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Cellular and molecular characterization of *Phytophthora parasitica* appressorium-mediated penetration

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Summary

• Data on plant pathogenic oomycetes are scarce and little is known about the early events leading to the onset of infection. The aim of this work was to analyze the penetration process of the soil-borne plant pathogen *Phytophthora parasitica*, which has a wide host range.

• Here, we performed a cytological analysis of the colonization of the first plant cell and developed an inoculation assay for characterizing the entire penetration process through cellular and molecular analyses. We showed that *P. parasitica* infects roots by producing a specialized structure, the appressorium.

• We produced the first cDNA library for the penetrating stage of a *Phytophthora* species and showed it to be highly enriched in pathogenicity-related sequences. These included coding sequences for many cell-degrading enzymes, effectors such as RXLR-containing proteins and proteins involved in protection against plant defense responses.

• Characterization of the appressorium cDNA library and identification of genes overrepresented early in *P. parasitica* infection provided us with an unprecedented opportunity to decipher the molecular mechanisms involved in penetration of the plant cells during the initiation of infection by a soil-borne oomycete.

Introduction

Filamentous plant pathogens – comprising true fungi and oomycetes – have evolved various strategies for entering their hosts (Mendgen *et al.*, 1996). Some enter via wounds or natural openings, such as stomata. Others penetrate their host by enzymatic or mechanical means, using specialized structures called appressoria. Most of the information about appressorium differentiation and function has been obtained from true fungi, such as the ascomycete *Magnaporthe grisea*. Differentiation of the *M. grisea* appressorium is induced by the perception of surface hardness and hydrophobicity, which activates the cyclic AMP (cAMP) and mitogen-activated protein kinase (MAPK) signaling pathways (Lee *et al.*, 2003; Caracuel-Rios & Talbot, 2007). Autophagic cell death is then required to mobilize lipids and carbohydrates from the spore and to generate the mechanical force of the appressorium (Veneault-Fourrey & Talbot, 2007). However, the function of the appressorium has not yet been fully characterized and whole-genome microarrays are currently being used to investigate overall changes in gene expression (Oh *et al.*, 2008).

Little is known about host penetration by oomycetes. They were long considered to be fungi, because of their filamentous growth. Consequently, the data obtained for ascomycetes were generally extrapolated to oomycetes. Phytopathogenic oomycetes include more than 85 *Phytophthora* species, hundreds of *Pythium* species and downy mildews. They infect many different host plants, including crops, ornamental plants and natural populations (Erwin & Ribeiro, 1996). Only a few studies have investigated the penetration process of oomycetes. It was shown that poor nutrient content, surface hydrophobicity and topography induce appressorium differentiation in *Phytophthora palmivora* (Bircher & Hohl, 1997). However, appressorium differentiation in *Phytophthora*, unlike that in true fungi, requires calcium but is not induced by cAMP (Bircher & Hohl, 1999). This difference suggests that different signaling pathways are activated in fungi and oomycetes. Differential screening and proteomic studies in *Phytophthora infestans* and *Phytophthora sojae* have shown that

a specific genetic program is activated during appressorium differentiation (Krämer *et al.*, 1997; Avrova *et al.*, 2003; Ebstrup *et al.*, 2005; Chen *et al.*, 2007). Genes involved in protein synthesis and amino acid and energy metabolism are induced during appressorium development and early infection (Ebstrup *et al.*, 2005; Grenville-Briggs *et al.*, 2005). A gene expression profiling study performed on *P. infestans* demonstrated the accumulation of transcripts encoding protein kinases, cell wall-degrading enzymes (CWDEs) and various effectors – proteins used to manipulate plant cells – during appressorium differentiation (Judelson *et al.*, 2008). Only three *Phytophthora* gene functions have been shown to be specifically required for the penetration process. Silencing of the Pibzp1 transcription factor from *P. infestans* abolishes appressorium differentiation (Blanco & Judelson, 2005). The RNAi-mediated silencing of a family of four cellulose synthase genes from *P. infestans* has also been shown to impair appressorium differentiation and plant infection (Grenville-Briggs *et al.*, 2008). The *PIHMP1* gene encodes a membrane protein that accumulates in appressoria and haustoria and is required for early infection (Avrova *et al.*, 2008).

The oomycete penetration process has been analyzed on artificial substrates that cannot be pierced (Bircher & Hohl, 1997). Thus, functions occurring during plant cell wall breaching and the initial exchanges with the host may not have been detected. Moreover, such studies were performed using pathogens infecting aerial parts of plants while most pathogenic oomycetes infect plants via the roots. The few studies of root infection have generated conflicting results. *P. sojae* infects soybean (*Glycine max*) via hyphae rather than appressoria (Enkerli *et al.*, 1997). Conversely, an apical swelling at the penetration site has been observed in *P. parasitica*/citrus, *Phytophthora palmivora*/citrus, *Phytophthora megasperma/Cicer arietinum* and *Phytophthora cryptogea/Chrysanthemum morifolium* interactions (Swiecki & Donald, 1988; Dale & Irwin, 1991; Enkerli *et al.*, 1997; Widmer *et al.*, 1998). Root penetration by an oomycete has yet to be fully characterized.

A new inoculation assay was used to characterize the *P. parasitica* penetration process. This species infects the roots of a wide range of plants and is thus representative of most pathogenic oomycetes. A cytological analysis of early infection showed that *P. parasitica* entered host cells using an appressorium. We generated a cDNA library for this oomycete at the penetration stage and showed the accumulation of sequences encoding functions associated with pathogenicity. We then monitored the accumulation of transcripts encoding CWDE and 'RXLR-EER' effectors.

Materials and Methods

Phytophthora parasitica and plant culture conditions

P. parasitica strain 310 was isolated from tobacco in Australia. *Phytophthora parasitica* strain 149 expressing constitutive cytoplasmic green fluorescent protein (GFP) was described previously (Le Berre *et al.*, 2008). Cultures were performed on V8 medium and zoospore production was induced as previously described (Galiana *et al.*, 2005).

Tomato plantlets (*Lycopersicum esculentum* cv. Microtom; Meissner *et al.*, 1997) were grown, inoculated and observed as previously described (Le Berre *et al.*, 2008) with minor modifications indicated in Supporting Information Methods S1.

Onion epidermis inoculations

Onion epidermis was soaked for 1 min in chloroform, rinsed with water and placed on damp Whatman paper. Zoospores from *P. parasitica* were immobilized by vortexing. Drops (50 μl) of encysted zoospores (10^5 cysts ml^{-1}) were deposited on onion epidermis. Inoculated onion epidermis was recovered after incubation at 24°C for 1–24 h in the dark. For the analysis of the penetration kinetics, the epidermis was stained with trypan blue (0.5%). Three independent experiments were performed.

RNA extractions and cDNA library construction

RNA extraction from inoculated plant tissues was performed as described by Laroche-Raynal *et al.* (1984). *Phytophthora parasitica* RNA was extracted using Trizol reagent (Invitrogen, France).

Poly(A^+) RNA from appressoria was purified using the Oligotex mRNA purification kit (Qiagen, France). Appressorium-derived cDNA was synthesized from 500 ng of mRNA with the BD smart PCR cDNA synthesis kit (BD Biosciences, San Jose, California, USA) using 18 cycles of amplification. Amplified fragments were ligated into the pGEM-T easy vector (Promega, France) and cloned into *Escherichia coli* DH5α cells.

Sequencing and analysis of the expressed sequence tag (EST) data set

Sequencing, vector trimming and EST clustering were performed by GATC Biotech (Konstanz, Germany) using Cross Match and Seqman (DNA Star software, Madison, Wisconsin, USA), respectively. Sequences were submitted to GenBank (FK934624 to FK938820). Uni-sequence composition is detailed in Table S1. Blast analyses were performed to differentiate *P. parasitica* and contaminant onion sequences (see Methods S2; Altschul *et al.*, 1990). Functional annotation was performed following blastx (E-value < 1E-05) searches against the National Center for Biotechnology Information (NCBI) nonredundant (NR) protein database and searches against the Interpro database (Apweiler *et al.*, 2001).

Prediction of open reading frames (ORFs) was performed using the ORF predictor program from Concordia University (https://fungalgenomics.concordia.ca/home/index.php).

Proteins containing the RXLR-EER motif were searched using the method described by Whisson *et al.* (2007). Similarly to these authors, putative secreted proteins were predicted according to SignalP3.0-HMM method (Sprob cut-off: 0.7; Krogh *et al.*, 2001; Bendtsen *et al.*, 2004; Emanuelsson *et al.*, 2007).

Real-time RT-PCR analyses

RNA used for quantitative RT-PCR was obtained from two biological replicates of the different developmental stages as indicated in Methods S3. Total RNA was treated with DNAse I (Ambion, Austin, TX, USA), reverse transcribed (1 μg) to cDNA using I Script cDNA synthesis (BioRad, Hercules, CA, USA). Real-time PCR experiments were performed using 5 μl of a 1 : 50 dilution of first-strand cDNA and SYBRGreen (Eurogentec SA, Seraing, Belgium) and Opticon 3 (BioRad). All assays were carried out in triplicate. Gene-specific oligonucleotides were designed using PRIMER3 (http://frodo.wi.mit.edu) and their specificity was validated by the analysis of dissociation curves. The gene encoding a ubiquitin conjugating enzyme (Ubc; CK859493) was selected as a constitutive internal control (Yan & Liou, 2006). Quantification of gene expression was performed using GENESPRING GX software (Agilent, Santa Clara, California, USA).

Results

Use of onion epidermis as a substrate for studying the process of penetration by *P. parasitica*

To characterize the *P. parasitica* penetration process, we analyzed early root infection in tomato host plants. We inoculated plantlets with *P. parasitica* strain 149 expressing the GFP gene (Le Berre *et al.*, 2008). After cyst germination at the root surface, round appressoria were differentiated and these structures penetrated the epidermis 2–4 h after inoculation (Fig. 1a). Appressoria generally differentiated at the junction between two cells (data not shown). Numerous swimming zoospores and germinating cysts were observed at the root surface, consistent with tomato root infection being an asynchronous process (data not shown).

We used onion epidermis as a surface for the induction of appressorium differentiation and penetration in order to carry out molecular investigations of the early stages of *P. parasitica* infection. Onion is a natural host of *P. parasitica* from which the epidermis is easily obtained as a single-cell layer (Erwin & Ribeiro, 1996). Chloroform-treated onion epidermis was inoculated with a high concentration (2×10^5 zoospores ml^{-1}) of mechanically encysted zoospores and incubated in the dark at 24°C. This led to the differentiation of appressoria, which breached the cell wall and penetrated the onion cells (Fig. 1b). The appressoria differentiating on onion and tomato roots were highly similar, demonstrating the validity of this system for studying the penetration process. A septum separating the appressorium from the germ tube was clearly visible (Fig. 1b). Furthermore, penetration was mediated by a restricted area of the appressorium similar to the penetration peg of ascomycetes such as *M. grisea* (Bourett & Howard, 1990).

The kinetics of *P. parasitica* penetration into plant cells was assessed (Fig. S1). Inoculation of onion epidermis made it possible to synchronize the entire developmental sequence. Penetration of the plant cell wall was always appressorium mediated and occurred mainly between 2 and 4 h after inoculation. Penetration occurred at random sites on the plant cell surface, rather than only at cellular junc-

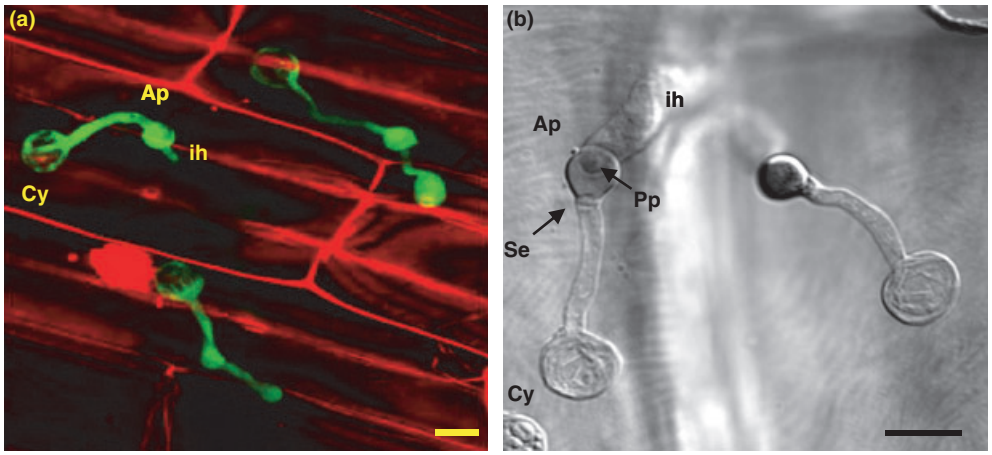

Fig. 1 Comparison of appressoria on onion epidermis and tomato root. *Phytophthora parasitica* was observed with a confocal laser scanning microscope 3 h after inoculation. (a) Green fluorescent protein (GFP)-transformed strain on a tomato root stained with propidium iodine (Z-stack images). (b) Wild-type strain on onion epidermis. Cy, cyst; Ap, appressorium; ih, infection hypha; Pp, penetration peg; Se, septum. Bars, 10 μm. The appressoria formed on onion epidermis and tomato roots are similar. A septum separates the appressorium from the germ tube during penetration.

tions as observed in tomato. This difference may be a consequence of the softening of the onion cell wall by chloroform treatment. The epidermal layer was then colonized and constrictions of the mycelium were observed at sites of cell-to-cell transfer. This may reflect the differentiation of specific structures enabling the oomycete to move between cells or the use of plasmodesmata for the invasion of adjacent cells, as observed for *M. grisea* (Kankanala *et al.*, 2007). The penetration process was completed within 10 h of inoculation.

Construction of a cDNA library from *P. parasitica* at the appressorium stage

We extracted mRNA from the onion cells 3 h after inoculation (hai) with *P. parasitica,* to identify genes expressed during the appressorial penetration of host plant cells. A cDNA library was constructed and 4421 ESTs were generated. These ESTs were assembled into 628 contigs and 1921 singlets, constituting a 2549 single-sequence set (referred to as 'sequences' (seq) hereafter; Fig. S2). For the identification of *P. parasitica* sequences, we compared the data set with *Phytophthora* databases and onion ESTs available in db-EST (see Methods S2). We attributed 2022 sequences (79%) to *P. parasitica* and 204 sequences (8%) to onion and the remaining sequences (13%) were considered to be of unknown origin.

The 2022 *P. parasitica* sequences were analyzed. A blastn search (E-value = 1E-50) revealed that only 40% of the appressorium-derived sequences were present in *P. parasitica* data sets generated from zoospores, germinated cysts, vegetative mycelium or infected tomato (Shan *et al.*, 2004; Skalamera *et al.*, 2004; Panabières *et al.*, 2005; Le Berre *et al.*, 2008). Tblastx searches (e-value cut-off: 1E-20) revealed that 42 sequences (2%) had no homolog in the three sequenced *Phytophthora* genomes, and may correspond to *P. parasitica*-specific genes. Nine of them matched a sequence in the NR database (blastx, E = 1E-05) and corresponded to proteins of unknown function. A large proportion of appressorium sequences (1334/2022; i.e. 66%) had significant matches in the NCBI NR protein database (blastx, E = 1E-05). Together with a comparison with *Phytophthora* proteomes, this analysis facilitated functional classification (Fig. 2). The most prominent functions were metabolism (12%), protein synthesis (9%) and fate (7%), transport (8%) and cell defense and virulence (7%); 30% of the sequences were of unknown function. Putative pathogenicity factors were classified into major categories (Table 1 and Table S2).

Putative pathogenicity factors: entering the host

We detected 36 sequences with similarity to putative CWDEs, constituting by far the most abundant class of candidate pathogenicity proteins (Table 1). Eight sequences potentially belonged to glycoside hydrolase families 5, 6, 7 and 12. Fourteen sequences encoded additional glucanases, such as xylanases, mannanases or arabinanases. Lastly, enzymes involved in pectin degradation were also abundant (11 sequences). All CWDE-encoding sequences had a homolog in the complete *Phytophthora* genomes (Table S2). However, most of the appressorium CWDEs were not observed in the other *P. parasitica* data sets (Shan *et al.*, 2004; Panabières *et al.*, 2005; Le Berre *et al.*, 2008).

We also checked for the expression of genes previously shown to be required for *Phytophthora* appressorium morphogenesis and function. We found no homolog of the Pi-bzp1 transcription factor, but one EST corresponded to a homolog (66% identity and 76% similarity) of the Pihmp1 protein from *P. infestans* (Table 1 and Table S2 (Blanco & Judelson, 2005; Avrova *et al.*, 2008)). Eleven ESTs assembled into six sequences encoding proteins closely resembling (95–100% identity) the four *P. infestans* cellulose synthases (Table 1 and Table S2; Grenville-Briggs *et al.*, 2008).

Putative pathogenicity factors: dealing with plant defense responses

Many genes involved in protection against reactive oxygen species (ROS) were identified (Table 1). Thirty-one seq-

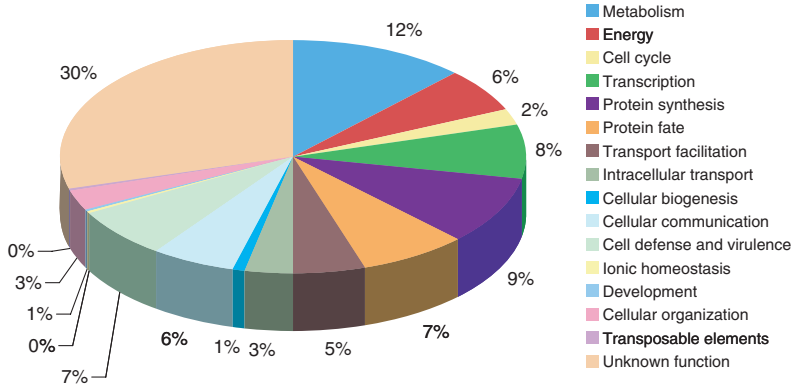

Fig. 2 Functional classification of *Phytophthora parasitica* sequences. Sequences were classified by carrying out blastx analysis against the NR protein database (e-value cut-off: 1E-05) and transposing the annotations for *Phytophthora infestans*. Categories are based on the MIPS (Munich Information Center for Protein Sequences) classification, modified as described by Panabières *et al.* (2005).

Table 1 *Phytophthora parasitica* sequences corresponding to putative pathogenicity factors

Putative pathogenicity factors	Number of sequences
Adhesion	
CBEL protein	**1**
Cellulose synthases	**6**
Membrane protein	
Pihmp1	**1**
Hydrolases	
Proteases	**14**
Serine proteases	8
Aspartyl protease	2
Cysteine protease	4
CWDE	**36**
Cutinase	3
Pectinases	11
Cellulases	8
Glucanases	14
Proteases inhibitors	**5**
Effectors	**30**
RXLR motif	13
Elicitin and elicitin-like	7
CRN	4
Necrosis-inducing peptide	3
Others	3
Cystein-rich protein	**2**
Detoxification, drug resistance and metabolite transport	**22**
Cytochrome P450	1
Putative ABC transporter	14
Others	7
Protection against ROS	**31**
Glutathione s transferase	**10**
Glutathione reductase	1
Peroxidases	6
Catalase	3
SOD	3
Glutaredoxin	3
Peroxiredoxine, thioredoxin	3
Thioredoxin reductase	1
Others	1
Signal transduction and signalling	**3**
MAP kinases	1
CPKA	2

See Supporting Information Table S2 for more details on each sequence.
CWDE, cell wall-degrading enzyme; MAP, mitogen-activated protein; ROS, reactive oxygen species.

uences encoded catalases, superoxide dismutases, and peroxidases. A total of 15 sequences encoded proteins with glutathione-related functions. One superoxide dismutase, three catalases and six enzymes related to glutathione function were absent from the *P. parasitica* mycelium and interaction libraries and were therefore proposed to play an important potential role in the early stages of infection (Table S2).

Sequences relevant to the export or degradation of plant antimicrobial molecules were also abundant in the appressorium library. Fourteen sequences, six of which were not found in other *P. parasitica* libraries, encoded putative ATP-binding cassette (ABC) transporters, whereas five sequences, of which three were present only in the appressorium database, corresponded to putative xenobiotic reductases (Table 1 and Table S2).

We also identified homologs of *P. infestans* protease inhibitors involved in protection against plant defense proteins (Tian *et al.*, 2004, 2007). Three of these sequences, homologous to EPI4 and EPI7 genes, encoded kazal-like serine protease inhibitors, whereas two homologs of the EPIC1 and EPIC4 genes encoded cystatin-like cysteine protease inhibitors (Table 1).

Putative pathogenicity factors: exchanges with plant cells

Identification of effectors started with a search for putative secreted proteins. We identified 114 candidate proteins (5.7% of the data set). In total, 29 sequences corresponded to previously characterized effectors (Table 1). These effectors included apoplastic effectors, such as elicitins and elicitin-like proteins, the Crinkling and Necrosis proteins (CRN7 and CRN13 homologs) and necrosis-inducing (NEP) peptides (Table 1). We also identified three sequences related to the M81 (transglutaminase) family (Fabritius & Judelson, 2003). These sequences were homologs of M81C and M81E, which are differentially expressed during the *P. infestans* life cycle (Fabritius & Judelson, 2003). Fifteen sequences encoded aspartyl, cysteine and serine proteases (Table 1). All of these sequences had a homolog in other *Phytophthora* genomes, but most (10/15) were absent from the other *P. parasitica* libraries (Table S2).

Secreted proteins with an N-terminal RXLR-EER motif have been shown to be targeted to the plant cell and the inhibition of programmed cell death by two such proteins was recently demonstrated (Bos *et al.*, 2006; Morgan & Kamoun, 2007; Whisson *et al.*, 2007; Dou *et al.*, 2008). We screened the appressorium-derived protein data set for the RXLR-EER motif, as described by Whisson *et al.* (2007). We recovered 10 candidates, six of which had a signal peptide (SignalP-HMM; Sprob > 0.9) and satisfied the requirements for RXLR effectors (Table 2). Three RXLR effector candidates apparently lack a signal peptide and sequence confirmation is required before any conclusion can be drawn about these transcripts. We also used the RXLR protein catalog established for other *Phytophthora* species (Whisson *et al.*, 2007; Jiang *et al.*, 2008) and identified three additional sequences related to *Phytophthora* RXLR effectors (blastx, E < 1E-10; Tables 2, S3). Thus, at least six proteins with RXLR motifs are produced in the *P. parasitica* appressorium, and seven other candidates require validation. As expected for such rapidly evolving genes, seven of these 13 sequences had no homolog in the three *Phytophthora* genomes (Table S2) and may therefore correspond

Table 2 RXLR-containing proteins produced at the appressorium stage

Contig	Motif	Position	SignalP probability	Translation initiation codon (AUG)	blastx RXLR *Phytophthora infestans*		blastx RXLR *Phytophthora sojae* and *Phytophthora ramorum*	
					Subject	E-value	Subject	E-value
1								
77	RFLR-EER	52–70	0.995	Yes	33_11093_−2_1439860	1E-03	PrAvh_157	9E-04
350	RLLR-EER	53–71	0.994	Yes	23_14883_−2_2018588	1E-20	PrAvh_283	8E-05
412	RFLR-EER	52–69	0.999	Yes	67_3872_−1_244558	6E-26	PsAvh_238	2E-17
1641	RRLR-EER	48–59	0.997	Yes	33_15803_−3_266325	9E-10	PrAvh_122	1E-01
2086	RLLR-EER	55–70	1	Yes	1436_14_+2_4376	3E-57	PrAvh_202	6E-11
2473	RFLR-EER	50–65	0.951	Yes	7_45042_−3_778066	2E-21	PrAvh_190	8E-14
2								
60	RNLR-EER	38–57	0	Yes	No hit	No hit	No hit	No hit
353	RNLR-EER	96–105	0	Yes	No hit	No hit	No hit	No hit
2264	RFLR-EER	69–83	0.001	Yes	28_1679_+1_1067410	2E-19	PsAvh_243	2E-16
3								
2006	RKLR-EER	/	ND	No	11_20502_−1_1869676	1E-01	No hit	No hit
1201	/	/	ND	No	38_1983_+1_1263022	6E-14	PrAvh_260	2E-15
2137	/	/	ND	No	29_18469_−3_860581	4E-20	PsAvh_284	3E-12
2382	/	/	ND	No	11_426_+1_359710	7E-18	PsAvh_191	2E-08

1. Proteins satisfying all the requirements for membership of the RXLR family (RXLR-EER motif and signal peptide).
2. Proteins with the RXLR-EER motif but with no signal peptide according to SignalP-HMM.
3. Proteins with an RXLR-EER motif but isolated from a partial cDNA lacking the start codon or partial proteins homologous to previously identified RXLR proteins. ND, partial protein sequences (no start codon) were not subjected to SignalP-HMM analysis.
Occurrence of the RXLR-EER motif in the *P. infestans*, *P. sojae* and *P. ramorum* proteins was checked (see Supporting Information Table S3).

to *P. parasitica*-specific effectors. Furthermore, nine of these sequences were detected only in the *P. parasitica* appressorium library (Table S2).

Quantification of CWDE- and RXLR-encoding transcripts during the penetration process

Analysis of the EST library suggested specific accumulation of some transcripts in appressoria. We monitored the relative expression levels of 24 CWDE and four RXLR candidate genes – found only in the *P. parasitica* appressorium cDNA library – by quantitative RT-PCR. Our analyses focused on zoospores, cysts germinated in half-strength V8 medium, vegetative mycelium and infection stages, selected as representative of the penetration (onion epidermis and tomato; 3 hai) and necrotrophy during root colonization (4 d after inoculation). The gene encoding Ubc was used for normalization (Yan & Liou, 2006).

Transcripts of most of the 28 genes accumulated in appressoria differentiated on onion and tomato (Fig. 3a). Only four of these genes (seq1641, 1512, 1792 and 1283) were weakly detected in all samples. Transcripts seq83 and 2027, encoding a glucanase and a cellulase, respectively, were accumulated to similar levels during vegetative growth and during early infection (zoospores, cyst germination and appressoria). Thus, transcripts corresponding to 23 of the 28 (82%) sequences were more abundant during the early infection process than in axenic mycelium or during the necrotrophic stage. These sequences displayed four principal expression patterns, with transcripts accumulating: (1) throughout the early infection stages (see seq375; Fig. 3a); (2) in cysts germinated in V8 medium and in appressoria (see seq306; Fig. 3b); (3) in zoospores and appressoria (see seq412 and 488; Fig. 3a,b); (4) only in appressoria (see seq2531 and 1691; Fig. 3b). In most cases, the pattern of transcript accumulation was similar in appressoria differentiated on onion epidermis and tomato roots. However, a few sequences displayed differences in mRNA accumulation in the two plants (see seq1691; Fig. 3b).

Most of the CWDE-encoding transcripts accumulated during appressorium-mediated penetration of the host. The transcripts encoding cutinases were all abundant during early infection (Fig. 3a). Only one of the five cellulase-encoding sequences (seq515) was strongly accumulated only in appressoria. Such specific expression was observed for a higher proportion of pectinase- and glucanase-encoding sequences (see seq2531 (pectinase) and seq197 and 1691 (arabinase and arabinofuranosidase, respectively); Fig. 3a,b; Table S2).

Similarly, three of the four 'RXLR-EER' effectors accumulated mostly in appressoria (Fig. 3a). Seq412 mRNA was highly abundant in appressoria differentiated on onion and also accumulated in significant amounts in appressoria differentiated on tomato. Seq350 transcript accumulated in

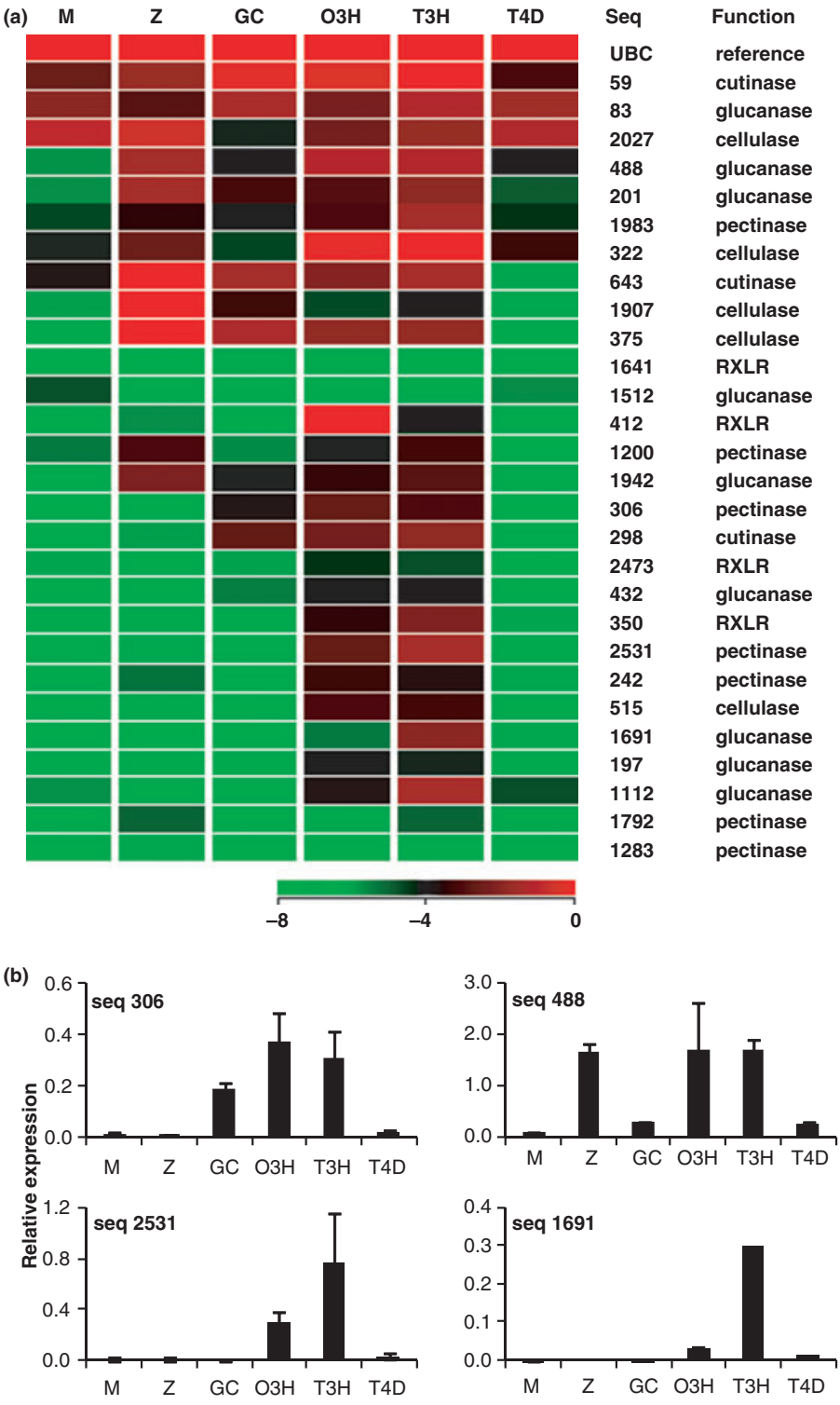

Fig. 3 Quantification of mRNAs encoding cell wall-degrading enzymes and RXLR motif-containing proteins. Relative mRNA levels were quantified by quantitative RT-PCR in samples corresponding to mycelium grown in V8 medium (M), swimming zoospores (Z), cysts germinated in half strength V8 medium for 3h (GC), appressoria differentiated on onion epidermis (O3H), appressoria differentiated on tomato root (T3H) or late tomato root infection (T4D). (a) Hierarchical clustering of 28 sequences found only in the *Phytophthora parasitica* appressorium cDNA library (the ubiquitin conjugating enzyme (UBC) gene is the reference gene). Data are presented as logarithmic values for gene expression relative to the UBC reference gene according to the scale in the right-hand corner. (b) Expression levels of four sequences. Data are presented as expression ratios relative to the UBC reference gene ($2^{-\Delta CT}$).

much greater amounts in appressoria (differentiated on onion or tomato) than in other samples. Finally, seq2473 was weakly expressed, but its mRNA accumulated in significantly larger amounts in appressoria.

Discussion

We describe the process by which *P. parasitica* penetrates its host. We show that the initial invasion of the plant root is mediated by an appressorium. This structure, which differentiates at the tip of the germ tube, develops as a round cell that both redirects growth and breaches the plant cell wall. This is consistent with previous cytological analyses describing hyphal swellings at the penetration sites of soil-borne *Phytophthora* species (Swiecki & Donald, 1988; Dale & Irwin, 1991; Enkerli *et al.*, 1997) and shows that *P. parasitica* invades its hosts by differentiating a specialized structure. Like Kramer and coworkers, we observed a septum separating the appressorium from the germ tube, showing that this structure is definitively different from a hyphae (Krämer *et al.*, 1997).

As in most filamentous root pathogens, analyses of the *P. parasitica* penetration process are limited by the asynchronous development of infection structures and low pathogen biomass at early stages. By means of a penetration assay based on onion epidermis, we synchronized the early events of infection and obtained appressoria highly similar to those observed on tomato roots.

Using chloroform-treated dead onion epidermis to induce appressorium differentiation, we obtained a cDNA library from *P. parasitica* at the penetration stage with a higher proportion of pathogen sequences (79%) than that reported for other plant–*Phytophthora* EST collections, even from later stages of infection during which the oomycete biomass is much greater (Qutob *et al.*, 2000; Randall *et al.*, 2005).

Sequences related to protein synthesis and fate were highly overrepresented in the *P. parasitica* appressorium library, as already reported for *P. infestans* and *M. grisea* appressoria differentiated *in vitro* (Ebstrup *et al.*, 2005; Panabières *et al.*, 2005; Le Berre *et al.*, 2008; Oh *et al.*, 2008). This may reflect the need for *de novo* protein synthesis during penetration.

Putative pathogenicity-related sequences appeared to be relatively abundant (7% of the single-sequence set). The transcripts of genes encoding proteins potentially involved in protection against ROS or in the export of plant toxic compounds were overrepresented with respect to the *P. parasitica* or *P. sojae* libraries generated from mycelia isolated from the plant or grown *in vitro* (Torto-Alalibo *et al.*, 2007; Le Berre *et al.*, 2008). Already described for *P. infestans* or *M. grisea* appressoria differentiated *in vitro*, the expression of such functions during early infection would enable the pathogen to deal with the plant's early defense responses (Ebstrup *et al.*, 2005; Judelson *et al.*, 2008; Oh *et al.*, 2008).

We identified 36 CWDE-encoding sequences, most of which were absent from the published *P. parasitica* mycelium and late infection-derived EST libraries (Le Berre *et al.*, 2008). Quantitative RT-PCR experiments confirmed that the transcripts of 21 of these genes accumulated preferentially early in infection (zoospore to appressorium stages), with preferential accumulation in the appressoria observed for nine of these sequences. Similar results were obtained for some *P. infestans* CWDE (Judelson *et al.*, 2008). Thus, expression of a specific set of CWDEs in the *P. parasitica* appressorium may be required to soften the plant cell wall or may be involved in pathogen cell wall remodeling during the penetration process, as suggested for an *M. grisea* cutinase mutant impaired in penetration peg formation (Skamnioti & Gurr, 2007). The *P. parasitica* appressorium library contains sequences encoding cellulose synthases. As cellulose synthesis is required for *P. infestans* host penetration (Grenville-Briggs *et al.*, 2008), it would be of interest to determine the mechanisms governing the balance between cellulose degradation and synthesis in the early stages of infection with *Phytophthora*.

We also tried to identify effectors potentially exported to the plant cell wall or cytoplasm to favor pathogen development. The proportion of secreted proteins (5.6%) was similar to that deduced from the *P. infestans*, *P. sojae* and *P. ramorum* proteomes (4.5, 7.8 and 7.5%, respectively). Apoplastic effectors, such as elicitin-like proteins, CRN-like proteins and Nep1-like proteins, were as abundant in our cDNA library as in libraries for the necrotrophic stages of *P. parasitica* and *P. sojae*, indicating that these genes may be expressed very early in infection (Kamoun, 2006; Torto-Alalibo *et al.*, 2007; Le Berre *et al.*, 2008). We also identified at least six sequences encoding proteins containing the RXLR-EER motif found in potential effectors and oomycete avirulence genes, and identified seven other sequences that may also belong to this family. Transcripts corresponding to three of these effectors preferentially accumulated in the appressoria, as already observed for similar proteins from *P. infestans* (Whisson *et al.*, 2007; Judelson *et al.*, 2008). At least two RXLR proteins are exported from the haustoria into the plant cytoplasm and abolish plant cell death (Bos *et al.*, 2006; Whisson *et al.*, 2007; Dou *et al.*, 2008). The secretion of such proteins from appressoria remains to be confirmed, but may be involved in the abolition of plant defense responses or the modification of plant cells to facilitate invasion by the pathogen.

According to our quantitative RT-PCR experiments, most of the transcripts accumulating in *P. parasitica* appressoria differentiated on tomato also accumulated in appressoria differentiated on onion epidermis, showing that our inoculation assay is reliable for analyses of the penetration process. The few exceptions observed could acc-

ount for host-specific responses or for loss of induction of some genetic pathways as a consequence of penetration of dead onion tissues. This limitation will have to be considered in future studies.

Sixty per cent of the appressorium-derived sequences were not present in previous *P. parasitica* EST collections (Shan *et al.*, 2004; Skalamera *et al.*, 2004; Panabières *et al.*, 2005; Le Berre *et al.*, 2008). Furthermore, our quantitative RT-PCR experiments showed that some transcripts accumulate specifically in the appressoria, and that genes expressed at the penetration stage are not always expressed early in zoospores or in artificial cyst germinations (diluted culture medium) as sometimes proposed (Shan *et al.*, 2004; Torto-Alalibo *et al.*, 2007). We show here that a specific genetic program operates at the appressorium stage.

The onion epidermis penetration assay provides molecular and cytological data concerning appressorium-mediated host penetration by *P. parasitica*. The synchronization of this process and the strong enrichment in pathogen sequences observed render this assay particularly useful for the identification of transcripts involved in the initial infection of plant cells. Several putative pathogenicity factors were identified and future studies will focus on identification of the genes required for host cell penetration by *P. parasitica*. Most of the ESTs identified in the *P. parasitica* appressorium library were also present in *P. sojae,* which is thought to penetrate host cells without the need for an appressorium (Enkerli *et al.*, 1997). It would therefore be of interest to determine the role of these sequences in host penetration by *P. sojae* and in *P. parasitica*, in a comparative study.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Methods S1. Tomato inoculations with *Phytophthora parasitica*.

Methods S2. Identification of *Phytophthora parasitica* and contaminant onion sequences.

Methods S3. Biological samples for quantitative RT-PCR analyses.

Fig. S1 *Phytophthora parasitica* penetration into onion epidermis.

Fig. S2 Expressed sequence tag (EST) redundancy among the 2549 single sequences obtained from appressoria differentiated on onion epidermis.

Table S1 Expressed sequence tag (EST) composition

Table S2 Automatic analysis of putative pathogenicity factors

Table S3 Analysis of *Phytophthora infestans*, *Phytophthora sojae* and *Phytophthora ramorum* effectors homologous to *Phytophthora parasitica* proteins

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