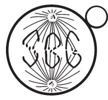
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Research Article

Phytophthora parasitica transcriptome, a new concept in the understanding of the citrus gummosis

Daniel D. Rosa[1], Magnólia A. Campos[2], Maria Luisa P.N. Targon[3] and Alessandra A. Souza[3]

[1]*Setor de Defesa Fitossanitária, Departamento de Produção Vegetal, Faculdade de Ciências Agronômicas, Universidade Estadual Paulista, Botucatu, SP, Brazil.*
[2]*Departamento de Biologia, Universidade Federal de Lavras, Lavras, MG, Brazil.*
[3]*Centro APTA Citros Sylvio Moreira, Instituto Agronômico de Campinas, Cordeirópolis, SP, Brazil.*

Abstract

Due to the economic importance of gummosis disease for the citriculture, studies on *P. parasitica-Citrus* interaction comprise a significant part in the Brazilian Citrus genome data bank (CitEST). Among them, two cDNA libraries constructed from two different growth conditions of the *P. parasitica* pathogen are included which has generated the PP/CitEST database (CitEST - Center APTA Citros Sylvio Moreira/IAC- Millennium Institute). Through this genomic approach and clustering analyses the following has been observed: out of a total of 13,285 available in the *Phytophthora parasitica* database, a group of 4,567 clusters was formed, comprising 2,649 singlets and 1,918 contigs. Out of a total of 4,567 possible genes, only 2,651 clusters were categorized; among them, only 4.3% shared sequence similarities with pathogenicity factors and defense. Some of these possible genes (103) corresponding to 421 ESTs, were characterized by phylogenetic analysis and discussed. A comparison made with the COGEME database has shown homology which may be part of an evolutionary pathogenicity pathway present in *Phytophthora* and also in other fungi. Many of the genes which were identified here, which may encode proteins associated to mechanisms of citrus gummosis pathogenicity, represent only one facet of the pathogen-host *Phytophthora* - Citrus interaction.

Key words: Citrus disease, elicitins, plant pathogen, gene expression profiles.

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Introduction

In the evolutionary history of eukaryotes, oomycetes are the only organisms with a history of self-sufficiency, due mainly to the genetic distinction and the biochemical mechanisms of interactions with their hosts (Kamoun *et al.*, 1999). Throughout the world, there are many species of *Phytophthora* described as pathogenic to plants, and are present in over 200 botanical families. The *Phytophthora* complex in citrus crops is a very important disease that belongs to this group (Erwin and Ribeiro, 1996). *Phytophthora parasitica* Dastur (= *Phytophthora nicotianae* Breda de Haan var.p*arasitica* (Dast.) Waterh.) is an oomycete that belongs to the kingdom *Stremenopiles*, which comprises a diverse group of organisms which has recently been consolidated as a result of mitochondrial analysis and ribosomal DNA sequences (Gunderson *et al.*, 1987; Förster *et al.*, 1990; Alexopoulos *et al.*, 1996). *P. parasitica* is the agent which causes brown rot, foot rot, gummosis and root rot of Citrus species, and the common diseases at high temperatures, above 35 °C. It was first reported in 1832 by the Arab botanist Ibn el Awan (Fawcett, 1936). The first to describe the Citrus gummosis in the Brazil was Averna-Saccá (1917), later identified as *P. parasitica* by Müller (1933). The *P. parasitica* attack on citrus crops led to drastic losses in the field, since the varieties possessing good agronomical characteristics have a low resistance to gummosis (Siviero *et al.*, 2002).

Despite the investigations related to the biological development of the citrus gummosis disease, little is known about the pathogenic determinants of *P. parasitica*. Molecular studies on pathogenicity and virulence of oomycetes are relatively rare when compared to those on plant pathogenic fungi, bacteria, and viruses, mainly because they differ in their cell composition, reproduction cycle and also in the genetic composition (Judelson, 1997). In this context, the use of expressed sequence tag (EST) analysis represents an approach that might contribute to the understanding of the basic biology of *P. parasitica,* through the production of a large volume of sequence information, not available

Send correspondence to Daniel Dias Rosa. Setor de Defesa Fitossanitária, Departamento de Produção Vegetal, Faculdade de Ciências Agronômicas, Universidade Estadual Paulista, Fazenda Lageado, Rua José Barbosa de Barros 1780, Caixa Postal 237, 18610-307 Botucatu, SP, Brazil. E-mail: danieldr@hotmail.com.

previously. The information thus generated may also assist to establish a database to facilitate further research on *P. parasitica* and other related organisms, like *P. sojae* (Waugh *et al.*, 2000). The understanding of the genetics and physiology of *P. parasitica* might lead to the development of control techniques and also provide information for the elucidation of the pathogen during the interaction with citrus hosts.

Due to the economic importance of the gummosis disease in citriculture, studies on *P. parasitica-Citrus* interaction were shown to play a significant role in the Brazilian Citrus genome data bank (CitEST). Among them, two cDNA libraries constructed from two different growth conditions of the *P. parasitica* pathogen are included, which generated the PP/CitEST database. This genomic approach is reported in this paper with a number of identified EST characterizations, possibly involved in *P. parasitica*-host interaction, in the PP/CitEST database.

Materials and Methods

Culture, growth conditions, library construction and sequencing

The isolation of *Phytophthora parasitica*-IAC 01/95 was cultivated in a medium carrot liquid (50g of triturated cooked carrot, 10 g of the dextrose and distilled water to complete 1liter) for 7 days at 28 °C. Mycelium mass was then cultivated 40 times under the same conditions. The mycelium mass was then filtered through a paper filter and used for RNA extraction. In an attempt to activate the pathogenicity, *P. parasitica*-IAC 01/95 was also inoculated in oranges, recovered from symptoms and cultivated in carrot medium under the same conditions. In the same way, mycelium mass was filtered through a paper filter and used for RNA extraction. The total RNA was extracted by using Trizol reagent (Life Technologies, Gaithersburg, MD) (10 mL/g of mycelium) and the poly(A+) RNA was isolated from 1 mg of the total RNA through the polyATtract mRNA Isolation System (Promega Corporation, Madison, WI). The method is based on a biotinylated oligo(dT) primer to hybridize in solution to the 3' poly(A) region of the mRNA. The hybrids were retrieved and washed at high stringency using streptavidin coupled with paramagnetic particles and a magnetic separation stand. The mRNA was eluted from the solid phase by adding RNAse-free deionized water.

Two libraries were constructed by using the SuperScript Plasmid System with Gateway Technology for cDNA Synthesis and Cloning (Life Technologies, Gaithersburg, MD). Complementary DNA (cDNA) was formed from mRNA using a primer consisting of a poly (dT) sequence with a *Not* I restriction site. *Sal* I adapters were connected to the blunt-ended cDNA fragments followed by a *Not* I digestion. The cDNA fragments were fractionated by Sephacryl S cDNA Size Fractionation Columns (Life Technologies, Gaithersburg, MD) and cloned into the *Not* I-*Sal* I restriction site of the pSPORT 1 vector. The pSPORT 1 vector (Life Technologies, Gaithersburg, MD) carries an ampicillin-resistance gene necessary for clone selection. The cloned cDNA fragments can be amplified by one of the following pairs of primer vector: SP6 promoter and T7 promoter or M13/pUC forward and M13/pUC reverse. The connected cDNA fragments were transformed into *E. coli* DH5α bacteria through the ice-cold $RbCl/CaCl_2$ solution method (Hanahan, 1983).

The colonies were inoculated into 200 mL of CG medium liquid containing 8% of (v/v) glycerol and 100 mg/mL of ampicillin in 96-well-microtiter plates, incubated overnight at 37 °C and stored at -80 °C. The sequence reactions were prepared according to the instructions of Applied Biosystems for the DNA sequencing Kit Big Dye Terminator cycle sequence ready reaction. The sequence was accomplished in the ABI 3700-Perkin Elmer.

Trimming and assembly of *Phytophthora parasitica* ESTs into sequence clusters

P. parasitica expressed sequence tags (ESTs) were obtained from two cDNA libraries formed by two different growth conditions, and grouped in the PP/CitEST database (Center APTA Citros Sylvio Moreira/IAC- Millennium Institute). The clustering of ESTs from PP/CitEST was performed in order to estimate the level of redundancy in the libraries. Clustering was the most critical step of the sequence analyses due to its importance in the reduction in the amount of sequence data. This reduces and organizes the reads into a less redundant set. In an attempt to minimize artifacts, the readings were trimmed prior to clustering. Through the cross-match program, the trimming procedure was initiated with vector masking, followed by removal of poly-A signals, vector and adapter regions. A quality trimmer was also applied, removing bases from the sequence ends, one by one, until there were at least 12 bases with quality phred above 15, in a window of 20 bases at the end.

Trimmed readings were assembled using the phrap program for the PhredPhrap package (Ewing *et al.*, 1998), with quality and stringent arguments (-penalty -15 -bandwidth 14 -minscore 100 -shatter greedy). The last assembly was accomplished using phrap program and included all trimmed readings. After the trimming, clustering of the *P. parasitica* 13,285 readings was performed using the CAP3 assembler (Huang and Madan, 1999) and its qualities. After clustering, all clusters were analyzed using the BLAST program and all information was stored in the database.

Database analysis

All the sequences analyzed were obtained from the *P. parasitica* CitEST database. Sequence analyses were performed using the BLAST program (Altschul *et al.*, 1997) facilities. The protein sequences were preferably analyzed

through the BLASTX, version 2.2.10, in the NR database and nucleotide sequences were analyzed through the BLASTN in the EST database, except human ESTs. The results were filtered, restricting the hits to an E-value < 1e^{-05}.

The PP/CitEST database was categorized using a protein database with known functions and defined by 40,000 proteins which had been selected from databases with examples of each category. The MIPS *Arabidopsis thaliana* database, Clusters Orthologous Groups-functional annotation, and EGAD cellular roles are included. Categorization was achieved through the automatic method followed by the construction of a database containing the proteins selected from public databases. Then a BLAST search was performed in contrast to this database using *P. parasitica* ESTs clusters as input. A cluster was considered to be categorized when matched with a sample protein of that category with an E-value = 1e^{-05} and coverage = 60%.

The comparative genomic analysis was performed between the *P. parasitica* database and EST collections COGEME, which comprises 59,765 ESTs obtained from thirteen species of plant pathogenic fungi, two species of phytopathogenic oomycete and three species of saprophytic fungi (Soanes and Talbot, 2006). For a comparative analysis between *P. parasitica* database and *Saccharomyces cerevisiae* genome, the blastx program was used to compare the databases and also used to categorize pathogenicity-related genes.

For the phylogenetic analysis, multiple alignments between PP sequences and homologies were performed using ClustalX 1.83 (Thompson *et al.*, 1997) on a workstation running Linux (Mandrake 10) with the ToolKit 6.1 (NCBI). Phylogenetic dendrograms were obtained by neighbor-joining analysis using the p-distance method and confidence levels assigned at various nodes determined after 10,000 replications or permutation also present in the MEGA (Molecular Evolutionary Genetics Analysis) software, version 2 (Kumar *et al.*, 2004) running Windows 2000.

Results and Discussion

The distribution of PP ESTs into clusters and functional annotation

A genomic approach was used to discover novel genes in *P. parasitica* that infect citrus. Out of a total of 15,942 clones sequenced from PP libraries, the 13,285 which expressed sequence tags were grouped into 4,567 clusters, comprising 2,649 singlets and 1,918 contigs, with a novelty of 58.0% and a success rate of 83.3%. Then the clusters were submitted to categorization. Among them only 2,651 clusters were categorized. As a result, above 20% were putatively involved with the protein metabolism. These ESTs were linked with the ribosomal proteins and also with other factors which are required for proteins synthesis. Also highly expressed were: around 15% related with carbohydrate metabolism and bioenergetics, 9% with amino acid metabolism, dynamic cell and cellular communication, and 4.5% with the metabolism related to the defense system, stress and virulence (Figure 1).

P. parasitica ESTs were distributed between known proteins or hypothetical proteins based on deduced amino acid sequences homologies. It was discovered during the annotation process that 1,915 (41.95%) of all of ESTs did not share sequence similarity with any sequence from the GenBank non-redundant database. This relative portion is consistent with reports of other fungus EST databases. It also depends on other points of the organism such as: the experimental design and the developmental stage (Kamoun *et al.*, 1999). On the other hand, clusters with E-value < 1e^{-5} added a total of 2,651 clusters (58.05%), indicating a satisfactory value of known sequences. This percentage highlights that less than half of the *P. parasitica* transcriptome is currently unknown. In addition, clusters with full homology with other sequences were spotted, 91 ESTs (0.2%) and 2,641 ESTs (57.83%) with E-values that varied from 1e^{-5} to 1e^{-200}. These clusters represent probable genes (Figure 2).

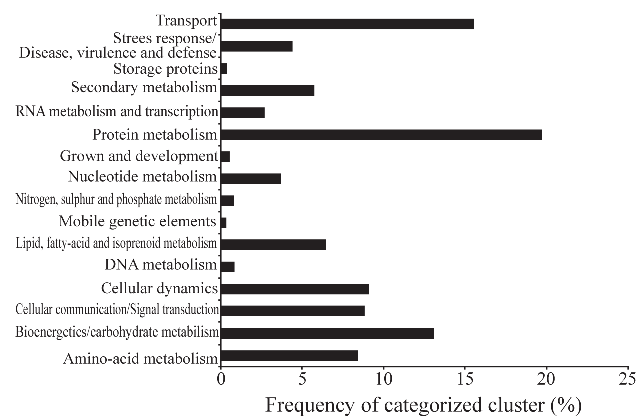

Figure 1 - Distribution of categorized clusters according to putative biological function defined by MIPS. Protein matches resulting from BLASTX searches were assigned to one of seven functional categories for comparisons involving life cycle. Percentage of frequency of clusters was shown in each category from a total set of categorized PP clusters.

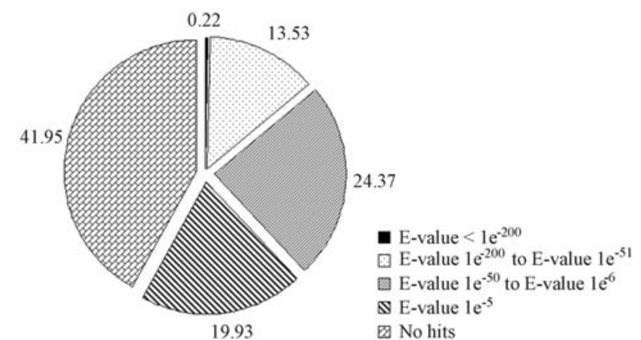

Figure 2 - Frequency of the distribution, in percentages, of *P. parasitica*/CitEST ESTs, based on E-value from BLASTX results.

Comparison of PP/CitEST database with expressed sequence tag collections

The clusters of *P. parasitica*/CitEST database were used to search for homologies in the COGEME EST database, which consist of 59,765 ESTs from 15 species of phytopathogens and three species of saprophytic fungi. Based on the number of unique sequences found in each species present in the COGEME database, it was possible to individually identify the number and the unique percentages with homology to *P. parasitica* clusters, as well as the number of unique putatively involved in the pathogenicity functions which matched the PP clusters (Table 1).

As a result, it was observed that about 54 unique sequences from *S. cerevisiae* genome have homologies in PP/CitEST. Comparative analyses with phytopathogen EST database led to the discovery that about 1.2% of unigenes from *Blumeria graminis* ESTs have homologies in PP/CitEST clusters. Similarly, about 56.51% and 23.84% of *P. infestans* and *P. sojae* unigenes, respectively, have homologies with PP clusters, among which 54 and 68 homologies are putatively involved with the pathogenicity functions (Table 1).

Through the comparison with saprophytic fungi ESTs, it was discovered that about 2.43% and 6.66% from *Emericella nidulas* and *Aspergillus niger*, respectively, have homologies with PP ESTs. Among the *E. nidulans* homologies, 21 unigenes were discovered putatively involved with the pathogenicity functions, whereas in the *A. niger* nine were found (Table 1).

Since the pathogenicity system in a parasite is never single gene-dependent, these data indicate that many genes putatively involved with tpathogenicity functions share sequence similarities among themselves, and they may have a common ancestor. Unlike some fungi, no pathogenicity unigenes were found with homologies in *P. parasitica*. Examples of this are *Sclerotinia sclerotiorum* and *Leptosphaeria maculans* with only a few sequences analyzed and *S. cerevisiae* which is not a plant pathogen.

Table 1 - Comparison of *P. parasitica* clusters with COGEME database.

Organism	Total	N_u	%	N_p
Aspergillus niger	1,577	105	6.66	9
Blumeria graminis	3,253	39	1.20	4
Botryotinia fuckeliana	2,901	123	4.24	11
Cladosporium fulvum	513	47	9.16	6
Colletotrichum trifolii	550	42	7.64	5
Cryphonectria parasitica	2,185	322	14.74	21
Emericella nidulans	4,805	117	2.43	7
Fusarium sporotrichioides	3,448	111	3.22	10
Gibberella zeae	4,688	511	10.90	19
Leptosphaeria maculans	118	22	18.64	0
Magnaporthe grisea	12,465	275	2.21	15
Mycosphaerella graminicola	2,926	440	15.04	18
Neurospora crassa	5,142	186	3.62	9
Phytophthora infestans	1,414	799	56.51	54
Phytophthora sojae	7,311	1,743	23.84	68
Sclerotinia sclerotiorum	738	53	7.18	0
Ustilago maydis	4,276	580	13.56	21
Verticillium dahliae	1,455	141	9.69	11
Saccharomyces cerevisiae	24,129	54	0.22	0

Total: Total of unigenes; N_u: number of unigenes homology; %: % of unigenes homology; N_p: number of pathogenic unigenes homology.

Genes in *P. parasitica* involved with pathogenicity, host colonization process and defense

As an approach to studying genes possibly involved with the *P. parasitica* colonization process, a number of clusters coding for wall cell degradation proteins, necrosis-inducer proteins, elicitins, among others were identified in the analysis. The breakdown of physical barriers during an infection process, penetration process and host tissue colonization involve the secretion of a vast range of degradative enzymes. During the process, several ESTs with significant similarity to degradative enzymes such as amidase, cutinase protein, endo- and exoglucanases, and chitinases have been identified (Table 2).

The degradation of the host cell wall is one of the first steps in disease. The process needs many enzymes, such as phospholipases, ß-glucosidase/ß-xylosidase, exo-1, 3-ß-glucanases, endo-1, 3-ß-glucanase, and endopolygalacturonases (endo-PGs) (Kamoun *et al.*, 1999). Clusters putatively encoded by all of these enzymes were found in the *P. parasitica*/CitEST database (Table 2). In addition, two clusters were found sharing sequence similarity with pectin lyase F isolated from *A. niger* and *A. nidulans* (Table 2). Pectin lyase F has been described in many plant-pathogenic bacteria and fungi as an enzyme used to break into the host tissues (Chen *et al.*, 1998). Moreover, pectolytic enzymes are essential in the decay of dead plant material through nonpathogenic microorganisms and thus assist carbon compound recycling in the biosphere (Chen *et al.*, 1998). The low frequency of these genes in the PP/CitEST database indicates that *P. parasitica* might not be a pathogenic fungi with great affinity to pectin degradation. This might be related to the reduced attack of *P. parasitica* in citrus fruit.

The important gene that was found in PP/CitEST databases is the CBEL (cellulose binding elicitor lectin) gene (Table 2), with four clusters in the database. This gene encodes a protein that binds to cellulose *in vitro*, suggesting that CBEL participates in the adhesion of *Phytophthora* to cellulosic substrates (Tucker and Talbot, 2001). Adherence to solid surfaces is a common feature in both saprophytic and parasitic microorganisms. In fungi and oomycetes, adherence is mediated by secreted adhesins which are part of the cell wall or it might be physically associated to it (Gaulin *et al.*, 2002).

Table 2 - *Phytophtora parasitica* ESTs and their known or predicted function, based on BLASTX results.

Cluster	Reads	Product	Organism	Score	E-value	Identity (%)
Metabolite resistance						
Contig936	6	heat shock protein Hsp88	*Neurospora crassa*	203	$9e^{-51}$	32
Contig553	4	heat shock protein Hsp80	*Oryza sativa*	476	$1e^{-133}$	95
Contig1866	9	heat shock protein Hsp90	*Achlya ambisexualis*	369	$1e^{-101}$	90
Contig1851	6	heat shock protein Hsp90	*Achlya ambisexualis*	772	0.0	92
Contig744	3	heat shock protein 70	*Phytophthora nicotianae*	263	$8e^{-69}$	86
Contig906	9	heat shock protein 70	*Botryllus schlosseri*	194	$2e^{-48}$	45
Contig451	3	heat shock protein 70	*Phytophthora nicotianae*	879	0.0	95
Contig1498	3	heat shock protein 70	*Phytophthora nicotianae*	1,166	0.0	91
PP14-C7-801-101-C08-CT.F	1	heat shock protein	*Arabidopsis thaliana*	253	$3e^{-66}$	57
PP14-C7-801-068-D08-CT.F	1	heat shock protein	*Shigella flexneri*	70	$3e^{-11}$	39
PP14-C7-801-085-G11-CT.F	1	heat shock transcription factor 2, putative	*Cryptococcus neo.* var. *neoformans*	88	$3e-^{16}$	40
Contig16	7	Mn superoxide dismutase	*Chlamydomonas reinhardtii*	253	$5e^{-66}$	58
PP14-C7-802-020-E08-CT.F	1	superoxide dismutase 2, mitochondrial	*Gallus gallus*	207	$2e^{-60}$	62
Contig1524	9	Superoxide dismutase	*Methylobacillus flagellatus*	148	$7e^{-35}$	78
Contig1351	2	glutathione peroxidase	*Sorghum bicolor*	181	$5e^{-44}$	53
PP14-C7-802-017-G12-CT.F	1	glutathione peroxidase	*Phytophthora infestans*	172	$1e^{-41}$	52
Contig757	6	glutamine synthetase	*Phytophthora infestans*	738	0.0	98
PP14-C7-802-138-E12-CT.F	1	glutathione reductase	*Bordetella parapertussis*	104	$3e^{-21}$	83
PP14-C7-801-079-H03-CT.F	1	glutathione S-transferase GST 23	*Glycine max*	84	$4e^{-15}$	37
PP14-C7-802-115-F05-CT.F	1	putative glutathione S-transferase OsGSTT1	*Oryza sativa*	103	$6e^{-21}$	33
Contig1155	8	putative glutathione S-transferase OsGSTT1	*Oryza sativa*	99	$8e^{-20}$	33
Contig9	9	glutathione s-transferase	*Xenopus laevis*	111	$2e^{-23}$	34
PP14-C7-801-102-B03-CT.F	1	cystein proteinase	*Citrus sinensis*	314	$2e^{-84}$	100
Contig710	11	cysteine protease	*Daucus carota*	280	$9e^{-74}$	47
Contig351	2	thioredoxin peroxidase	*Phytophthora infestans*	278	$6e^{-74}$	71
Contig359	5	acidic chitinase	*Phytophthora infestans*	273	$5e^{-72}$	69
Contig964	6	thioredoxin peroxidase	*Phytophthora infestans*	412	$1e^{-114}$	98
Contig1816	9	L-carnitine dehydrogenase	*Acinetobacter* sp.	226	$2e^{-63}$	77
PP14-C7-802-105-B02-CT.F	1	ornithine carbamoyl-transferase	*Bordetella parapertussis*	210	$5e^{-53}$	83
PP14-C7-802-095-F08-CT.F	1	ornithine cyclodeaminase	*Bordetella pertussis*	114	$2e^{-24}$	71
PP14-C7-801-079-F03-CT.F	1	ornithine decarboxylase	*Mucor circinelloides* fsp. *lusitanicus*	96	$1e^{-18}$	38
Contig1121	9	pleiotropic drug resistance transporter	*Phytophthora sojae*	356	$1e^{-102}$	91
Contig1569	6	pleiotropic drug resistance transporter	*Phytophthora sojae*	141	$1e^{-32}$	42
PP14-C7-801-077-C07-CT.F	1	pleiotropic drug resistance transporter	*Phytophthora sojae*	223	$5e^{-57}$	56
Contig353	4	multidrug resistance-associated protein 2	*Oryctolagus cuniculus*	216	$5e^{-55}$	46
PP14-C7-801-028-F11-CT.F	1	putative caffeine-induced death protein 1	*Oryza sativa*	70	$7e^{-11}$	39
Contig77	4	polyketide synthase	*Clostridium thermocellum*	143	$5e^{-33}$	34

Table 2 (cont.)

Cluster	Reads	Product	Organism	Score	E-value	Identity (%)
Contig735	7	pepsinogen C	*Monodelphis domestica*	57	$6e^{-07}$	36
PP14-C7-801-042-F12-CT.F 6	1	elicitin protein	*Phytophthora parasitica*	180	$7e^{-45}$	100
Contig1181	2	elicitin protein	*Phytophthora sojae*	134	$2e^{-30}$	57
Contig739	11	elicitin protein	*Phytophthora sojae*	55	$1e^{-06}$	35
Contig133	13	elicitin gamma-megaspermin protein	*Phytophthora megasperma*	210	$3e^{-53}$	86
Contig888	9	elicitin INF2A protein	*Phytophthora infestans*	201	$1e^{-50}$	85
Contig987	6	elicitin INF7 protein	*Phytophthora infestans*	113	$4e^{-24}$	56
PP14-C7-801-101-H07-CT.F	1	wound-inducible basic protein	*Phaseolus vulgaris*	88	$1e^{-16}$	85
Contig1413	6	necrosis-inducing-like protein	*Phytophthora sojae*	107	$2e^{-22}$	32
PP14-C7-802-125-A07-CT.F	1	crinkling and necrosis-inducing protein CRN2	*Phytophthora infestans*	66	$1e^{-09}$	34
PP14-C7-802-082-A05-CT.F	1	crinkling and necrosis-inducing protein CRN2	*Phytophthora infestans*	100	$9e^{-20}$	38
Contig1550	4	crinkling and necrosis-inducing protein CRN1	*Phytophthora infestans*	90	$4e^{-17}$	47
Contig1422	3	crinkling and necrosis-inducing protein CRN1	*Phytophthora infestans*	72	$4e^{-11}$	30
Hydrolytic enzymes						
Contig1603	3	acidic chitinase	*Phytophthora infestans*	387	$1e^{-106}$	81
PP14-C7-801-048-C04-CT.F	1	acidic chitinase	*Phytophthora infestans*	238	$1e^{-61}$	66
Contig1114	3	amidases related to nicotinamidase	*Burkholderia cepacia*	177	$2e^{-43}$	59
PP14-C7-801-047-A01-CT.F	1	amidases related to nicotinamidase	*Burkholderia cepacia R1808*	109	$1e^{-22}$	49
PP14-C7-801-032-F02-CT.F	1	beta(1-3)endoglucanase	*Aspergillus fumigatus*	80	$5e^{-14}$	30
PP14-C7-802-111-C10-CT.F	1	beta(1-3)endoglucanase	*Aspergillus fumigatus*	61	$1e^{-08}$	30
Contig201	6	beta-glucosidase	*Phytophthora sojae*	104	$1e^{-21}$	44
PP14-C7-802-030-F10-CT.F	1	beta-glucosidase	*Bacillus clausii*	62	$2e^{-08}$	34
PP14-C7-801-042-E05-CT.F	1	beta-glucosidase precursor	*Tenebrio molitor*	77	$5e^{-13}$	46
Contig659	6	beta-glucosidase/xylosidase	*Phytophthora infestans*	213	$9e^{-60}$	60
Contig618	9	CBEL protein	*Phytophthora parasitica*	505	$1e^{-142}$	89
Contig1583	11	CBEL protein	*Phytophthora parasitica*	448	$1e^{-125}$	87
PP14-C7-801-040-C07-CT.F	1	CBEL protein, formerly GP34	*Phytophthora parasitica*	224	$3e^{-57}$	45
Contig381	2	CBEL protein, formerly GP34	*Phytophthora parasitica*	76	$7e^{-13}$	27
Contig543	6	cutinase (CutB)	*Phytophthora brassicae*	402	$8e^{-12}$	83
Contig373	3	endo alpha-1,4 polygalactosaminidase	*Idiomarina loihiensis*	89	$3e^{-34}$	43
Contig1609	4	endo-1,3-beta-glucanase	*Phytophthora infestans*	388	$1e^{-127}$	79
PP14-C7-801-016-D05-CT.F	1	endo-1,3-beta-glucanase	*Phytophthora infestans*	140	$3e^{-32}$	41
PP14-C7-801-016-D05-CT.F	1	endo-1,3-beta-glucanase	*Phytophthora infestans*	140	$3e^{-32}$	41
Contig1624	8	endo-1,4-beta-glucanase	*Pyrococcus horikoshii*	127	$7e^{-28}$	26
Contig1622	9	endo-1,4-beta-glucanase	*Pyrococcus horikoshii*	82	$3e^{-14}$	25
PP14-C7-802-135-B12-CT.F	1	endoglucanase	*Clostridium thermocellum*	62	$1e^{-08}$	39
Contig383	4	esterase/lipase	*Burkholderia cepacia R18194*	62	$5e^{-09}$	72

Table 2 (cont.)

Cluster	Reads	Product	Organism	Score	E-value	Identity (%)
Contig1330	3	exo-beta-1,3-glucanase	*Magnetospirillum magnetotacticum*	79	$3e^{-13}$	25
Contig764	2	exopolyphosphatase	*Bordetella parapertussis*	230	$6e^{-60}$	87
PP14-C7-801-079-C06-CT.F	1	exopolysaccharide biosynthesis protein	*Mesorhizobium loti*	116	$9e\text{-}^{25}$	38
PP14-C7-802-098-H03-CT.F	1	lipase, putative	*Paramecium tetraurelia*	89	$1e^{-16}$	32
PP14-C7-801-105-A03-CT.F	1	pectine lyase F	*Aspergillus niger*	492	$4e^{-48}$	44
Contig420	6	pectine lyase F	*Aspergillus nidulans*	135	$8e^{-31}$	42
Contig1852	6	putative 1,3-beta-glucan synthase	*Oryza sativa*	325	$2e^{-87}$	56
PP14-C7-802-076-B03-CT.F	1	putative beta-1,3-glucan synthase	*Nicotiana alata*	171	$2e^{-41}$	38
Contig585	7	putative endo-1,3;1,4-beta-glucanase	*Phytophthora infestans*	58	$5e^{-08}$	96
PP14-C7-801-064-A02-CT.F	1	putative esterase	*Oryza sativa*	117	$3e^{-25}$	38
PP14-C7-801-007-E03-CT.F	1	putative exo-1,3-beta-glucanase	*Phytophthora infestans*	345	$5e^{-95}$	74
PP14-C7-802-074-B12-CT.F	1	putative exo-1,3-beta-glucanase	*Phytophthora infestans*	325	$3e^{-88}$	98
Contig1478	16	putative exo-1,3-beta-glucanase	*Phytophthora infestans*	270	$3e^{-71}$	65
Contig258	23	putative exo-1,3-beta-glucanase	*Phytophthora infestans*	919	0.0	88
Contig258	3	putative exo-1,3-beta-glucanase	*Phytophthora infestans*	919	0.0	88
PP14-C7-801-023-D06-CT.F	1	putative exo-1,3-beta-glucanase	*Phytophthora infestans*	100	$3e^{-20}$	40
PP14-C7-801-013-E08-CT.F	1	related to amidase	*Neurospora crassa*	81	$4e^{-14}$	39
Contig1326	3	urea amidolyase	*Xanthomonas axonopodis* pv. *citri*	50	$1e^{-05}$	44
Others						
Contig1574	8	CTR1-like kinase kinase kinase	*Oryza sativa*	196	$1e^{-48}$	43
Contig946	5	CTR1-like protein kinase	*Oryza sativa*	135	$2e^{-30}$	29
Contig336	3	CTR1-like kinase kinase kinase	*Brassica juncea*	79	$3e^{-13}$	35
Contig810	5	CTR1-like kinase kinase kinase	*Brassica juncea*	97	$6e^{-19}$	30
Contig1781	8	CTR1-like kinase kinase kinase	*Oryza sativa*	87	$3e^{-16}$	38
PP14-C7-801-036-D03-CT.F	1	MAPK-related kinase	*Tetrahymena thermophila*	129	$1e^{-28}$	39
PP14-C7-802-140-G08-CT.F	1	MAP kinase 4	*Zea mays*	84	$3e^{-15}$	59
PP14-C7-802-010-E02-CT.F	1	MAP kinase kinase	*Yarrowia lipolytica*	77	$1e^{-13}$	43
PP14-C7-801-066-F02-CT.F	1	MAP3K beta 1 protein kinase	*Brassica napus*	82	$2e^{-14}$	37
Contig1084	5	cyst germination specific acidic protein	*Phytophthora infestans*	92	$3e^{-17}$	42
PP14-C7-801-090-C09-CT.F	1	ascus development protein 1	*Neurospora crassa*	100	$1e^{-21}$	33
PP14-C7-801-099-F07-CT.F	1	ethylene-inducible CTR1-like protein kinase	*Lycopersicon esculentum*	87	$7e^{-16}$	34

Six putative genes which belonged to the complex family of elicitin-like proteins were also found in the PP/CitEST database (Table 2). Elicitin-like genes encode putative extra cellular proteins which share the 98 amino-acid elicitin domains, which correspond to the mature INF1 elicitin. Five *inf* genes (*inf*2A, *inf*2B, *inf*5, *inf*6, and *inf*7) encode predicted proteins with a C-terminal domain in addition to the N-terminal elicitin domain. The elicitins genes are classified into four classes, class IA, class IB, class II and class III, based on peptide signal sequence (Baillieul *et al.*, 2003). These proteins may form a `lollipop on a stick' structure, formed by disulfide bonds in cysteine residues (Figure 3), on which an O-glycosylated domain forms an extended rod that holds the protein to the cell wall causing the extra cellular N-terminal domain to be left exposed on the cell surface. Therefore, these atypical INF proteins may be associated with the surface or cell wall glycoprotein that interacts with plant cells during infection (Kamoun *et al.*, 1997).

Elicitins are extracellular proteins with still unknown functions, but it has already been proven that they induce a hypersensitive reply in the host, as already proven in tobacco by Qutob *et al.* (2003). It is believed that elicitins are lipid binding-related proteins and that they present functions of phospholipid; thus they are able to cross cell membranes, by an interaction with ergosterol in residues present in amino acid sequences (Kamoun *et al.*, 1997). Other studies suggest that multiple layers of INF elicitin recognition and late blight resistance occur in *Nicotiana tabacum* (Baker *et al.*, 1997).

Experiments with elicitins in tobacco have shown that elicitins are either proteins which cause hypersensitive responses in the plant or they are virulence factors. Such molecules are typically secreted into the intercellular interface between the pathogen and the plant, or they are taken up into the host cell to reach their cellular target. Interactions between plants and microbial pathogens involve complex signal exchange on the plant surface and in the intercellular space interface. The elicitins are considered only one signal in this complex communication (Parniske, 2000; Hahn and Mendgen, 2001).

Phylogenetic analysis of the six PP/CitEST elicitins and homologies has grouped the sequences into four clades, except for the outgroup (Figure 4). One PP elicitin cluster (PP14-C7-801-042-F12-CT.F) was grouped in the clade of the class IA which has 75.4% of homology with *P. cinnamomi.* A second clade consisting of two PP clusters (Contig 987, Contig 739) similar to elicitins class IB was close to *P. megasperma*, with 82% and 79% sequence identity, respectively (Figure 4). In a third clade, one PP cluster (contig 133) grouped with class II *P. cinnamomi* elicitins, with 75% sequence similarity. In the last clade, two PP clusters, consisting of contig 888 and contig 1181, were grouped together with the class III *P. infestans* and *P. brassicae* elicitins, which share 78% and 84% similarity,

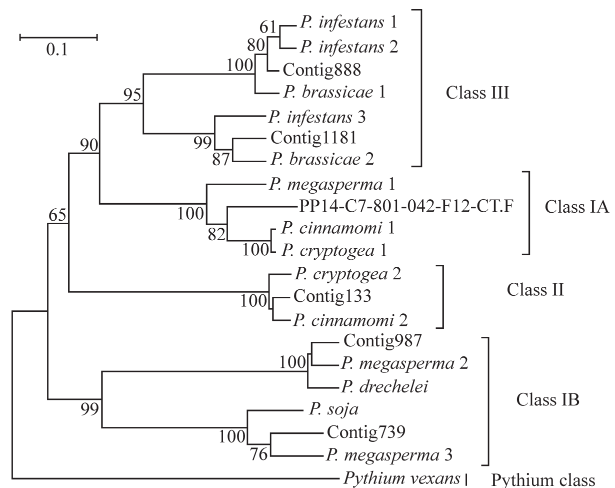

Figure 4 - Phylogenetic dendrogram of elicitin amino acid sequences. Multiple alignment of selected elicitin amino acid sequences from PP/CitEST and homology was performed in Clustal X. Dendrogram was constructed and visualized by Mega programs using neighbor-joining method. The following sequences were obtained from EMBL GeneBank: *Phytophthora megasperma* 1 (AJ493606), 2 (AJ493607), 3 (gi|544239), *P. cryptogea* 1 (gi|599947), 2 (gi|21466142), *P cinnamomi* 1 (gi|4469292), 2 (gi|4469290), *P. dreschslei* (gi|544238), *P. sojae* (gi|27922903), *P. infestans* 1 (gi|51832281), 2 (gi|2707621), 3 (gi|16225870), *P. brassicae* 1 (gi|29838396), 2 (gi|29838400), *Pythium vexans* (gi|945184). There were six clusters from *Phytophthora parasitica*/CitEST: one sharing sequence similarity with elicitin protein class IA (PP14-C7-801-042-F12-CT.F), two clusters with elicitins class IB (Contig 987, Contig 739), one cluster with elicitin class II (Contig 133) and two clusters with elicitins class III (Contig 888, Contig 1181). The numbers indicate percentages supporting the branches by 10,000 bootstrap replicates (bar corresponds to 0.1 substitutions per site).

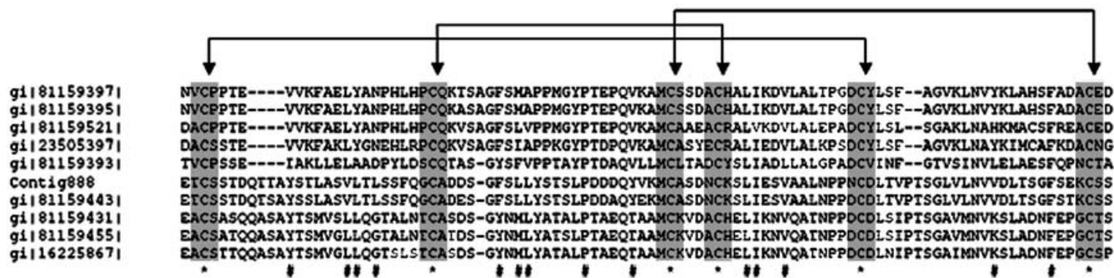

Figure 3 - Multiple alignment of *Phytophthora* elicitin amino acid sequences and homologies. Cysteine residues involved in disulfide bonds are showed in shaded box. The three conserved disulfide bonds are represented by arrows. Residues that putatively interact with ergosterol are indicated by #.

respectively (Figure 4). Huitema *et al.* (2005) demonstrated that elicitins class III induced hypersensitive response activity that led to cell death and showed a resistance character. The class I elicitins are known by their interaction with non-host and they are probably used by *P. parasitica* to survive in the saprophytic form.

Necrosis-inducer proteins are related to the necrotic responses in plant. Many genes related to this induction are characterized as *avr* genes (MacGregor *et al.*, 2002). There are countless Avr loci, but there are only few *avr* gene sequences known. *P. sojae* has more than 13 *avr* genes, but they have not been isolated yet, although a recent study has shown that Avr loci may be successfully identified by positional cloning methods (Tyler *et al.*, 1995). This has led to both the isolation of the Avr1a and Avr1b/Avr1k loci, and also the identification of the Avr1b protein (Tyler *et al.*, 1995; MacGregor *et al.*, 2002). No work has reported that these molecules have been described to elicit host and non-host responses, although they seem to be specific Avr gene for definitive races (Cheong *et al.*, 1991; Nürnberger *et al.*, 1994).

The analyses of the responses induced by the crinkling and necrosis-inducers (CRN) cDNAs in many plants, suggest that they are general elicitors that trigger necrotic responses nonspecifically, both in resistant species and also in the susceptible host (Kamoun *et al.*, 1998; Kamoun *et al.*, 1999; Qotub *et al.*, 2002). It is believed that CRNs differ from specific elicitors, such as INF1, which induce defense responses only in specific plant genotypes (Kamoun *et al.*, 1998; Kamoun *et al.*, 1999), but it also resembles NIP, which functions in several dicotyledonous plants (Qutob *et al.*, 2002). The general elicitors of plant pathogens were recently compared to pathogen-associated molecular patterns (PAMPs), which are surface-derived molecules that induce the expression of defense-response genes as well as the production of antimicrobial compounds in both animal and plant cells (Gomez-Gomez and Boller, 2002; Nürnberger and Brunner, 2002). Whether the CRN proteins function as PAMPs still remains unclear. It is supported by observation that CRN genes are found in several *Phytophthora* species. In addition, CRNs could aid in a colonization process of plant tissue during the late necrotrophic phase of the infection, as proposed for the NIP protein (Qutob *et al.*, 2002).

Four EST clusters similar to CRN proteins (Figure 5, Table 2) were found in the *P. parasitica*/CitEST database. Three of these clusters (Contig 1422, PP14-C7-802-082-A05-CT.F and PP14-C7-802-125-A07-CT.F) were phylogenetically related to CRN8 of *P. infestans*;, whereas the fourth cluster (Contig 1550) showed similarity to CRN6 of *P. infestans* (Figure 5). Strange as it may seem, the observation that CRN genes were found in the *P. parasitica* virulent strain during colonization of the media culture indicates that it is only expressed during the infection process is incorrect. In fact, what is correct is the importance of the factor for the expression and secretion during the infection

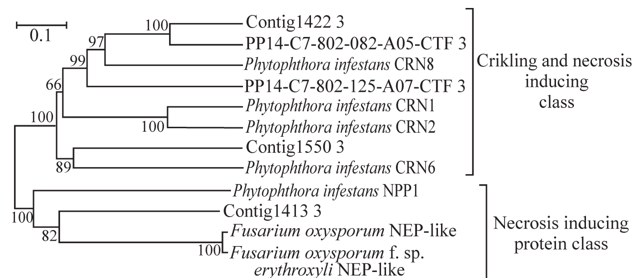

Figure 5 - Analysis of amino acid sequences of necrosis-inducing proteins. Dendrogram built by alignment of the selected amino acid sequences of the necrosis-inducing proteins. The tree was constructed and visualized by Mega using the Neighbor-joining method. The following sequences were obtained from EMBL GeneBank: the crinkling and necrosis inducing class clade showed *Phytophthora infestans* CRN1 gi|23394425, CRN2 gi|23394429, CRN6 gi|66270141 and CRN8 gi|66270145 with the clusters Contig1433, Contig 1550, PP14-C7-802-082-A05-CT.F and PP14-C7-802-125-A07-CT.F. In necrosis inducing protein clade showed the cluster contig1413 with the *P. infestans* NPP1 gi|66270095, *F. oxyxporum* NEP-like gi|86371279 and *Fusarium oxysporum* f. sp. *erythroxyli* gi|2697132. The scale is corrected for multiple substitutions. The numbers indicate percentages supporting the branches by 10,000 bootstrap replicates (bar corresponds to 0.1 substitutions per site).

process. In the future, additional functional analyses of the CRN genes in *P. parasitica* and the *P. parasitica*-Citrus system will aid in determining the nature of the contribution of these genes in the infection process

Another necrosis-inducing protein in the *P. parasitica*/CitEST database was NIP (necrosis-inducing protein), which is a secreted protein of 60 amino acids. This protein was detected in other pathogens, and besides that, there is the hypothesis that this gene product has a dual function in both fungal avirulence and virulence (Tyler, 2002). In barley cultivars expressing the *R* gene *Rrs1,* the protein elicits defense reactions ofin the plant (avirulence function). However, in a concentration dependent manner, and without considering the plant resistance genotype, the formation of necrotic lesions is induced similar to the scald symptoms. This occurred in barley cultivars. as well as in other cereal species; however, it did not occur in the dicotyledonous species *Arabidopsis thaliana* (virulence-associated function). This toxic activity seems to be mediated by the stimulation of the plant plasma membrane-localized H^+-ATPase (Tyler, 2002).

In *Fusarium oxysporum* f. sp. *erythroxyli,* a NEP1 protein (necrosis-inducing protein) was found which causes cell death in many different plant species when applied as a foliar spray. In other studies, orthologues of NEP1 gene were cloned and characterized in *Phytophthora megakarya*; indicating that it is a fungal agent for black pod disease in *Theobroma cacao* (cacao). After observing the necrotic lesions in cacao leaves sprayed with NEP1 (Bae *et al.*, 2005) for 10 days, the constitutive expression of this protein was noted. This is directly involved with the transition between the hemibiotrophic and the necrotrophic phases.

In the *P. parasitica* database, one cluster with homology to *P. sojae* protein (Table 2) was detected, but in the phylogenetic analyses this cluster appeared in the clade with NEP of *F. oxysporum* with 64% similarity (Figure 5).

The production of polypeptides and polyamines is also an important factor in pathogenicity. In the PP/CitEST database, one singleton read was found sharing a sequence similar to the ornithine decarboxilase (ODC) of *Mucor circinelloides* f. *lusitanicus*, (Table 2), and other enzymes of this pathway. ODC is an important enzyme in polyamine production. The inhibition of this enzyme is an effective therapy in the treatment of Trypanosomiasis and also other diseases caused by *Plasmodia*, *Giardia,* and *Leishmania* and in *Stagonospora (Septoria) nodorum*, a phytopathogenic fungi. This is probably a target for chemical control because of the need for this enzyme in virulence and growth (Bailey *et al.*, 2000).

In yeast, a pleiotropic drug resistance transporter system is responsible for the protection of microorganism cell against antibiotic and heavy metals, such as cadmium. In *P. parasitica,* three clusters that have homology with these genes were noted. These were also found in *P. sojae* (Table 2). This system probably aids in its survival in soil with high levels of heavy metal or exposure to fungicides.

Another group of expressed genes found in *P. parasitica* transcripts are heat shock proteins (HSP), also called stress proteins. This is a group of proteins that is present in all cells in all kinds of organisms. They are induced when a cell undergoes different kinds of environmental stresses such as heat, cold and oxygen deprivation.

Heat shock proteins are molecular chaperones. They are usually cytoplasmic proteins and they perform functions in many of the intra-cellular processes. They play an important role in protein-protein interactions, such as folding and assisting in the establishment of proper protein conformation (shape) and also in the prevention of undesirable protein aggregation. Through the partial stabilization of the unfolded proteins, HSPs aid to transport proteins across intreacellular membranes. Some members of the HSP family are expressed from low to moderate levels in all organisms due to their essential role in protein maintenance (Lund, 2001).

Here, eleven clusters related to HSPs were found in the *P. parasitica* database, with evidence pointed out by Jacobson *et al.* (1994) that *P. parasitica* probably uses these proteins in melanin metabolism and in the infection process (Table 2). Moreover, several clusters were also found in the *P. parasitica* database (Table 2), showing significant sequence similarity to other genes related to infection and host colonization processes, such as cystein proteinase, pepsinogen, proteases and acidic chitinase.

There is evidence linking melanin biosynthesis to virulence in *Aspergillus fumigatus* conidia. Superoxide dismutases, glutathione S-transferase GST, glutathione peroxidase and glutamine synthetase are important cleaning antioxidants and they have an additional hypothetical role in virulence. However, although these enzymes have been biochemically characterized in *Aspergillus* and *Cryptococcus*, there is no concrete evidence that these enzymes are involved in pathogenicity. Catalase production may play some role in the virulence of *Candida albicans,* but this enzyme has not yet been proven to have some kind of influence in the virulence of *A. fumigatus*. There is data supporting an antioxidant function of the acyclic hexitol mannitol in *C. neoformans*, however, further investigation is required. Research on the putative antioxidant activities in a range of other fungal enzymes, like acid phosphatases, currently still limited (Hamilton and Holdom, 1999).

Eleven genes of this group were detected in the *P. parasitica* database (Table 2). These genes are important in the pathogen's defense system because their products protect the organism against reactive oxygen species or induce cell death in the host. Jacobson *et al.* (1994) report the production of superoxide dismutase (SOD) and melanin in pathogenic fungi as important factors for basidiomycetes, considering that melanin production is an established virulence factor and that pathogenic fungi produce melanin (Jacobson *et al.*, 1994).

Three clusters spotted in the *P. parasitica* database share sequence similarity with a superoxide dismutase family in *P. infestans* (67%) and in *C. reinhardtii* (58%). In addition, we found: four clusters showing similarity with glutathione S-transferase, two clusters similar to glutathione peroxidase, one cluster similar to glutamine synthetase, one glutathione reductase and two similar to thioredoxin peroxidase (Table 2). The expression of oxidative stress-related genes *in vitro* could be related to *P. parasitica* melanin production, and it could also be related to an increase in expression during the infection process.

This is the first report on global gene expression in *P. parasitica,* a causal agent of gummosis in citrus. Here, several genes were identified which may contribute to the understanding of pathogenicity mechanisms of *P. parasitica* and which also may represent new possible tags for chemical control. The understanding of this pathogenicity could aid in the development of new methods or new chemical control tags for citrus gummosis. For instance, the development of molecules that deactivate the pathogenicity factors presented here.

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Internet Resources

http://citest.centrodecitricultura.br - Center APTA Citros Sylvio Moreira/IAC - Millennium Institute, Database webpage (verified January 28, 2005).

http://www.phrap.org - PhredPhrap package (verified March 25, 2004).

http://www.ncbi.nlm.nih.gov - GenBank database and BLAST tools (verified January 28, 2005).

http://www.ncbi.nlm.nih.gov/COG/ - Clusters of Orthologous Groups of proteins (verified February 28, 2005).

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Associate Editor: Raquel Luciana Boscariol-Camargo