevant qPCR primers. No amplification was obtained in pea genome at 60°C. Marker is GeneRuler DNA ladder mix.

For three candidate genes *Avr1b-1*, *Nip1* and *Pdh1* no transcripts were detected in any of the infected root samples or in mycelium grown in liquid, whereas other monitored genes showed distinct patterns of expression in roots over the experimental period (Fig. 6). The transcript profiles of *Pro1*, *Glu1*, *Pdr1* and *Crn1* showed a significant up-regulation following inoculation ($P \leq 0.049$). The most significant up-regulation was observed in transcript profile of *Pro1* which showed 160 fold more expression at 72 hpi as compared to its expression at 2 hpi. Transcript levels of *Glu1* increased by four-fold at 48 hpi comparing with the levels at 2 hpi. *Crn1* showed a constant expression pattern up to 27 hpi, followed by an up-regulation at 48 and 72 hpi.

In contrast to other genes, no transcripts for *Gip1* or *Epi1* were found in mycelium grown in liquid culture, whilst a notable up-regulation was observed during the first 2-6 hours following inoculation ($P = 0.001$ and 0.053 respectively). Later during infection the transcript levels of *Epi1* declined at 20 hpi, approaching zero by 27 hpi. Transcript levels of *Gip1* decreased over the experimental period. Transcript profile of *Pck1* showed a significant up-

regulation early during infection at 2 hpi in comparison with its expression in mycelium grown in liquid culture, followed by a reduction at 6 hpi and a transient increase up to 48 hpi ($P < 0.019$).

Four candidate genes were selected for a second experiment where expression was compared between dead and live pea roots at 20 hpi (Table 3). No significant difference in expression of *P. pisi* *Pro1* and *Crn1* between dead and live roots was measured. *Epi1* showed expression during infection of live roots but no transcripts were found for this gene during growth on dead roots at 20 hpi. No *Gip1* transcript was found during *P. pisi* growth on neither live nor dead roots.

Table 3. Expression levels of four *P. pisi* candidate genes in live and dead pea roots at 20 hpi.

gene	Live root (ratio) ^a	Dead root (ratio) ^a	<i>P</i> -value ^b
<i>Pro1</i>	290±168	1639±1394	0.10
<i>Crn1</i>	74±28.5	88±35	0.57
<i>Epi1</i>	2506±379	–	0.05
<i>Gip1</i>	–	–	–

^a Expression ratio was calculated as the ratio between the transcript number of the target gene and 1000 *S3a* transcript copies. The value following ± represent the standard deviation of four biological replicates. ^b *P*-value from Student's *t*-test.

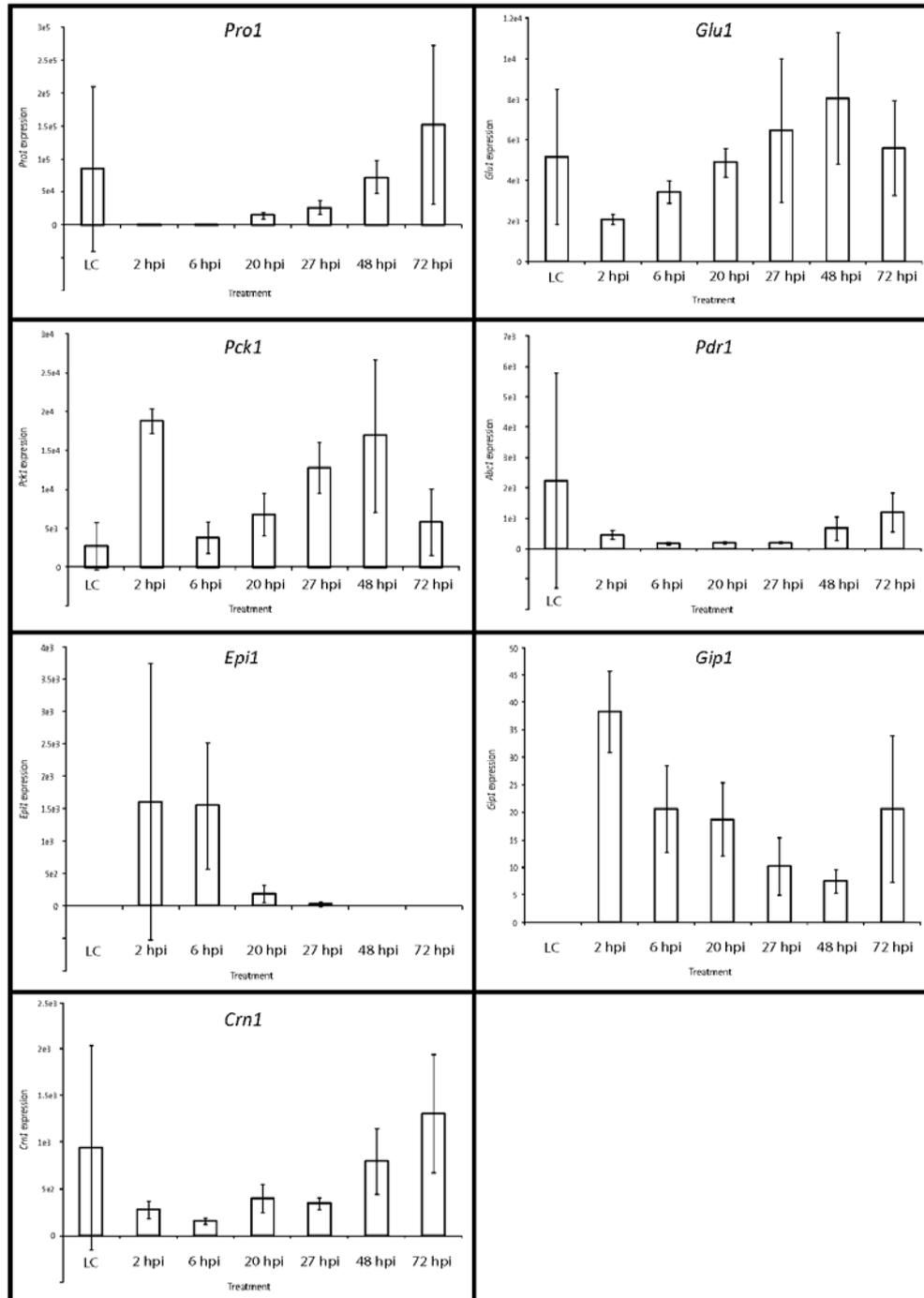


Fig. 6. Expression profiles of seven candidate genes of *Phytophthora pisi* during pea root infection and in mycelium grown in liquid media. Gene expression (y-axis) was calculated as the ratio between the transcript number of the target gene and 1000 *S3a* transcript copies. Error bars represent the standard deviation of four biological replicates.

Domain structure and phylogeny analysis of PRO1:

The 1599 bp open reading frame of *Pro1* encoded a protein sequence of 533 amino acids that showed high similarity (96 % identical amino acids) to *P. sojae* cysteine proteinase (CF844845). The protein sequence included a signal peptide for secretion, a peptidase_C1A super family domain (cd02248), an inhibitor_129 super family domain (cl07031) and a ML super family (MD-2-related lipid-recognition) domain (cl00274) (Fig. 7).

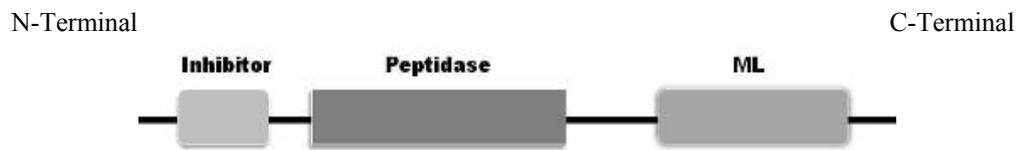


Fig. 7. Domain organisation of cysteine protease (PRO1) of *Phytothophora pisi*. One inhibitor_129 super family domain, one peptidase_C1A super family domain and one MD-2-related lipid-recognition domain.

BLASTX analysis of *P. pisi Pro1* against the *P. sojae* genome indicated the presence of 24 similar proteases in *P. sojae*. Among these 24 proteases, the ML super family (MD-2-related lipid-recognition) domain was present in only one *P. sojae* cystein proteinase (protein ID 142383). Phylogenetic analysis of *P. pisi* PRO1, *P. sojae* proteases and a selected set of other cysteine proteases revealed three separate groups (Fig. 8); one consisting of cysteine peptidases from family C1A subclass cathepsin-L-like, another consisting of peptidases from family C1A subclass cathepsin-B-like and finally a group consisting of cysteine peptidases from family C1B. According to this phylogenetic analysis *P. pisi* PRO1 belongs to family C1A subclass cathepsin-L-like.

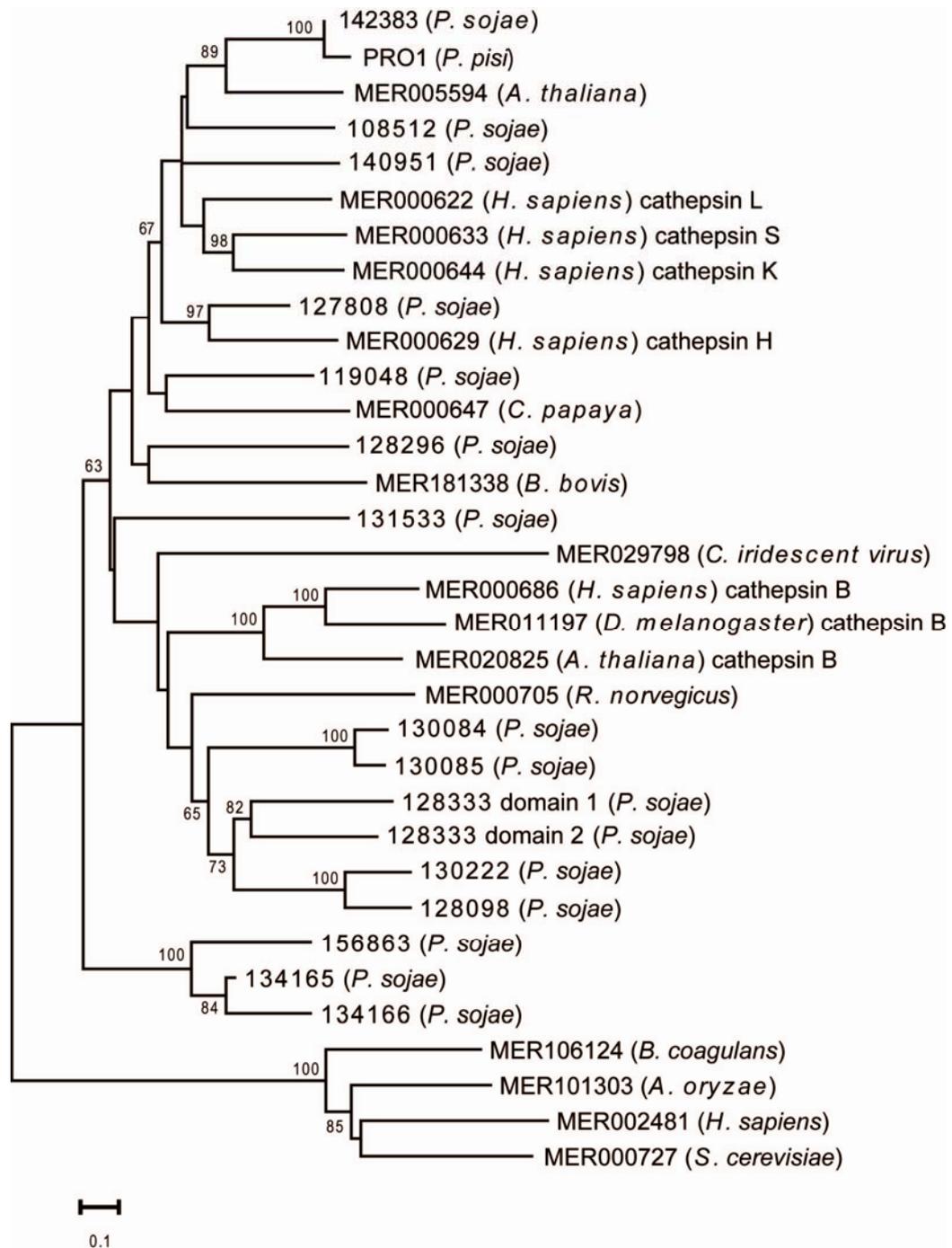


Fig. 8. Phylogeny of *P. pisi* cystein protease based on a selected set of cystein proteases. Analysis was performed using neighbour-joining implemented in MEGA version 4 with the poisson correction of substitution rates and pair wise deletion of gaps, based on a Clustal W alignment of protease peptidase domain amino acid sequences. Branch support values (bootstrap proportions ≥ 60) are associated with nodes. The bar marker indicates number of amino acid substitutions. Protein identifiers include protein accession numbers in MEROPS database or protein ID in the *P. sojae* genome database.

DISCUSSION:

Inoculation system and infection monitoring:

The initial requirement toward monitoring colonization level and pathogenicity factors of *P. pisi* during infection of pea roots was to establish an efficient inoculation system. To this goal the inoculation system was designed using supporter racks to place tip of pea roots in the zoospore suspension. By 20 hpi most pea roots showed soft roots, suggesting the efficiency of this system for inoculation of pea roots.

Stereomicroscopic observations confirmed that in *P. pisi*, infection can be initiated by zoospores as has been reported in *P. sojae* (Torto-Alalibo et al., 2007), the known species closest related to this pathogen. The motile zoospores attracted notably to the tip of pea roots. Like in *P. sojae* during soybean infection, approximately within 3 hpi, the zoospores form adhesive encysts that germinate to produce hypha and penetrate the host tissue. It has been reported that in *P. sojae*, by 6 hpi, six to eight cell layers of the root cortex are colonized by hyphae which then produce haustoria to absorb nutrients. By 24 hpi, hyphae cause the death of many plant cells. In a compatible interaction, at 48 hpi, collapse of host tissue has occurred, and the pathogen has shifted to necrotrophic growth (Torto-Alalibo et al., 2007). Based on our microscopic observations, in *P. pisi* during the compatible interaction, approximately by 48 hpi, sporangia might form and by 72 hpi the motile zoospores might release.

Pathogen colonization:

Colonization by *P. pisi* in pea seedlings roots showed a gradual increase of the pathogen up to 48 hpi, while it dropped by 72 hpi. However there was no statistically significant difference between colonization levels at 48 hpi and 72 hpi. Since the *P. pisi* / pea DNA ratio was measured at approximately 1 cm from the root tips, the colonization decrease at 72 hpi might have been a result of root growth by that time. Alternatively, it can be speculated that the root tips that is the first place of colonization is not a proper area for continuing more colonization during later infection.

Expression of candidate pathogenicity factors:

In this study the *P. pisi* candidate genes were selected based on previous studies on different species of *Phytophthora*.

Hydrolytic enzymes (Protease and Glucanase):

Genes encoding hydrolytic enzymes such as proteases and glucanases, which are involved in degradation of host tissues for nutrient acquisition, constitute a large group of pathogenicity factors in oomycetes (Judelson et al., 2008; Torto-Alalibo et al., 2007). The significant up-regulation of *P. pisi Pro1* during pea root infection suggests the importance of this enzyme in energy generation during colonization of host. Proteases, also known as peptidases, catabolise proteins and polypeptides through cleavage of the peptide bond. Thus pathogen proteases are thought to have important roles in adhesion to host cells, penetration of cell wall, colonization, manipulate the host immune system and catabolism of the host proteins for nutrition (Atkinson et al., 2009; Bindschedler et al., 2003). The notable 160 fold expression increase of *Pro1* at 72 hpi in comparison with 2 hpi, suggest that its biological activity is most likely degradation of host proteins for nutrient acquisition. This function is further supported by the higher *Pro1* expression in dead roots as compared with live roots at 20 hpi, as more proteins are available to *P. pisi* during necrotrophic growth than during the establishment of infection. The *P. sojae* ortholog to *Pro1* is highly induced during infection of soybean (Torto-Alalibo et al., 2007).

In oomycetes, glucanases have several different biological roles. They play a role in hyphal tip growth and branching where there is a delicate balance between cell wall synthesis and hydrolysis (Stössel and Hohl, 1981). Furthermore they have role in Ca^{2+} induced sporulation and in degradation of the host cell wall (McLeod et al., 2003). Endo-1,3;1,4-beta-glucanases are glycoside hydrolases that act on 1,3;1,4-beta-glucan, which is one component of plant, oomycete and fungal cell walls (Bartnicki-Garcia and Wang, 1983; Harvey et al., 2001). It has been shown that in *P. infestans*, glucanases are developmentally regulated during different *in vitro* growth stages and are also expressed *in planta*. Higher expression of *P. infestans Piendo2* was obtained late during a compatible infection (McLeod et al., 2003). Also expressed sequence tag (EST) analysis in *P. sojae* showed a high frequency of *Piendo2* in an infected soybean hypocotyl library. In this study *P. pisi Glu1* showed a gradual up-

regulation during infection, suggesting a possible role in nutrient acquisition or growth during pathogenesis. This four-fold up-regulation at 48 hpi compared to its expression at 2 hpi, indicate that more activity of GLU1 is required late during the necrotrophic phase of infection for degrading host cell walls, hyphal growth or sporangia germination.

Gluconeogenesis and glycolysis (Phosphoenolpyruvate carboxykinase and Pyruvate dehydrogenase):

Pathogen genes associated with glycolysis and gluconeogenesis play an important role in establishment of a profitable feeding relationship with a host. Adaption of a pathogen's metabolism to the changes in nutrient availability during the interaction with the host is important during the infection process. During the initial stages of infection, stored nutrients are the major source of energy for most fungi and oomycetes, but after penetration they start to utilize host components. Sugars and amino acids are the major plant nutrients which are mobilized through membrane transporters on hyphae or haustoria (Judelson et al., 2009). Phosphoenolpyruvate carboxykinase (PEPCK), is an enzyme used in the metabolic pathway of gluconeogenesis through converting oxaloacetate into phosphoenolpyruvate and carbon dioxide. In the current study, a significant six-fold increase in expression of *Pck1* by 2 hpi compared to liquid culture growth can suggest that the pathogen relies on stored nutrients for energy generation during the initial infection stage. The decrease of *Pck1* expression at 6 hpi can indicate a successful establishment of feeding structures and a switch to host-derived nutrients at 6 hpi. In *P. sojae*, formation of haustoria by 6 hpi has been reported (Torto-Alalibo et al, 2007). The gradual up-regulation of *Pck1* up to 48 hpi may indicate pathogen anabolism and build-up of nutrient storage compounds.

Pyruvate dehydrogenase is an enzyme associated with glycolysis (converting of glucose into pyruvate). The failure to detect expression of this gene in *P. pisi* either during pea root infection or during liquid culture growth may be explained by a low requirement of the *Pdh1* protein for glycolysis or that there are additional *Pdh1* gene copies in the *P. pisi* genome that are expressed.

Enzymes inhibitors (GIP1, EPI1):

Secretion of suites of proteins involved in attack, defence and counter defence is a remarkable characteristic of extracellular interactions between pathogens and their host plants. Among these proteins, glucanase and protease inhibitors that are produced by *Phytophthora* spp. to counter plant pathogenesis-related (PR) proteins are well studied. For instance, the glucanase inhibitors GIP1 and GIP2 are secreted by *P. sojae* into the apoplast to target the soybean endo- β -1,3 glucanase, EGaseA, inhibiting its hydrolytic activity (Bishop et al., 2005; Damasceno et al., 2008; Kamoun, 2006; Misas-Villamil and AL van der Hoorn, 2008; Rose et al., 2002). *Epi1* and *Epi10* encode serine protease inhibitors of *P. infestans* that both are up-regulated during infection of tomato. Also, PsojEPI1 is a predicted extracellular effector from *P. sojae* that belongs to the diverse family of Kazal-like serine protease inhibitors (Kamoun, 2006; Tian et al., 2004; Tian et al., 2005). There is no information available about *PsojEPI1* expression patterns.

In the current study, expression of *P. pisi Gip1* and *Epi1* was detected during infection but not in the mycelium grown in liquid culture, suggesting that expression of these enzyme inhibitors is induced by host factors. The transcript levels of *P. pisi Epi1* decreased by 27 hpi and no transcripts were found later during infection. These results suggest that EPI1 may play an important role in counter defence of *P. pisi* during the early biotrophic phase of pea root infection, followed by down-regulation during the necrotrophic phase. In addition, the *Epi1* transcript profile may suggest that the necrotrophic phase of *P. pisi* pea infection starts approximately between 20-27 hpi. The detection of *Epi1* transcripts in live but not dead roots at 20 hpi in the second gene expression experiment further supports this hypothesis.

Although very low *P. pisi Gip1* transcript numbers were detected, the differential expression during infection suggests that more expression of this protein is required early during infection as its function is to counteract host defence through targeting host hydrolytic enzymes,. In the second experiment no transcripts was found either in the live or in the dead roots.

RXLR effector (Avr1b-1):

The outcome of infection in many plant-pathogen interactions is determined by the interactions between resistance genes in plants and avirulence genes in pathogens. The

avirulence genes are among the cytoplasmic effectors that are associated with race specific resistance. The *Avr1b-1* gene of *P. sojae* was the first defined oomycete avirulence gene cloned by a map-based strategy (Shan et al., 2004). So far, four Avr genes from oomycetes have been cloned. These genes carry a conserved motif termed RXLR (Arg-X-Leu-Arg) that might function as a signal to mediate trafficking into host cells (Dou et al., 2008; Qutob et al., 2009). In this study expression of *P. pisi Avr1b-1* was not detected during pea root infection or in the mycelium. The result suggests that this particular gene in *P. pisi* might have become a pseudogene and some other avirulence genes are involved during infection. Another possibility is that although the virulent isolates of *P. pisi* contain the *Avr1b-1* gene, expression of this particular effector gene might be switched off to avoid host recognition, as has been reported for some virulent isolates of *P. sojae* (Shan et al., 2004).

Necrosis-inducing protein (PsojNIP):

Occurrence of host cell death is a casual feature of most plant-pathogen interactions that is associated with both susceptible and resistance interactions and might be caused by necrosis-inducing proteins. Nep1-like proteins (NLPs), are apoplastic effectors that are broadly distributed in *Phytophthora* and induce defence response in as many as 20 dicotyledonous plants, including both susceptible and resistant plants. PsojNIP of *P. sojae* (Qutob et al., 2002) and PiNPP1 of *P. infestans* (Kanneganti et al., 2006) are among the well studied examples that are shown to be expressed late during host infection and thus may function as toxins to facilitate colonization of host tissue during the necrotrophic phase. No expression of PsojNIP has been detected in encysted or germinating zoospores. In the current study *P. pisi Nip1* transcripts were not detected during infection of pea roots or in the mycelium. This result suggests that either there is no need for *P. pisi* to express this gene strongly during colonization of pea roots, or the function is replaced by a paralogous gene which was not detected with the primers, as *PsojNIP* has been reported to be part of a gene family in *P. sojae* (Qutob et al., 2002).

Crinkler protein (CRN2):

Another group of pathogenicity factors are members of the crinkler family that act as cytoplasmic effectors. The function of CRN proteins during pathogenesis is unknown (Tyler,

2009). CRN1 and CRN2, which first were identified in *P. infestans*, function as general elicitors and induce necrosis, chlorosis and cell death nonspecifically in both resistant and susceptible host plant (Torto et al., 2003). *P. infestans* *Crn* genes are constitutively expressed during colonization of host plant and *in vitro* grown mycelium (Torto et al., 2003; Torto-Alalibo et al., 2007). In the current study *P. pisi* *Crn1* was up-regulated significantly at 72 hpi, suggesting that it might aid in colonization of plant tissue during the late necrotrophic phase of infection. On the other hand, no difference was obtained between the expression of this gene between live and dead roots at 20 hpi which is consistent with the constitutive expression of *Crn* genes reported in *P. infestans* (Torto et al., 2003). Considering its role in inducing necrosis and cell dead late during infection, higher expression of this gene in live roots than dead roots might be obtained late during necrotrophic phase of infection (at 48 to 72 hpi).

ABC transporter (PDR):

Genes associated with detoxification, drug resistance and metabolic transporters form a large group of pathogenicity factors in fungi and oomycetes. ATP binding cassette (ABC) transporters are among these factors that play a role in protection of pathogen from fungicides and a wide variety of plant-derived toxins (Connolly et al., 2005). Members of the pleiotropic drug resistance (PDR) family are among the ABC transporters that have been described in yeast (Bauer et al., 1999) fungi (Del Sorbo et al., 2000) and plants (Sanchez-Fernandez et al., 2001). *Pdr1* of *P. sojae* is the first characterized PDR transporter from oomycetes and was reported to have the highest expression level of all ABC transporters in a *P. sojae* zoospore EST library (Connolly et al., 2005). PDR1 in the zoospore might function to protect cells from a group of exogenous toxins which are produced as a consequence of expended energy to sustain locomotion (Connolly et al., 2005). In the current study the expression profile of *P. pisi* *Pdr1* revealed significant up-regulation of this gene late during infection. Considering zoospore formation during late infection, as the pathogens life cycle is completed, it can be speculated that higher expression of *Pdr1* is required at this time to protect the zoospores from produced toxins.

Different expression patterns of these candidate genes represent valuable information for identifying putative pathogenicity factors of *P. pisi* during biotrophic and necrotrophic phases

of infection. The results show the efficiency of the inoculation system used in this study. However, high variation between biological replicates was observed in mycelium grown in liquid culture and in 72 hpi infection samples. This variation might suggest that the mycelium samples might not have been harvested at the same stage of fungal growth and some samples could have included zoospores. Also, microscopy observations show that the pathogen have completed its life cycle at 72 hpi and the production of new zoospores could be the reasons of this notable variation among 72 hpi replicates.

Domain and phylogeny analysis of PRO1:

Three conserved domain structures were found in PRO1. The peptidase domain indicate that the protein is a cystein peptidase (CPs) belonging to clan CA. Cysteine peptidases are classified into nine clans which have no common ancestor. Most parasite cysteine peptidases are within clan CA, which contain 24 families. Family C1 is a large and diverse family that splits into two broad classes in the MEROPS database: family C1B, a smaller group that contains no parasite proteins and is associated with bleomycin hydrolase activity, and family C1A, the cathepsin-like group which is mainly divided into the cathepsin-L-like and cathepsin-B-like groups (Atkinson et al., 2009; Shindo and Van Der Hoorn 2008). Phylogenetic analysis of *P. pisi* PRO1 suggests that this protein most likely belongs to a cathepsin-L-like group from the C1A subfamily of peptidases.

P. pisi PRO1 also contain a cathepsin propeptide inhibitor domain (I29) at the N-terminus. This domain is found in certain C1 peptidases where it functions as a propeptide that needs to be proteolytically removed to activate the protease. In addition, a MD-2-related lipid-recognition domain (ML) is situated in the C-terminal part of PRO1. This domain is predicted to interact with specific lipids to mediate different biological functions. One possible function is to target the PRO1 peptidase to the cell wall – plasma membrane interface of the host. Analysis of the *P. sojae* genome revealed that among 24 cystein protease paralogs, only the ortholog to *P. pisi* PRO1 has a ML-domain. In addition, an ortholog to PRO1, including the ML-domain, is also present in *P. infestans*. These results suggest that the PRO1 orthologs are the result of a gene fusion event (preceding the split between *P. pisi*, *P. sojae* and *P. infestans*) between a cathepsin-L-like peptidase and a ML-domain and that this unique domain-structure provided a selectable advantage during pathogenesis.

Conclusion:

Experimental data on oomycete pathogenicity factor evolution and function play a key role towards better understanding of oomycete-plant interactions, which eventually may result in identification of host resistance genes for breeding programs. This study provides a brief insight into the identification of some putative *P. pisi* pathogenicity factors during biotrophic and necrotrophic phases of pea root infection. Further functional analyses are essential to improve our overall understanding of the *P. pisi* pathogenic lifestyle.

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