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Gene identification in the oomycete pathogen *Phytophthora parasitica* during in vitro vegetative growth through expressed sequence tags ☆

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Abstract

Phytophthora parasitica is a soilborne oomycete pathogen capable of infecting a wide range of plants, including many solanaceous plants. In a first step towards large-scale gene discovery, we generated expressed sequence tags (ESTs) from a cDNA library constructed using mycelium grown in synthetic medium. A total of 3568 ESTs were assembled into 2269 contiguous sequences. Functional categorization could be performed for 65.45% of ESTs. A significant portion of the transcripts encodes proteins of common metabolic pathways. The most prominent sequences correspond to members of the elicitin family, and enzymes involved in the lipid metabolism. A number of genes potentially involved in pathogenesis were also identified, which may constitute virulence determinants.

Keywords: Expressed sequence tags; Gene identification; Oomycete; *Phytophthora*

1. Introduction

The genus *Phytophthora* comprises approximately 70 species, causing together most of devastating plant diseases worldwide (Erwin and Ribeiro, 1996). They belong to the oomycetes, a class of lower eukaryotes far from fungi but clustered with brown algae and diatoms, though they exhibit a fungus-like morphology (Baldauf et al., 2000). Infection traditionally occurs through the release from sporangia of motile, flagellate zoospores that spread on the surface of plants or in water films in the soil. Zoospores then rapidly encyst after detachment of flagella, produce a cell wall and develop a germ tube. Subsequent host colonization occurs through the growth of a coenocytic mycelium. This vegetative state continues until sporulation (Erwin and Ribeiro, 1996). The various steps of the life cycle of *Phytophthora* have been studied at the cytological level, especially the early steps from sporangia cleavage to cyst germination (Hardham, 2001). Most of these steps require the specific expression of many genes (Avrova et al., 2003; Fabritius et al., 2002; Kim and Judelson, 2003; Kramer et al., 1997; Van West et al., 1998). As the success of disease relies on the development of the various physiological stages of the pathogen, the unraveling of the molecular processes regulating the life cycle of *Phytophthora* is important to identify determinants of pathogenesis and develop appropriate control strategies.

Molecular studies on the life cycle of *Phytophthora* have lagged behind studies on *Phytophthora*–plant interactions, where there has been an emphasis on the characterization of proteinaceous effectors of plant defense responses. They include *Phytophthora*-specific proteins such as the members of the elicitin family, which are

☆ Sequences of the contigs and singletons are hosted in the Genoplante-Info database at the following URL: http://urgi.infobiogen.fr/data/gpi_seq/run.php. All ESTs will appear in GenBank.

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considered as avirulence factors on the non-host tobacco (Kamoun, 2001; Ponchet et al., 1999), elicitors of plant responses in both hosts and non-hosts (Brunner et al., 2002; Khatib et al., 2004), and molecules that are found in oomycetes as well as true fungi and bacteria, and that elicit innate immune response in plants (Fellbrich et al., 2002; Pemberton and Salmond, 2004; Qutob et al., 2002). This focusing on elicitors led to a lack of knowledge on the biology and physiology of the pathogen. Hence, crucial stages such as the hyphal growth or infection process are poorly documented at the molecular level. This justified the recent engagement of considerable efforts in the generation of genomic resources, mainly on two model species, *Phytophthora infestans*, the potato late blight agent, and *Phytophthora sojae*, the causal agent of stem and root rot of soybean (Birch and Whisson, 2001; Judelson, 1997; Kamoun, 2003; Tyler, 2001). This includes generation of expressed sequence tags (ESTs, Kamoun et al., 1999; Qutob et al., 2000; Randall et al., 2005), the construction of BAC libraries (Randall and Judelson, 1999; Whisson et al., 2001), the development of transformation protocols (Kamoun, 2003) and gene silencing (Latijnhouwers et al., 2004; van West et al., 1999). More recently, the genomes of *P. sojae* and the sudden oak death pathogen *Phytophthora ramorum* have been sequenced (http://www.jgi.doe.gov), and the genome sequencing project is under way for *P. infestans*, despite its large size (237 Mb). In parallel, efforts have been developed to identify *P. infestans* genes expressed at particular physiological stages (Kim and Judelson, 2003; Tani et al., 2004) or whose expression is altered during interaction (Avrova et al., 2003; Beyer et al., 2002).

Unlike *P. infestans* and *P. sojae*, that have a very narrow host range, many *Phytophthora* species attacks a broad spectrum of plants. Among them, *Phytophthora parasitica* Dastur (syn. *Phytophthora nicotianae* Breda de Haan), a major pathogen of numerous plant genera of importance, among which solanaceous species and other cultivated crops worldwide (Erwin and Ribeiro, 1996). Some genomic resources were recently developed on this species, such as a BAC library (Shan and Hardham, 2004) and the characterization of genes up-regulated in motile zoospores (Skalamera et al., 2004) or after cyst germination (Shan et al., 2004), resulting in the identification of 240 and 146 genes, respectively. To investigate the transcriptome of *P. parasitica*, we developed a large-scale approach of gene identification during mycelium growth. We describe here more than 3500 ESTs, corresponding to 2269 genes.

2. Materials and methods

2.1. Phytophthora strain and culture conditions

Phytophthora parasitica strain 149 was isolated from tomato in Spain. It was maintained at the INRA Antibes collection on malt-agar (1, 1%, wt/vol) at 24 °C. For RNA isolation, liquid glucose asparagine medium (Hall et al., 1969) was inoculated with *Phytophthora* zoospores and the culture was harvested after a 4-day-period at 24 °C in the dark.

2.2. RNA isolation, cDNA library construction, and sequencing

Total RNA was extracted from in vitro-grown mycelium using the method of Logemann et al. (1987). Poly $(A)^+$ RNA was purified on an oligo dT-cellulose column according to (Aviv and Leder, 1972). The cDNA library was constructed using the ZAP Express cDNA synthesis kit and the ZAP Express cDNA Gigapack III Gold cloning kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions with slight modifications. Briefly, [α-^{32}P]dATP was added to the 1st-strand cDNA mixture. After synthesis of the 2nd strand, cDNA was fractionated on a Sepharose 4B column (2 ml in TE). Fifty microliters fractions were collected and an aliquot of each fraction was analyzed in 8% polyacrylamide gels in 0.5× TBE, and visualized by autoradiography. Fractions containing cDNA shorter than 500 bp were discarded. Selected fractions were pooled and the cDNA was precipitated before ligation in ZAP vector. Plasmid DNA miniprep and sequencing were performed by Genome Express SA (Meylan, France).

2.3. Sequence processing and analysis

The ABI formatted chromatogram sequences were processed into a FASTA file. This file was edited for local analyses using BioEdit (v. 5.0.8 for Windows 98). Sequences were processed manually to remove vector sequences. The redundancy of ESTs was determined iteratively by comparing all sequences with one another. Sequences were assembled into contigs using the CAP program (Huang, 1992) on line on the infobiogen site (http://www.infobiogen.fr/services/analyseq/cgi-bin/cap_in.pl). Similarities were searched against the GenBank database using the BLASTX algorithm (Altschul et al., 1997) with an *E* value cut-off $\leqslant 10^{-4}$. ESTs were also compared to the *Phytophthora* Functional Genomics Database (www.pfgd.org), the *P. ramorum* and *P. sojae* sequences recently released in the public domain (http://www.jgi.doe.gov) and the COGEME database, which gathers ESTs from several fungal pathogens and from *P. infestans* and *P. sojae* (http://cogeme.ex.ac.uk, (Soanes et al., 2002)). The putative cellular roles of the transcripts identified by ESTs were assigned manually using the functional categories defined at the Munich Information Centre for Protein Sequences (MIPS, www.mips.gsf.de). However, new categories relevant to *Phytophthora* were added, such as "avirulence factors and elicitin-related proteins" and "disease and

virulence." Protein motifs were searched against the Interpro database (Mulder et al., 2003) at the EBI web site (www.ebi.ac.uk/InterProscan/). For alignments and phylogenetic reconstructions, sequences were edited using SeqPup (version 0.6 for Macintosh) and alignment, tree construction and bootstrap (1000 trials) were performed using ClustalW for Macintosh (Thompson et al., 1994).

3. Results and discussion

3.1. EST sequence analysis

The library created from 4-day-old mycelium contained 1×10^6 primary clones. After amplification, more than 100,000 clones were plated and 4000 colonies were randomly picked and cultivated in 96-well plates before sequencing. Sequencing reactions were performed for 3648 cDNA clones and 3626 sequences were obtained. Clones without insert were removed, as well as clones that produced low quality sequences, giving rise to 3613 ESTs. The average read length of ESTs, after vector trimming and removal of low quality sequences was 648 bp. Nuclear and mitochondrial ribosomal RNA were identified in 45 ESTs that were removed, and a total of 3568 sequences (97.8% of the initial sample) were retained for subsequent analysis.

The ESTs were aligned and represented 2269 contigs, organized in 468 clusters of 2–72 ESTs and 1801 singletons (Fig. 1). This corresponds to a redundancy of 49.5% (the number of ESTs assembled in clusters/total number of ESTs). This is roughly similar to the redundancy rate observed in other EST projects of similar scale developed on fungal organisms, such as *Mycosphaerella graminicola* (43%; Keon et al., 2000) or *Blumeria graminis* (37.7%, Thomas et al., 2001). cDNAs corresponding to 25 genes (1.1% total contigs) took into account for 10.67% of total ESTs, and were considered as highly expressed. Conversely, more than 97 % of ESTs corresponded to singletons or contigs of 2–5 ESTs. So, further sequencing random cDNA clones from this library has a great potential to identify novel sequences. It has yet to be noted that sequencing was performed only from the 5′ end of cDNAs. So it is possible that the redundancy was under estimated, as ESTs defined as singletons could correspond to non-overlapping sequences derived from the same transcript.

3.2. Functional classification of ESTs and analyses

Using the BLASTX program, 302 (8.5%) ESTs would correspond to hypothetical proteins of unknown function, and 930 (26%) were designed as "no hit." This latter category was composed of 788 unigenes, among which 718 singletons. On the other hand, 73.9% of the ESTs displayed significant similarity to known sequences in GenBank, and 65.5% of total ESTs could be assigned to a putative cellular function, according to the categorization scheme outlined in the MIPS database, modified as described in Section 2 (Fig. 2). Genes related to energy and metabolism formed the largest category, corresponding to more than 25% of ESTs. Sequences assigned to protein synthesis or fate represented 13.9 and 8.1% of total ESTs, respectively. Among them, ribosomal proteins were represented by more than 8% of total ESTs (294/3568). Translation elongation factors were identified in 29 contigs constituted by 62 ESTs. Among them, *Tef1*, encoding the elongation factor 1α (van't Klooster et al., 2000), was represented by 18 ESTs. The ubiquitin/

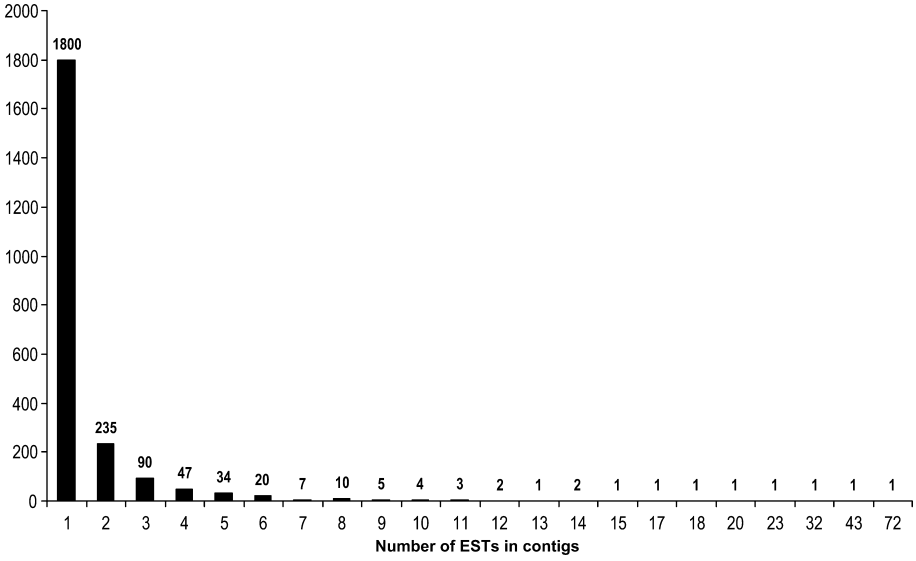

Fig. 1. EST redundancy among 3568 sequences obtained from in vitro-grown mycelium cDNA library *of P. parasitica*. Number of ESTs is indicated above each bar.

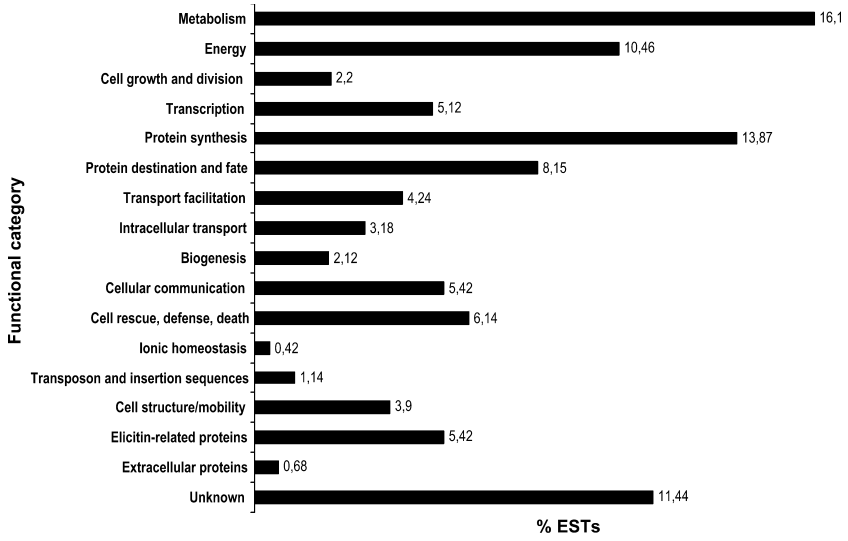

Fig. 2. Functional classification of ESTs. Categories are based on the MIPS Comprehensive Yeast genome Database classification catalogue, with modifications as described in Section 2. The "no hits" category was discarded from analysis. Frequency is presented as percentage of ESTs that match known sequences in GenBank.

proteasome pathway was represented by 75 ESTs (2.1% of the library), among which seven clones corresponding to ubiquitin, and 39 ESTs constituting 23 cDNAs encoding components of the proteasome.

The representation of sequences corresponding to protein synthesis or fate is significantly lower than those encountered in a 632 ESTs-library derived from *P. infestans* mycelium (27 and 12%, respectively, Kamoun et al., 1999), or a 1002 EST-library from *P. sojae* mycelium (ca. 25%, Qutob et al., 2000). These discrepancies may be the outcome of different sequencing scales. Nevertheless, the overall high redundancy of sequences related to energy metabolism and translation in the *P. parasitica* gene repertoire is not surprising. So it is unlikely to represent an artifact of library construction, but rather reflects the expression of the corresponding genes during filamentous growth.

3.3. ESTs relevant to major cellular functions

The availability of a large set of sequences allowed exploring the major biological functions in *P. parasitica*. As an example, *P. parasitica* appears to possess most of the major regulators of cell cycle found in all eukaryotes examined so far, among which two cyclin-dependent kinases related to the cdc2/cdc28 family, the main cell cycle control gene in yeast (Lees, 1995), cdc16 and cdc27, which are associated in an anaphase-promoting complex (King et al., 1995), cyclins, a member of the NIMA-related kinase family, which is required for entry in mitosis (O'Connell et al., 2003), and wos2, which interacts with cdc2 in the mitosis control of *Schizosaccharomyces pombe* (Munoz et al., 2002).

Among the major metabolic pathways, the glycolysis was reconstructed through identification of 119 ESTs (3.33% of total sequences). Contigs were generally represented by 1–9 ESTs, excepted enolase and GA3PDH (see below). A key feature was the identification of a contig that exhibited significant similarity (63% at the protein level, with e value $= 3e^{-79}$) with pyrophosphate (PPi)-dependent phosphofructokinases, while no match was found with ATP-dependent enzymes. PPi has been proposed to be of great importance in the metabolism of *Phytophthora* (Marshall et al., 2001). Its concentration is particularly high in *Phytophthora* (Niere et al., 1994), and the pyrophosphate-utilizing pyruvate phosphate dikinase (PPDK) in *P. cinnamomi* (Marshall et al., 2001), plays a significant role in the glycolytic flux of sporulating hyphae in *P. cinnamomi* as an alternative to pyruvate kinase (Marshall et al., 2001). However, we found only 2 PPDK ESTs in the mycelium library, while four distinct cDNAs represented by 9 ESTs showed extensive similarities to pyruvate kinase. The overall similarity between the deduced peptides ranged from 86.8 to 95.9%, and they all possessed typical signatures of pyruvate kinase. Whether PPi plays a major role in *Phytophthora* during vegetative growth remains an open question. So the interplay between ATP-dependent and PPi-dependent enzymes, especially in the various life cycle stages, has to be further investigated in *Phytophthora*.

Enolase and GA3PDH were among the 25 genes highly represented in the mycelium library. Enolase was represented by a single contig of 32 ESTs, whereas 46 GAPDH-related sequences were clustered into three contigs of 20, 14, and 8 ESTs, respectively, and four singletons. Two genomic organizations for GA3PDH-encoding sequences have been described in oomycetes (Liaud et al., 2000). A first gene, called Gap-C1 in the nomenclature, encodes a 36 kDa cytosolic protein, while another gene, called Gap-CIII, encodes a fusion

transcript comprising triose phosphate isomerase (TPI) in the 5′ moiety and GA3PDH in its 3′ moiety (Liaud et al., 2000; Unkles et al., 1997). The resulting fusion transcription unit, called *tigA*, is translated as a single peptide which is imported into mitochondria prior to its assembly into a tetrameric bifunctional enzymatic complex (Liaud et al., 2000). We found a single EST matching to *tigA*, while the 45 others shared extensive similarities to the cytosolic Gap-C1 from *A. bisexualis* and displayed features of discrete transcription units. So at least seven genes would encode various forms of GA3PDH in *P. parasitica*. This result differs from a recent work indicating that *P. parasitica* GAPDH is encoded by a single-copy gene (Shan et al., 2004). We also found one EST corresponding to a discrete form of TPI. This contrasts previous observations on *P. infestans* suggesting that *tigA* is the single source of TPI (Unkles et al., 1997).

Sequences related to fatty acid synthesis are also abundant as they reach 3.84% (137/3568) of total ESTs. They comprise redundant ESTs encoding enzymes providing acetyl CoA, such as acetyl CoA synthetase, ATP citrate lyase, and enzymes of the pyruvate dehydrogenase complex, as well as sequences directly involved in fatty acids synthesis, such as acetyl CoA carboxylase (ACC) and desaturases. Most of these enzymes were represented by 9–11 ESTs. It thus indicates that vegetative growth of *Phytophthora* in vitro is accompanied by transcriptional activity for formation of lipid reserves.

3.4. ESTs relevant to hyphal development

Filamentous growth is characterized by a highly polarized apical synthesis that involves localized exocytosis of vesicles carrying components necessary for cell wall biosynthesis. However, the basic machinery of vesicle transport was poorly represented in our database. Sequences corresponding to the various elements of coat protein complexes were identified in only 14 ESTs. Small Ras-related GTPases (Rabs), which mediate the vesicle transport, and vary in an 11–60 families range from yeast to human (Bock et al., 2001), were represented by only two contigs, encoding Rab1/ypt1, previously identified in *P. infestans* (Chen and Roxby, 1996), which is essential for trafficking from the endoplasmic reticulum to the Golgi apparatus, and ypt5, which regulates transport to endosomes (Gupta and Brent Heath, 2002). In a same way, only three out of the ~20–35 SNAREs, which mediate vesicle fusion to membranes (Gupta and Brent Heath, 2002) possessed analogs in the *P. parasitica* library, represented each by a single EST. A hypothesis is that the genes assuming these functions, if present in the *Phytophthora* genome, may be expressed at a level too low to be represented in our database, or substantially diverged to be identified in Blastx analyses.

Among the cytoskeletal components, actin plays a major role in the establishment and the maintenance of hyphal tip growth (Heath et al., 2000). It was also shown to be of prime importance in the zoosporogenesis of *Phytophthora* (Jackson and Hardham, 1998). It was among the most prominent sequences, as 23 ESTs (0.6% of total ESTs) constituted a single contig, displaying 99% identity at the amino acid level with the *P. infestans* actin-A sequence. Besides actin, 14 ESTs encode various actin binding or capping proteins, which regulate the structure of actin filaments (Carlier, 1998). The relative abundance of sequences involved in actin depolymerization is likely to reflect the dynamics of actin microfilaments in elongating hyphae. Myosins, the actin-associated motors, were represented by 11 ESTs. A continuous growth of the hyphal tip requires a balance between wall-synthesizing and wall-lyzing enzymes. Five ESTs would encode various β-glucan synthases, while 14 ESTs corresponded to *PiEXO1*, encoding an exo-1,3-β-glucanase, and 3 ESTs corresponded to *PiENDO1*, an endo-1,3-β-glucanase from *P. infestans* (McLeod et al., 2003). These genes are highly expressed in *P. infestans* mycelium (McLeod et al., 2003), reflecting a probable role in hyphal growth or branching. The recycling of cell wall material associated to hyphal growth may also be illustrated by the very important representation of ESTs relevant to proteins synthesis and degradation mentioned above.

3.5. Comparison to Phytophthora datasets

BLASTN searches were performed against *Phytophthora* sequences. A first investigation concerned *P. parasitica* cDNAs expressed in germinated cysts (Shan et al., 2004) or in motile zoospores (Skalamera et al., 2004). A total of 121 out of 149 sequences of germinated cysts corresponding to 107 genes were present in the mycelium library. In contrast, only 149 out of 404 sequences of zoospores, corresponding to 90 genes, were also present in the mycelium library. They corresponded to ribosomal proteins (35 genes), actin and tubulin, as well as sequences involved in cell–cell communication and signal transduction. This comparison was performed on a few available sampling of sequences. Nevertheless, it reveals important differences in EST content between mycelial and zoospore stages. Exploring other physiological stages with an EST approach is likely to reveal a number of stage-specific expressed sequences, and will permit to widen the *P. parasitica* gene catalog.

Mycelium ESTs were also compared to sequences from other *Phytophthora* species publicly available. They include ~23,000 ESTs from *P. sojae* located in the PFGD public database, more than 75,000 ESTs from *P. infestans* recently released in GenBank, and draft releases of the *P. ramorum* and *P. sojae* genomes. A total of 526 ESTs, corresponding to 28 contigs and 444 singlets, did not match to *P. infestans* or *P. sojae* databases. A large proportion corresponded to the "no hit" or

"unknown function" categories (386/526 and 28/526, respectively). In parallel analyses, Blastn queries to genomic sequences of *P. ramorum* and *P. sojae* revealed that a substantial proportion of *P. parasitica* ESTs (50.3%) matched to sequences in both genomes. Conversely, 383 ESTs, corresponding to 20 contigs and 327 singlets did not match on either *P. ramorum* or *P. sojae*. Here again, most of them (322/383) belonged to the "no hit" or "unknown function" categories. Last, 315 ESTs, corresponding to 14 contigs and 274 singlets, did not give any significant hit in any *Phytophthora* dataset, among which a vast majority of "no hit" and "unknown" sequences (287/315), and could be considered as *P. parasitica*-specific. So, a significant proportion (up to 12.7%) of *P. parasitica* unigenes do not appear to possess counterparts in other Phytophthora species. Complete sequencing of these ESTs and further comparisons with available data are required to confirm this result. Nevertheless, this observation highlights the limitation of concentrating efforts on a limited set of models and the relevance of developing appropriate genomic resources when needed. In addition, it indicates that the *P. parasitica* ESTs, beyond their importance for the understanding of the biology and pathogenicity of this species, will be useful for synteny analyses, and are a potential tool for studying the organization and evolution of *Phytophthora* genomes.

3.6. Identification of candidate genes for pathogenicity

Several *P. infestans* genes have been described as up-regulated during compatible interactions and may participate to the infection process. They include calmodulin (Pieterse et al., 1993), extracellular proteases (Paris and Lamattina, 1999), mucin-like proteins (Gornhardt et al., 2000), heat shock proteins (Avrova et al., 2003), and the ribosomal protein L10a (Avrova et al., 2003), as well as two genes families, named *ipiB* and *ipiO* (Pieterse et al., 1994b; Van West et al., 1998). All these sequences are present during the *P. parasitica* vegetative growth, excepted *ipiO*. This is not unexpected, as expression of *ipiO* was not observed in in vitro-grown mycelium (Van West et al., 1998). However, *ipiB1* was represented by 8 ESTs in the *P. parasitica* library, whereas *ipiB* expression was not detectable during in vitro growth of *P. infestans* on rich medium (Pieterse et al., 1994a).

A variety of genes were identified that may be associated with pathogenesis. They include molecules that counteract plant defense responses, such as an analog of GIP, which is a *P. sojae* member of a family encoding glucanase inhibitors that interacts with the soybean endo-β-1,3-glucanases during infection (Rose et al., 2002), and an EST similar to EPI1, a Kazal-like proteinase inhibitor from *P. infestans* that inhibits host proteases (Tian et al., 2004). Other candidates may be grouped in several categories (Table 1). They comprise a large array of hydrolytic enzymes, among which putative cell wall degrading enzymes and proteases, genes involved in protection against active oxygen species generated as part of host defense response, detoxification enzymes, and amino acid, peptide or sugar transporters which may function in the uptake of host degradation products. Other products may be secreted that are crucial for pathogenicity, such as molecules involved in the adhesion to host. At least 28 ESTs would encode proteins with similarities to mucin-like glycoproteins or surface-binding proteins. All these genes are candidate on the sole basis of similarity reports and analogies to other plant or animal pathogens. Nevertheless, they may participate to infection by achieving rapid penetration, assimilating nutrients, or disabling antifungal molecules. Hence, they may constitute quantitative factors that contribute to the overall virulence of *P. parasitica*.

In addition, several ESTs displayed similarities to various proteins involved in protein modifications, such as protein disulfide isomerases, ADP-ribosylation factors and other chaperones. Among them, they were several members of the *hsp70* gene family (Shan and Hardham, 2004) that have been recently shown to be up-regulated during the *P. infestans*/potato interaction (Avrova et al., 2003). As they have been implicated in animal diseases (Dobbin et al., 2002), their expression has to be investigated during the life cycle of *P. parasitica* and the infection process. Last, 30 ESTs, corresponding to three contigs and 14 singletons, display similarities with transposase-like sequences and potential retroelements. If functional, these sequences may impact genome stability with potential alterations in virulence and host range, as already observed in phytopathogenic fungi (Kang et al., 2001; Taylor et al., 1995).

3.7. ESTs relevant to the elicitin family and other glycoprotein elicitors

Up to 6% ESTs assigned to a given function corresponded to members of the elicitin gene family. The most redundant sequence of this family, represented by 87 ESTs, matched to *ParA* 1, which encodes parasiticein, or PARA1, the major secreted elicitin of *P. parasitica* (Kamoun et al., 1993; Ricci et al., 1993). Three distinct transcripts were shown to encode PARA1, and had been designated as *ParA1*, *ParA1*.1 and *ParA1*.2, the two latter differing from *ParA1* by three G–A transitions in the 3′ untranslated region (UTR, Colas et al., 2001). Seventy two out of 87 ESTs corresponded to *ParA1*.2 and constituted by far the most redundant sequence in the library, while *ParA1*.1 was represented by 13 ESTs. Two ESTs were highly related to *ParA1*.1 and *ParA1*.2, but displayed faint differences in the 3′ UTR, and were designed as *ParA1*.3 and *ParA1*.4. These four sequences were aligned with *parA1* described by Kamoun et al. (1993) Fig. 3. None of the ESTs found in our cDNA

Table 1
Phytophthora parasitica ESTs corresponding to cDNAs potentially associated with pathogenesis

EST	EST number	Best Blastx match	Organism	Accession Nos.	*E* value
Protection against oxidative stress					
EST149_14_C03_T3	1	Ascorbate peroxidase, putative	*P. yezoensis*	AAP37478	5.00E−35
EST149_37_A08_T3	1	Catalase	*X. laevis*	AAH54964	1.00E−86
EST149_21_E07_T3	2	Cytochrome *c* peroxidase	*S. cerevisiae*	AAA88709	9.00E−06
EST149_35_C08_T3	1	Glutaredoxin type I	*F. agrestis*	AAB92658	7.00E−14
EST149_07-A04_T3*	2	Gluthatione reductase	*P. polycephalum*	BAA93433	1.00E−45
EST149_01_C02_T3	2	Methionine sulfoxide reductase	*L. sativa*	AAF19789	9.00E−51
EST149_03_A01_T3*	1	Phospholipid hydroperoxide glutathione peroxidase-like	*M. crystallinum*	CAB96145	3.00E−13
EST149_07_C01_T3*	3	Superoxide dismutase	*R. norvegicus*	AAH70913	5.00E−69
EST149_22_A02_T3	6	Superoxide dismutase	*C. reinhardtii*	AAA80639	4.00E−63
EST149_04_H09_T3*	1	Superoxide dismutase	*C. reinhardtii*	AAA80639	1.00E−15
EST149_02_F07_T3*	1	Superoxide dismutase	*S. carnosus*	CAC14833	4.00E−15
EST149_04_E12_T3 EST149_22_B11_T3 EST149_23_E05_T3	1	Peroxiredoxin 2	*B. taurus*	AAG53659	3.00E−64
Detoxification, drug resistance and metabolite transport					
EST149_17_H09_T3	1	Bialaphos acetylhydrolase	*S. hygroscopicus*	A47031	5.00E−11
EST149_14_C03_T3	1	Cytochrome P450 like_TBP	*C. lanatus*	BAD26579	1.00E−09
EST149_37_A08_T3	1	Cytochrome P450-like protein	*O. sativa*	NP_922423	5.00E−20
EST149_02_F05_T3	1	Cytochrome P450-like protein	*O. sativa*	XP_470289	4.00E−18
EST149_20_C03_T3	1	Glutathione *S*-transferase, predicted	*P. fluorescens PfO-1*	ZP_00267498	3.00E−26
EST149_03_B09_T3*	4	Glutathione *S*-transferase	*A. thaliana*	BAB11100	3.00E−24
EST149_38_F03_T3	1	Glutathione *S*-transferase related	*A. thaliana*	NP_193723	7.00E−70
EST149_11_F04_T3	1	Lactoylglutathione lyase, putative	*L. interrogans*	AAN48616	1.00E−56
EST149_16_E08_T3	1	Penicillin-binding protein, putative	*B. cereus ATCC 10987*	NP_978605	3.00E−13
EST149_11_G06_T3	1	Similar to xenobiotic reductase	*Nostoc sp. PCC 7120*	NP_485905	4.00E−62
EST14907_H08_T3	1	Xenobiotic reductase B	*Pirellula sp.*	NP_869810	1.00E−64
EST149_10_A05_T3	1	ABC transporter	*P. infestans*	BAB20331	4.00E−04
EST149_20_H06_T3	1	ABC transporter	*S. oneidensis MR-1*	NP_719021	4.00E−65
EST149_24_A05_T3	1	ABC transporter	*B. fuckeliana*	BAC67160	7.00E−05
EST149_24_C01_T3	1	ABC transporter	*A. thaliana*	NP_850354	6.00E−08
EST149_24_C10_T3	1	ABC transporter	*B. japonicum*	NP_772047	2.00E−38
EST149_13_C01_T3*	2	ABC transporter	*A. thaliana*	AAM14842	4.00E−78
EST149_22_E06_T3*	4	ABC transporter	*O. sativa*	XP_450985	7.00E−81
EST149_07_E01_T3*	2	ATP-binding cassette transporter AtABCA1	*A. thaliana*	AAK39643	8.00E−05
EST149_04_F11_T3*	2	ATP-binding cassette transporter AtABCA1	*A. thaliana*	AAK39643	6.00E−27
EST149_28_G08_T3*	3	ABCA1.2 transporter	*L. tropica*	AAL73206	5.00E−71
EST149_35_F06_T3	1	MATE efflux protein-related	*A. thaliana*	NP_200058	8.00E−38
EST149_17_B07_T3*	2	MATE efflux family protein, putative	*O. sativa*	XP_462973	7.00E−12
EST149_05_G10_T3*	2	Similar to multidrug resistance-associated protein 5	*G. gallus*	XP_422754	3.00E−63
EST149_13_H02_T3	1	Pleiotropic drug resistance transporter	*P. sojae*	AAT85568	e−153
EST149_36_C05_T3	1	pleiotropic drug resistance transporter	*P. sojae*	AAT85568	e−104
EST149_36_C06_T3	1	Pleiotropic drug resistance transporter	*P. sojae*	AAT85568	e−157
EST149_03_E11_T3*	2	Pleiotropic drug resistance transporter	*P. sojae*	AAT85568	0.0
EST149_13_F07_T3*	2	PoxA (oxacillinase)	*P. aeruginosa*	AAT09607	2.00E−07
Hydolytic enzymes					
EST149_11_D02_T3*	2	Cellulase c	*Orpinomyces sp. PC-2*	AAB92679	7.00E−17
EST149_29_D01_T3*	2	Exocellulase	*I. lacteus*	BAA76364	7.00E−60
EST149_16_G09_T3*	2	2-deoxy-D-gluconate 3-dehydrogenase, pectin degradation	*B. japonicum USDA*	BAC46347	7.00E−51
EST149_30_C12_T3	1	β-Glucosidase	*M. degradans 2-40*	ZP_00316269	5.00E−88
EST149_10_A04_T3	1	β-Glucosidase	*C. hutchinsonii*	ZP_0038266	1.00E−29
EST149_09_H02_T3	1	β-Glucosidase	*S. rochei*	CAA52344	7.00E−09
EST149_27_G06_T3*	4	β-Glucosidase/xylosidase	*P. infestans*	AAK19754	e−128
EST149_02_H08_T3*	2	β-Glucosidase/xylosidase	*P. infestans*	AAK19754	2.00E−114
EST149_16_D06_T3	1	Strictosidine-*O*-β-D-glucosidase	*R. serpentina*	CAC83098	2.00E−50
EST149_17_D02_T3	1	Strictosidine-*O*-β-D-glucosidase	*R. serpentina*	CAC83098	7.00E−42

(*continued on next page*)

Table 1(*continued*)

EST	EST number	Best Blastx match	Organism	Accession Nos.	*E* value
EST149_05_C04_T3*	2	*O*-glycosyl hydrolase family 30	*T. tengcongensis*	NP_623885	9.00E−40
EST149_03_D05_T3*	3	CEL4a mannanase	*A. bisporus*	CAB76904	5.00E−51
EST149_11_C04_T3*	2	Acidic chitinase	*P. infestans*	AAN31509	e−107
EST149_02_B06_T3*	2	Acidic chitinase	*P. infestans*	AAN31509	e−109
EST149_02_B03_T3	1	Xylanase A	*S. tendae*	AAP72963	1.00E-03
EST149_02_B07_T3	1	Secreted endo-1,4-β-xylanase	*S. coelicolor A3*	CAB61191	1.00E−18
EST149_08_A03_T3	1	Endoglucanase	*E. desertorum*	AAM77702	2.00E−59
EST149_02_D03_T3*	3	Endo-1,3-β-glucanase, putative	*P. infestans*	AAM18482	2.00E−56
EST149_29_C06_T3	1	β(1-3)Endoglucanase	*A. fumigatus*	AAF13033	5.00E−40
EST149_37_B04_T3	1	Endo-1,4-β-glucanase	*P. horikoshii OT3*	BAA30271	6.00E−13
EST19_19_A04_T3*	3	Exo-β-1,3-glucanase	*M. magnetotacticum*	ZP_00052629	2.00E−13
EST149_02_A03_T3*	14	Exo-1,3-β-glucanase, putative	*P. infestans*	AAM18483	0.0
EST149_18_E09_T3	1	Cathepsin L-like (cysteine proteinase)	*T. molitor*	AAR05023	3.00E−22
EST149_12_C10_T3*	3	Toxopain-2 (cysteine proteinase)	*T. gondii*	AAT74529	7.00E−62
EST149_22_E08_T3	1	Cysteine proteinase	*T. foetus*	AAQ82649	2.00E−37
EST149_31_F03_T3	1	Cysteine proteinase precursor	*M. glutinosa*	AAF19630	3.00E−43
EST149_01_H08_T3*	2	Homologue of Sarcophaga 26, 29 kDa proteinase	*P. americana*	BAA86911	2.00E−07
EST149_14_G03_T3*	2	Membrane alanine aminotransferase	*M. acetivorans*	NP_616538	3.00E−41
EST149_33_F05_T3	1	Membrane alanine aminotransferase	*M. mazei Go1*	AAM29975	2.00E−06
EST149-35_A03_T3	1	Serine carboxypeptidase	*H. sapiens*	NP_112601	1.00E−34
EST149_05_D06_T3	1	Similar to carboxypeptidase y	*Y. lipolytica*	XP_502601	6.00E−07
EST149_03_A10_T3	1	Serine protease family member	*C. elegans*	NP_508170	3.00E−34
EST149_17_H09_T3	1	Esterase/lipase	*B. cepacia R18194*	ZP_00215798	3.00E−07
EST149_20_F08_T3	1	Phenolic acid decarboxylase	*G. zeae*	EAA71092	2.00E−71
Host degrading enzymes inhibitors					
EST149_23_H08_T3	1	Glucanase inhibitor protein 2	*P. sojae*	AAL11721	3.00E−62
EST149_35_A06_T3	1	Kazal-like proteinase inhibitor EPI5	*P. infestans*	AAT00504	5.00E−36
Toxin biosynthesis					
EST149_02_C11_T3	1	RTX toxin	*Magnetococcus sp. MC-1*	ZP_00290176	3.00E−05
EST149_14_H02_T3	1	Related to host-specific AK-toxin Akt2	*N. crassa*	T49722	9.00E−05
EST149_15_G11_T3	1	Related to host-specific AK-toxin Akt2	*N. crassa*	XP_323244	3.00E−05
Adhesion					
EST149_04_A05_T3*	6	Antigenic cell wall protein MP2	*A. fumigatus*	AAR22399	4.00E−28
EST149_12_F05_T3	1	CBEL protein, formerly GP34	*P. parasitica*	CAA65843	6.00E−13
EST149_31_E06_T3*	4	CBEL protein, formerly GP34	*P. parasitica*	CAA65843	0.0
EST149_05_A08_T3*	3	CBEL protein, formerly GP34	*P. parasitica*	CAA65843	0.0
EST149_07_H05_T3	1	CAR precursor (mucin-like)	*P. infestans*	AAC72309	3.00E−18
EST149_07_F05_T3*	2	Glue protein	*D. virilis*	CAA82672	2.00E−09
EST149-07_A10_T3	1	Mucin-like protein	*H. glycines*	AAC62109	1.00E−25
EST149_18_E008_T3	1	Mucin-like protein	*H. glycines*	AAC62109	5.00E−18
EST-149_11_E11_T3*	2	Mucin	*H. sapiens*	CAC83675	3.00E−04
EST149_01_H10_T3*	2	Mucin	*S. scrofa*	AAC48526	3.00E−05
EST149_06_A12_T3*	5	Platelet binding protein GspB	*S. scrofa*	AAL13053	2.00E−27

* Indicates the representative EST within a contig.

library corresponded to this allele. *ParA1*.3 and *ParA1*.4 differ from each other by three G–A transitions and may represent allelic variants of a single gene, or derive from distinct genes. Thus, at least four genes encoding parA1 are present in the *P. parasitica* genome.

Based on the classification previously defined for the elicitin family (Kamoun et al., 1999; Qutob et al., 2003), elicitin-encoding cDNAs were named *par2A*, *par4*, *par5*, *par6*, *par7*, and *par8*. Open reading frames corresponding to proteins of 195, 121, 183, 186, 199, and 118 amino acids, including potential signal peptides, were found for *par2A*, *par4*, *par5*, *par6*, *par7*, and *par8*, respectively. As already mentioned for INF2A, INF5 and INF6 (Kamoun et al., 1999), PAR2A, PAR5 and PAR6 display numerous potential sites for O-glycosylation. These three proteins were represented by 9, 15, and 17 ESTs, respectively. *Par4* and *par7* were both represented by 4 ESTs, while a single EST defined *par8* as a novel class, unobserved in the EST collections of *P. infestans* and *P. sojae*. All protein sequences were compared to their orthologs of *P. infestans* and *P. sojae*. The resulting dendrogram clearly indicates that sequences are clustered with high bootstrap

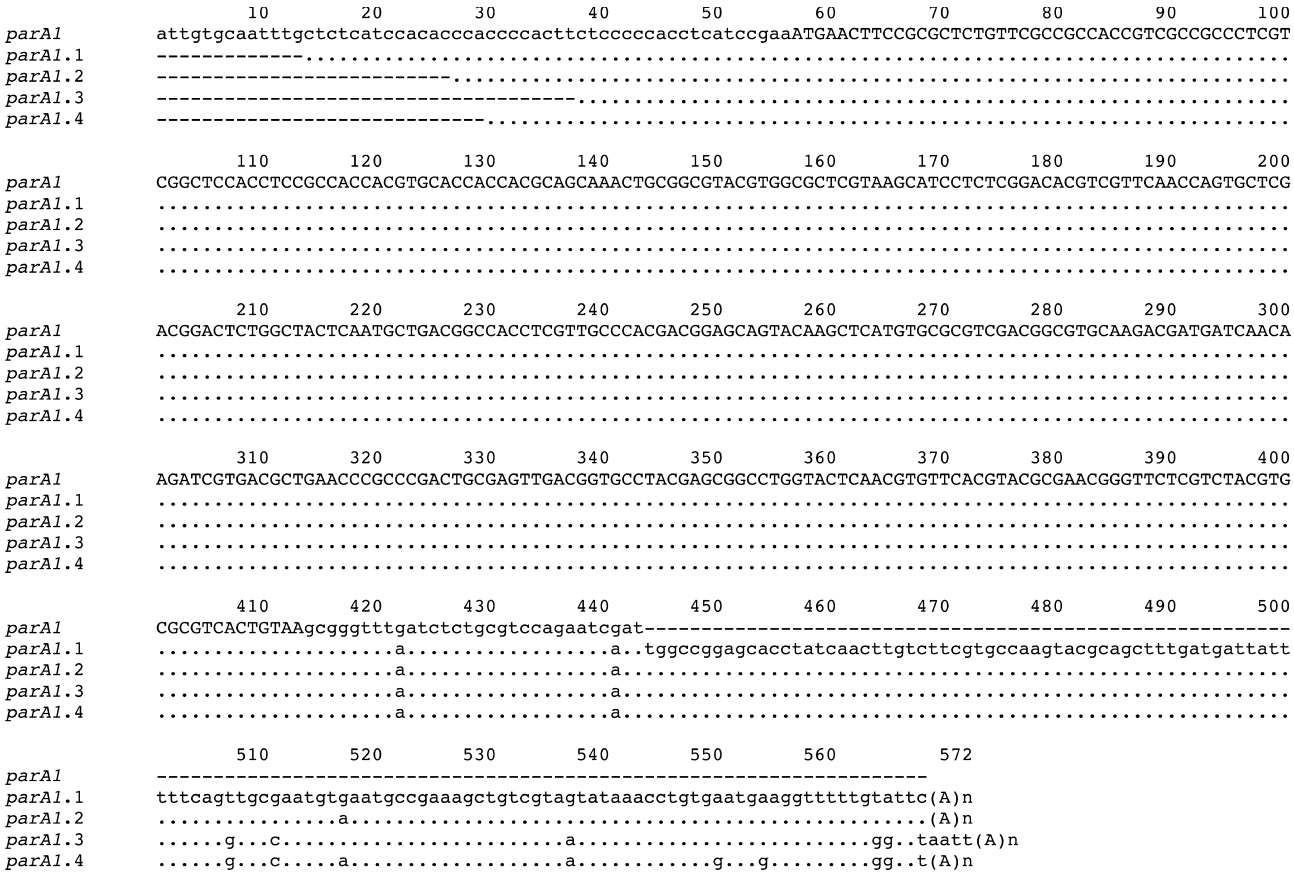

Fig. 3. Alignment of the genomic sequence of *parA1* [Kamoun et al., 1993 #108] and the various parA1 alleles identified in the EST library from *P. parasitica* 149. Coding regions are in capital letters. The putative polyadenylation site is underlined. Sequences are ParA1.1 (GenBank Accession No. AAK01624), ParA1.2 (AAK01625), ParA1.3 (EST149_15_C02_T3), and ParA1.4 (EST149_19_C06_T3).

values according to their class and not their species of origin (Fig. 4). The restriction of the analysis to the elicitin domain did not change the tree topology (not shown), confirming the hypothesis that the divergence of elicitin genes, generating the current multigene family, occurred before the speciation of *Phytophthora* (Qutob et al., 2003). Despite the abundance and diversity of elicitin sequences, no EST was related to the highly acidic elicitins that have been described in the soilborne species *P. cryptogea* (Panabieres et al., 1995) and *P. cinnamomi* (Duclos et al., 1998).

Taken together, all elicitin genes were highly more distributed in *P. parasitica* library than in those generated from *P. sojae* (Qutob et al., 2003). Hence, ESTs encoding SOJA-2, the analog of PARA1, only constituted 0.19% of a 5851 mycelium library, whereas *ParA1* ESTs reached 2.44% of the *P. parasitica* library. In a same way, ESTs corresponding to SOJ2, SOJ3, and SOJ6 were present in 3–7 copies, constituting 0.05–0.12% of total ESTs. Here again, these discrepancies may reflect different culture conditions, or may correspond to differences between *P. parasitica* and *P. sojae*, such as ecological niches or host range. However, these comparisons require validation by measurement of gene expression.

The different members of the elicitin gene family were also compared to the N-terminal sequence of elicitin-like phospholipases isolated from *Phytophthora capsici*, the pepper blight agent (Nespoulous et al., 1999). These glycoproteins of 22 and 32 kDa exhibit important sequence similarities with capsicein, the major elicitin of *P. capsici* (Nespoulous et al., 1999). The Fig. 5 shows the alignment of the phospholipase PlB2 with the N-terminal region of PAR5 and PAR6. The overall similarity was exceptionally high, indicating that PAR5 and PAR6, as well as their orthologs in *P. infestans* and *P. sojae*, are likely analogs of *P. capsici* phospholipases. All elicitin sequences share a common core domain whose structure has been determined in the case of cryptogein, the major elicitin of *P. cryptogea* (Fefeu et al., 1997; Lascombe et al., 2002). It was shown that it not only binds sterols, but also various fatty acids (Osman et al., 2001). The present identification of one or two elicitin members as likely interacting with lipid molecules lead to suggest a general function for the elicitin family as lipid binding proteins, through the elicitin core domain, in a variety of cellular processes, including plant recognition. In this context, the marked abundance of both elicitin sequences and ESTs relevant to lipid metabolism is interesting. A comparative analysis of

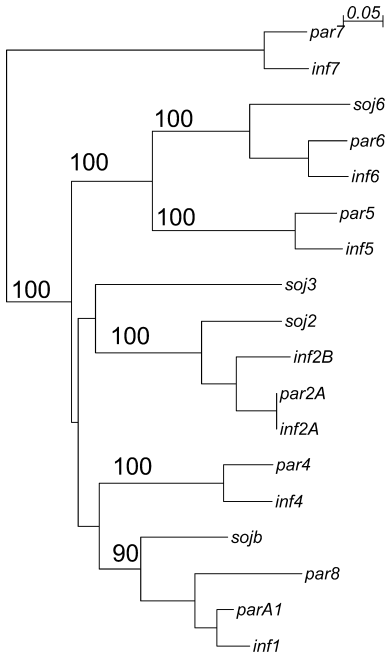

Fig. 4. Phylogenetic reconstruction of the elicitin gene family from *P. parasitica*, *P. infestans*, and *P. sojae*. Alignment included INF1 (GenBank Accession No. AAB49807), PARA1 (AAK01624), PAR8 (EST149_15_B03_T3), SOJB (AAO24640), INF4 (AAL16011), PAR4 (EST149_14_D12_T3), INF2A (AU10320), PAR2A (EST149_27_G10_T3), INF2B (AAB94815), SOJ2 (AAO24642), SOJ3 (AAO24642), INF5 (AAL16012), PAR5 (EST149_01_B02_T3), INF6 (AAL16013), PAR6 (EST149_01_F05_T3), SOJ6 (AAO24643), INF7 (AAL16014), and PAR7 (EST149_06_D03_T3). Protein sequences were compared using the neighbor-joining method of Clustal W1.4. The scale bar represents 5% sequence divergence. Bootstrap values from 1000 replications are indicated at the nodes.

Fig. 5. Alignment of the N-terminal sequences of the phospholipase PlB2, isolated from *P. capsici* (Nespoulous et al., 1999), PAR5 and PAR6 (this study). Dashes indicate potential cysteine residues (not identified), X indicates unidentified residues. Shading indicates blocks of identical (black) or similar (gray) amino acids. Conserved residues are indicated in black letters on a gray background.

their distribution in various physiological situations is necessary and finding an eventual coordinated expression of these sequences would be a step in the understanding of the precise role of elicitins in *Phytophthora* biology.

ESTs were also examined for genes homologous to known *Phytophthora* elicitors that act on host or non-host plants. A single EST matched to the 42 kDa transglutaminase (Brunner et al., 2002; Fabritius and Judelson, 2003). We identified seven ESTs encoding two closely related proteins corresponding to CBEL. The two contigs, named T-149-73.1 and T-149-73.2, only differed by a T–C transition in signal peptide region, and a 10-aa deletion in the Thr/Pro rich spacer of T-149-73.2. *Cbel* was proposed to be a single-copy gene on the basis of Southern experiments (Mateos et al., 1997). So the two contigs described here may represent alleles. Alternatively, the occurrence of two genes tandemly arrayed cannot be ruled out. A consequence of the 10 aa deletion is that the distance between the cellulose-binding domains is shortened. Whether the deleted protein displays different substrate specificity is a matter of conjecture.

4. Concluding remarks

This study constitutes a first step in the large-scale gene identification in *P. parasitica*. Although other sequencing projects have been engaged on mycelia of other *Phytophthora* species, they only fed databases without further annotation, or descriptive analyses concerned a limited set of sequences. So the present work constitutes to date the most comprehensive gene repertoire of the vegetative growth of a soilborne oomycete with a broad host range, and serves as a basis for the transcriptome definition during the vegetative growth. It constitutes a step for understanding the molecular bases of biology and pathogenicity of this species, and analysis of several thousands of ESTs from *P. parasitica*/plant interaction cDNA libraries is under way in our laboratory. It will help in increasing the gene catalogue of *P. parasitica*, already documented with zoospores and germinated cysts cDNAs (Shan et al., 2004; Skalamera et al., 2004). As we prepared non-normalized libraries, EST redundancy approximately reflects the abundance of the corresponding transcript. Hence, it will be possible to perform comparative gene-expression analyses, and possibly identify stage-specific gene expression. Some key results of this analysis may be summarized below.

The main cellular functions of *P. parasitica* were identified on the basis of EST annotation and functional analogies. The accumulation of sequences, as well as functional analyses through heterologous expression or complementation of yeast mutants will be necessary for a deeper investigation of the various metabolic pathways and cellular processes.

An important result is that elicitin-related sequences are over-represented in the mycelium library. Studies of these proteins were for a long time limited to their role in plant–*Phytophthora* interactions. Their ability to bind sterols, and to a larger extent lipid molecules, the modular diversity of the elicitin family already described in other species (Kamoun et al., 1999; Qutob et al., 2003), and confirmed in the present study, justify a thorough analysis of the properties of these proteins and their expression pattern, during interactions with plants but also in various in vitro situations in order to definitively unravel their fundamental function.

Another important result is that many genes otherwise up-regulated during compatible interactions with

the aerial *P. infestans* or potentially involved in pathogenicity of fungal microorganisms are already expressed during the in vitro vegetative growth of the soilborne *P. parasitica*. Whether these genes are actually induced during infection of host plants by *P. parasitica* has to be validated. In all cases, they may constitute preformed determinants, easily available for early stages of the infection process. So, the pathogen would adapt to plant recognition and responses at low transcriptional cost.

We found a substantial population of *P. parasitica* ESTs for which we found no counterpart in the *P. infestans* or *P. sojae* ESTs databases, or in the *P. ramorum* and *P. sojae* draft genomes. They can correspond to rapidly evolving genes and may contribute to the unique features of *P. parasitica*, or are a characteristic of soilborne pathogens with broad host range. They may reflect the wide diversity of biological, ecological and pathological features encountered within the genus *Phytophthora*.

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References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Aviv, H., Leder, P., 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid–cellulose. Proc. Natl. Acad. Sci. USA 69, 1408–1412.

Avrova, A.O., Venter, E., Birch, P.R., Whisson, S.C., 2003. Profiling and quantifying differential gene transcription in *Phytophthora infestans* prior to and during the early stages of potato infection. Fungal Genet. Biol. 40, 4–14.

Baldauf, S.L., Roger, A.J., Wenk-Siefert, I., Doolitle, W.F., 2000. A kingdom-level phylogeny of eukaryotes based on combined protein data. Science 290, 972–977.

Beyer, K., Jimenez Jimenez, S., Randall, T.A., Lam, S., Binder, A., Boller, T., Collinge, M., 2002. Characterization of *Phytophthora infestans* genes regulated during the interaction with potato. Mol. Plant Pathol. 3, 473–485.

Birch, P.R.J., Whisson, S.C., 2001. *Phytophthora infestans* enters the genomic area. Mol. Plant Pathol. 2, 257–263.

Bock, J.B., Matern, H.T., Peden, A.A., Scheller, R.H., 2001. A genomic perspective on membrane compartment organization. Nature 409, 839–841.

Brunner, F., Rosahl, S., Lee, J., Rudd, J.J., Geiler, C., Kauppinen, S., Rasmussen, G., Scheel, D., Nurnberger, T., 2002. Pep-13, a plant defense-inducing pathogen-associated pattern from *Phytophthora* transglutaminases. EMBO J. 21, 6681–6688.

Carlier, M., 1998. Control of actin dynamics. Curr. Opin. Cell Biol. 10, 45–51.

Chen, Y., Roxby, R., 1996. Characterization of a *Phytophthora infestans* gene involved in vesicle transport. Gene 181, 89–94.

Colas, V., Conrod, S., Venard, P., Keller, H., Ricci, P., Panabières, F., 2001. Elicitin genes expressed in vitro by some tobacco isolates of *Phytophthora parasitica* are down-regulated during compatible interactions. Mol. Plant Microbe Interact. 14, 326–335.

Dobbin, C.A., Smith, N.C., Johnson, A.M., 2002. Heat shock protein 70 is a potential virulence factor in murine toxoplasma infection via immunomodulation of host NF-kappa B and nitric oxide. J. Immunol. 169, 958–965.

Duclos, J., Fauconnier, A., Coelho, A.C., Bollen, A., Cravador, A., Godfroid, E., 1998. Identification of an elicitin gene cluster in *Phytophthora cinnamomi*. J. Seq. Mapp. 9, 231–237.

Erwin, D.C., Ribeiro, O.K., 1996. *Phytophthora* diseases worldwide. in: M.N. St. Paul (Ed.), American Phytopathological Society.

Fabritius, A.L., Cvitanich, C., Judelson, H.S., 2002. Stage-specific gene expression during sexual development in *Phytophthora infestans*. Mol. Microbiol. 45, 1057–1066.

Fabritius, A.L., Judelson, H.S., 2003. A mating-induced protein *of Phytophthora infestans* is a member of a family of elicitors with divergent structures and stage-specific patterns of expression. Mol. Plant Microbe Interact. 16, 926–935.

Fefeu, S., Bouaziz, S., Huet, J.C., Pernollet, J.C., Guittet, E., 1997. Three-dimensional solution structure of beta cryptogein, a beta elicitin secreted by a phytopathogenic fungus *Phytophthora cryptogea*. Protein Sci. 6, 2279–2284.

Fellbrich, G., Romanski, A., Varet, A., Blume, B., Brunner, F., Engelhardt, S., Felix, G., Kemmerling, B., Krzymowska, M., Nurnberger, T., 2002. NPP1, a *Phytophthora*-associated trigger of plant defense in parsley and Arabidopsis. Plant J. 32, 375–390.

Gornhardt, B., Rouhara, I., Schmelzer, E., 2000. Cyst germination proteins of the potato pathogen *Phytophthora infestans* share homology with human mucins. Mol. Plant Microbe Interact. 13, 32–42.

Gupta, G.D., Brent Heath, I., 2002. Predicting the distribution, conservation, and functions of SNAREs and related proteins in fungi. Fungal Genet Biol. 36, 1–21.

Hall, R., Zentmeyer, G.A., Erwin, D.C., 1969. Approach to the taxonomy of *Phytophthora* through acrylamide gel electrophoresis of proteins. Phytopathology 59, 770–774.

Hardham, A.R., 2001. The cell biology behind *Phytophthora* pathogenicity. Aust. Plant Pathol. 30, 91–98.

Heath, I.B., Gupta, G., Bai, S., 2000. Plasma membrane-adjacent actin filaments, but not microtubules, are essential for both polarization and hyphal tip morphogenesis in *Saprolegnia ferax* and *Neurospora crassa*. Fungal Genet. Biol. 30, 45–62.

Huang, X., 1992. A contig assembly program based on sensitive detection of fragment overlaps. Genomics 14, 18–25.

Jackson, S.L., Hardham, A.R., 1998. Dynamic rearrangement of the filamentous actin network occurs during zoosporogenesis and encystment in the oomycete *Phytophthora cinnamomi*. Fungal Genet. Biol. 24, 24–33.

Judelson, H.S., 1997. The genetics and biology of *Phytophthora infestans*: modern approaches to a historical challenge. Fungal Genet. Biol. 22, 65–76.

Kamoun, S., 2001. Nonhost resistance to *Phytophthora*: novel prospects for a classical problem. Curr. Opin. Plant Biol. 4, 295–300.

Kamoun, S., 2003. Molecular genetics of pathogenic oomycetes. Eukaryot. Cell 2, 191–199.

Kamoun, S., Hraber, P., Sobral, B., Nuss, D., Govers, F., 1999. Initial assessment of gene diversity for the oomycete pathogen *Phytophthora infestans* based on expressed sequences. Fungal Genet. Biol. 28, 94–106.

Kamoun, S., Klucher, K.M., Coffey, M.D., Tyler, B.M., 1993. A gene encoding a host-specific elicitor protein of *Phytophthora parasitica*. Mol Plant Microbe Interact. 6, 573–581.

Kang, S., Lebrun, M.H., Farrall, L., Valent, B., 2001. Gain of virulence caused by insertion of a Pot3 transposon in a *Magnaporthe grisea* avirulence gene. Mol. Plant Microbe Interact. 14, 671–674.

Keon, J., Bailey, A., Hargreaves, J., 2000. A group of expressed cDNA sequences from the wheat fungal leaf blotch pathogen, *Mycosphaerella graminicola* (Septoria tritici). Fungal Genet. Biol. 29, 118–133.

Khatib, M., Lafitte, C., Esquerre-Tugaye, M.T., Bottin, A., Rickauer, M., 2004. The CBELelicitor of *Phytophthora parasitica* var. *nicotianae* activates defence in *Arabidopsis thaliana* via three different signalling pathways. New Phytol. 162, 501–510.

Kim, K.S., Judelson, H.S., 2003. Sporangium-specific gene expression in the oomycete phytopathogen *Phytophthora infestans*. Eukaryot. Cell 2, 1376–1385.

King, R.W., Peters, J.M., Tugendreich, S., Rolfe, M., Hieter, P., Kirschner, M.W., 1995. A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. Cell 81, 279–288.

Kramer, R., Freytag, S., Schmelzer, E., 1997. In vitro formation of infection structures of *Phytophthora infestans* is associated with synthesis of stage specific polypeptides. Eur. J. Plant Pathol. 103, 43–53.

Lascombe, M.B., Ponchet, M., Venard, P., Milat, M.L., Blein, J.P., Prange, T., 2002. The 1.45 Å resolution structure of the cryptogein–cholesterol complex: a close-up view of a sterol carrier protein (SCP) active site. Acta Crystallogr. D Biol. Crystallogr. 58, 1442–1447.

Latijnhouwers, M., Ligterink, W., Vleeshouwers, V.G., van West, P., Govers, F., 2004. A G alpha subunit controls zoospore motility and virulence in the potato late blight pathogen *Phytophthora infestans*. Mol. Microbiol. 51, 925–936.

Lees, E., 1995. Cyclin dependent kinase regulation. Curr. Opin. Cell Biol. 7, 773–780.

Liaud, M.F., Lichtle, C., Apt, K., Martin, W., Cerff, R., 2000. Compartment-specific isoforms of TPI and GAPDH are imported into diatom mitochondria as a fusion protein: evidence in favor of a mitochondrial origin of the eukaryotic glycolytic pathway. Mol. Biol. Evol. 17, 213–223.

Logemann, J., Schell, J., Willmitzer, L., 1987. Improved method for the isolation of RNA from plant tissues. Anal. Biochem. 163, 16–20.

Marshall, J.S., Ashton, A.R., Govers, F., Hardham, A.R., 2001. Isolation and characterization of four genes encoding pyruvate, phosphate dikinase in the oomycete plant pathogen *Phytophthora cinnamomi*. Curr. Genet. 40, 73–81.

Mateos, F.V., Rickauer, M., Esquerre-Tugaye, M.T., 1997. Cloning and characterization of a cDNA encoding an elicitor of *Phytophthora parasitica* var. *nicotianae* that shows cellulose-binding and lectin-like activities. Mol. Plant Microbe Interact. 10, 1045–1053.

McLeod, A., Smart, C.D., Fry, W.E., 2003. Characterization of 1,3-beta-glucanase and 1,3;1,4-beta-glucanase genes from *Phytophthora infestans*. Fungal Genet. Biol. 38, 250–263.

Mulder, N.J., Apweiler, R., Attwood, T.K., Bairoch, A., Barrell, D., Bateman, A., Binns, D., Biswas, M., Bradley, P., Bork, P., et al., 2003. The InterPro Database, 2003 brings increased coverage and new features. Nucleic Acids Res. 31, 315–318.

Munoz, M.J., Daga, R.R., Garzon, A., Thode, G., Jimenez, J., 2002. Poly(A) site choice during mRNA 3′-end formation in the *Schizosaccharomyces pombe* wos2 gene. Mol. Genet. Genomics 267, 792–796.

Nespoulous, C., Gaudemer, O., Huet, J.C., Pernollet, J.C., 1999. Characterization of elicitin-like phospholipases isolated from *Phytophthora capsici* culture filtrate. FEBS Lett. 452, 400–406.

Niere, J.O., De Angelis, G., Grant, B.R., 1994. The effects of phosphonate on the acid-soluble phosphorus components in the genus *Phytophthora*. Microbiology 140, 1661–1670.

O'Connell, M.J., Krien, M.J., Hunter, T., 2003. Never say never. The NIMA-related protein kinases in mitotic control. Trends Cell Biol. 13, 221–228.

Osman, H., Mikes, V., Milat, M.L., Ponchet, M., Marion, D., Prange, T., Maume, B.F., Vauthrin, S., Blein, J.P., 2001. Fatty acids bind to the fungal elicitor cryptogein and compete with sterols. FEBS Lett. 489, 55–58.

Panabieres, F., Marais, A., Le Berre, J.Y., Penot, I., Fournier, D., Ricci, P., 1995. Characterization of a gene cluster of *Phytophthora cryptogea* which codes for elicitins, proteins inducing a hypersensitive-like response in tobacco. Mol. Plant Microbe Interact. 8, 996–1003.

Paris, R., Lamattina, L., 1999. *Phytophthora infestans* secretes extracellular proteases with necrosis inducing activity on potato. Eur. J. Plant Pathol. 105, 753–760.

Pemberton, C.L., Salmond, G.P.C., 2004. The Nep1-like proteins a growing family of microbial elicitors of plant necrosis. Mol. Plant Pathol. 5, 353–359.

Pieterse, C.M., Verbakel, H.M., Spaans, J.H., Davidse, L.C., Govers, F., 1993. Increased expression of the calmodulin gene of the late blight fungus *Phytophthora infestans* during pathogenesis on potato. Mol. Plant Microbe Interact. 6, 164–172.

Pieterse, C.M.J., Derksen, A.M.C.E., Folders, J., Govers, F., 1994a. Expression of the *Phytophthora infestans ipiB* and ipiO genes in planta and in vitro. Mol. Gen. Genet. 244, 269–277.

Pieterse, C.M.J., Van West, P., Verbakel, H.M., Brassé, P.W.H.M., Van Den Berg-Velthuis, G.C.M., Govers, F., 1994b. Structure and genomic organization of the *ipiB* and *ipiO* gene clusters of *Phytophthora infestans*. Gene 138, 67–77.

Ponchet, M., Panabieres, F., Milat, M.L., Mikes, V., Montillet, J.L., Suty, L., Triantaphylides, C., Tirilly, Y., Blein, J.P., 1999. Are elicitins cryptograms in plant–oomycete communications?. Cell. Mol. Life Sci. 56, 1020–1047.

Qutob, D., Hraber, P.T., Sobral, B.W., Gijzen, M., 2000. Comparative analysis of expressed sequences in *Phytophthora sojae*. Plant Physiol. 123, 243–254.

Qutob, D., Huitema, E., Gijzen, M., Kamoun, S., 2003. Variation in structure and activity among elicitins from *Phytophthora sojae*. Mol. Plant Pathol. 4, 119–124.

Qutob, D., Kamoun, S., Gijzen, M., 2002. Expression of a *Phytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy. Plant J. 32, 361–373.

Randall, T.A., Judelson, H.S., 1999. Construction of a bacterial artificial chromosome library of *Phytophthora infestans* and transformation of clones into *P. infestans*. Fungal Genet. Biol. 28, 160–170.

Randall, T.A., Dwyer, R.A., Huitema, E., Beyer, K., Cvitanich, C., Kelkar, H., Ah Fong, A.M.V., Gates, K., Roberts, S., Yatzkan, E., Gaffney, T., Law, M., Testa, A., Torto-Alalibo, T., Zhang, M., Zheng, L., Mueller, E., Windass, J., Binder, A., Birch, P.R.J., Gisi, U., Govers, F., Gow, N.A., Mauch, F., van West, P., Waugh, M.E., Yu, J., Boller, T., Kamoun, S., Lam, S.T., Judelson, H.J., 2005. Large-scale gene discovery in the oomycete *Phytophthora infestans* reveals likely components of phytopathogenicity shared with true fungi. Mol. Plant Microbe Interact. 18, 229–243.

Ricci, P., Panabières, F., Bonnet, P., Maia, N., Ponchet, M., Devergne, J.C., Marais, A., Cardin, L., Milat, M.L., Blein, J.P., 1993. Proteinaceous elicitors of plant defense responses. In: Fritig, B., Legrand, M. (Eds.), Mechanisms of Plant Defense Responses. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 121–135.

Rose, J.K., Ham, K.S., Darvill, A.G., Albersheim, P., 2002. Molecular cloning and characterization of glucanase inhibitor proteins: coevolution of a counterdefense mechanism by plant pathogens. Plant Cell 14, 1329–1345.

Shan, W., Hardham, A.R., 2004. Construction of a bacterial artificial chromosome library, determination of genome size, and characterization of an Hsp70 gene family in *Phytophthora nicotianae*. Fungal Genet. Biol. 41, 369–380.

Shan, W., Marshall, J.S., Hardham, A.R., 2004. Gene expression in germinated cysts of *Phytophthora nicotianae*. Mol. Plant Pathol. 5, 317–330.

Skalamera, D., Wasson, A.P., Hardham, A.R., 2004. Genes expressed in zoospores of *Phytophthora nicotianae*. Mol. Genet. Genomics 270, 549–557.

Soanes, D.M., Skinner, W., Keon, J., Hargreaves, J., Talbot, N.J., 2002. Genomics of phytopathogenic fungi and the development of bioinformatic resources. Mol. Plant Microbe Interact. 15, 421–427.

Tani, S., Yatzkan, E., Judelson, H.S., 2004. Multiple pathways regulate the induction of genes during zoosporogenesis in *Phytophthora infestans*. Mol. Plant Microbe Interact. 17, 330–337.

Taylor, J.L., Pedras, M.S., Morales, V.M., 1995. Horizontal transfer in the phytopathogenic fungal genus *Leptosphaeria* and host-range expansion. Trends Microbiol. 3, 202–206.

Thomas, S.W., Rasmussen, S.W., Glaring, M.A., Rouster, J.A., Christiansen, S.K., Oliver, R.P., 2001. Gene identification in the obligate fungal pathogen *Blumeria graminis* by expressed sequence tag analysis. Fungal Genet. Biol. 33, 195–211.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Tian, M., Huitema, E., Da Cunha, L., Torto-Alalibo, T., Kamoun, S., 2004. A Kazal-like extracellular serine protease inhibitor from *Phytophthora infestans* targets the tomato pathogenesis-related protease P69B. J. Biol. Chem. 279, 26370–26377.

Tyler, B.M., 2001. Genetics and genomics of the oomycete–host interface. Trends Genet. 17, 611–614.

Unkles, S.E., Logsdon Jr., J.M., Robison, K., Kinghorn, J.R., Duncan, J.M., 1997. The tigA gene is a transcriptional fusion of glycolytic genes encoding triose-phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase in oomycota. J. Bacteriol. 179, 6816–6823.

Van West, P., De Jong, A.J., Judelson, H.S., Emons, A.M.C., Govers, F., 1998. The *ipi* O gene of *Phytophthora infestans* is highly expressed in invading hyphae during infection. Fungal Genet. Biol. 23, 126–138.

van West, P., Kamoun, S., van't Klooster, J.W., Govers, F., 1999. Internuclear gene silencing in *Phytophthora infestans*. Mol. Cell 3, 339–348.

van't Klooster, J.W., van den Berg-Velthuis, G., van West, P., Govers, F., 2000. tef1, a *Phytophthora infestans* gene encoding translation elongation factor 1 alpha. Gene 249, 145–151.

Whisson, S.C., van der Lee, T., Bryan, G.J., Waugh, R., Govers, F., Birch, P.R., 2001. Physical mapping across an avirulence locus of *Phytophthora infestans* using a highly representative, large-insert bacterial artificial chromosome library. Mol. Genet. Genomics 266, 289–295.